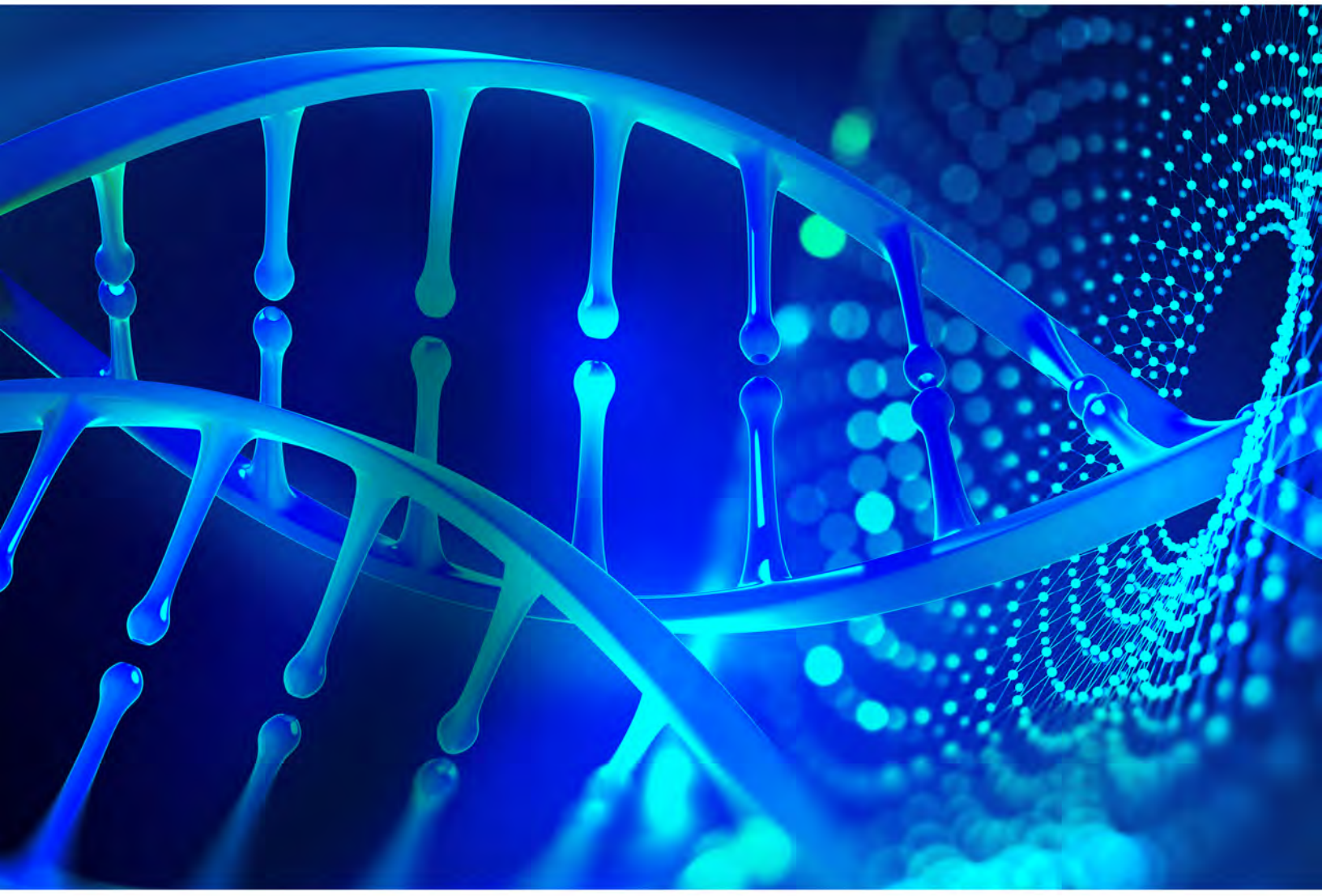
A PRACTICAL APPROACH TO MOLECULAR CLONING



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A Practical Approach to Molecular Cloning

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PREFACE

In this post-genomic era of modern biology, a large number of molecular biology manuals are available on the market. As the authors of this manual, we feel committed to addressing this logical question: **"What is the need to write another molecular biology manual?"** Although several excellent molecular biology manuals are available nowadays, most of these manuals are aimed at catering to the relatively trained and experienced workers/experimenters in the field. Notably, all of them extensively cover almost all the methodologies that are widely used not only in molecular cloning but also in wider areas of molecular biology, including genomic libraries, RNA and protein methodologies, molecular genetics, genomics, and proteomics. An apprentice in the field often gets lost when s/he is offered one such manual to consult for carrying out elementary experiments involving the isolation of genomic DNA from a common source organism or even carrying out a simple cloning experiment. Thus, we feel that, indeed, there is a genuine need for a manual that will cater to the essence of the key methodology involved in molecular cloning and help the relative newcomers in the field.

In composing the chapters in this book, we deliberately remain "cloning-centric" and focus on appending only those methods that are directly associated with molecular cloning processes and stay away from methods covering diverse areas of modern biology. We feel that the selection of the methods will make the manual simple and succinct, which will help the new students learn about a specific/selected method more easily. Given the risk of handling various laboratory elements, including chemical, biological, and radiological reagents, as well as safety precautions to deal with them, an introductory chapter addressing these issues is included. This chapter also provides various types of fundamental skills needed to train an individual adequately into an experienced experimenter. Considering the fact that during this training, a beginner must learn sufficient background of a given method, we provide sound explanations of rationale and modes of action for all individual steps. Moreover, after specific individual steps in all the protocols, we provide notes (presented in italics) that cover many critical parameters, modes of action, and do's and don'ts involved in that step, which we think will be very helpful for the new students to master the entire method. Furthermore, to provide sufficient background information, we present special information panels on selected topics, reagents, methods, *etc.*, in every chapter that would equip an apprentice with profound knowledge and information on various topics associated with molecular cloning. Finally, we included an appendix at the end that lists the composition and methods of preparation of all the reagents/buffers/solutions used in diverse protocols and the most commonly used methods that are involved in various techniques.

Finally, we would like to thank our students who provided various feedback and several sample photographs from their experiments that were used in different chapters in this manual. Last but not least, we would like to apologize for any undetected and inadvertent mistakes/errors that might still be present despite a rigorous attempt to eliminate them.

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CHAPTER 1

Getting Started in Molecular Biology Experiments

Abstract: This introductory chapter depicts our efforts to cover all the important aspects that a beginner should learn and know to work successfully in a molecular biology laboratory. Familiarity with all kinds of laboratory safety rules, including proper handling of various hazardous chemicals, do's and don'ts of various procedures, and storage of various chemical, biological, and radiological reagents and the hazards associated with them, is mandatory for every beginner in this area and hence the discussion about these topics is the foremost element, to begin with. Tips for personal protection and safety of the experimenters during the experimentations with these hazardous agents are also mentioned at different places in this chapter. Next, the authors include a virtual walk-through of the laboratory to provide knowledge of the location of the entire laboratory and departmental equipment and their handling. The importance and requirements of mathematical and other experimental skills, starting with cleaning glassware and autoclaving to designing a cloning experiment, are discussed categorically in an elaborate manner that should benefit the beginner experimenter.

Keywords: Autoclaving, Experiments, Hazards, Safety, Skills, Sterilization.

1.1. INTRODUCTION

Advances in science have revolutionized the barriers to different topics, giving rise to interdisciplinary subjects. Molecular biology involves knowledge related to biology, chemistry, physics, and mathematics. This discipline aims to investigate the properties of biomolecules like DNA, RNA, proteins, and lipids at the molecular level and how chemical and physical laws dictate their abundance and functions. To have an understanding of how these molecules function and communicate with other molecules, researchers in the field of molecular biology have designed and performed various "techniques of molecular biology" that consist of diverse experiments and methods to study these biomolecules. Medicine, agriculture, forensic science, diagnostics, and many other fields rely on these fundamental technologies, which in turn pave the way for the development of cutting-edge innovations. These include diagnostic tests for genetic diseases, forensic DNA analysis, crops with improved yields, genetically modified plants' resistance to disease, new cancer therapies, tracking pandemics, new treatment methods, novel approaches to the generation of energy, and much more.

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Although the molecular biology laboratory is puzzling to a beginner, learning some basic and essential skills and mastering fundamental biochemical and molecular techniques would be an absolute mandate for him/her to move on to the next level. Consequently, it is essential to follow the mantra that will set one up for success to avoid failures. Towards this end, the first task would be to familiarize the student with the overall laboratory ambiance,which includes laboratory safety, design of workbenches, chemical inventory, equipment, microbiological practices, rules, and guidelines [1, 2].

This introductory chapter will describe certain essential requirements that each student must know before starting to work in the laboratory. They are:

- The health hazards associated with the various chemicals and biological samples routinely used in experiments and the safety procedures to be exercised in molecular biology research.
- Various equipment and instrumental facilities routinely needed for research.
- Practical requirements to carry out various experimental procedures involving mathematical, analytical, and experimental skills.
- Diverse research strategies to be used when investigating and analyzing DNA, RNA, and proteins.
- Planning a project.

1.2. LABORATORY SAFETY RULES

1.2.1. Proper Handling and Storage of Chemical, Biological, and Radiological Reagents

Depending on the discipline and the nature of specific sets of experiments carried out, each laboratory is equipped with different reagents and chemicals. It is customary to mention here that the experimenter must exercise utmost care while handling them. Each laboratory facility and experiment present unique challenges, and hence, different rules and safety measures are assigned by the facility for the safety of the workers. However, working with organisms like pathogenic bacteria, infectious viruses, and hazardous chemicals definitely poses serious health issues. One must acquire a good amount of knowledge about these reagents and must follow the safety guidelines drafted by the government health departments to safeguard his/her health. The safety of an individual is of utmost importance, and one must ensure and identify the health hazards associated with specific experiments and, consequently, adopt all the safety measures before beginning the experiments. The following sections will cover the different areas of health hazards and safety procedures [1, 2].

1.2.2. Chemical Hazards and Chemical Safety

Every chemical is different, and so are its chemical properties. Some chemicals are reasonably harmless, while others pose a substantial threat to the experimenter's health. For example, inorganic acids and bases are extremely corrosive, which, upon exposure or contact with skin, may lead to serious injury, blindness, *etc.* In addition, many acids and organic chemicals produce corrosive vapors, which, if inhaled, can cause damage and injury to the respiratory tract and airways. Some of the important chemicals, such as 2-mercaptoethanol, acrylamide, *etc.*, are neurotoxins, whereas some other chemicals, such as ethidium bromide, are powerful mutagens. An experimenter of molecular biology cannot avoid using these chemicals but must learn how to protect himself/herself from their detrimental effects [1].

1.2.2.1. Diverse Hazardous Chemicals and General Features of Hazards Associated with Routinely used Chemicals in the Molecular Biology Laboratory

Many chemicals used in molecular biology are dangerous and hazardous for the general health of the user. The nature of the hazards involved in handling a specific chemical (such as irritant/neurotoxin/flammable/burnable) is usually described on the label of the container. Therefore, extreme care should be taken while working with them. Common hazardous chemicals can be categorized into three types [1, 2, 4].

- ***Organic solvents:*** Phenol is one of the most dangerous solvents used in molecular biology laboratories and is used for the removal of proteins during the isolation of DNA and RNA (see ***PROTOCOL A.4.1*** in the Appendix). Phenol is nowadays commercially available in Tris-buffered saturated form that can be readily used without any pre-treatment. Phenol causes severe burns, and therefore, it is advisable to always wear gloves when carrying out phenol extractions. Moreover, since it is volatile, phenol extraction should be carried out under a fume hood to avoid spreading its fume in the laboratory ambiance [1, 2, 4].
- ***Mutagens and carcinogens:*** Some chemicals used in molecular biology laboratories have mutagenic or carcinogenic properties. Among them, ethidium bromide, which binds to DNA *in vitro* for the detection of DNA in agarose/acrylamide gels, is a carcinogen (see special information panel on Ethidium Bromide in ***CHAPTER 5, UNIT 5.3***). Generally, ethidium bromide is added to agarose while casting a gel, but it can also be added to the electrophoresis buffer. The second option poses a risk of skin contamination to the user. There is, however, no advantage in staining DNA *in situ* in the agarose gel by adding ethidium bromide in the electrophoresis buffer over post-staining.

Gels stained with ethidium bromide should not be disposed of in the general laboratory waste but in a separate waste bin designated for it. Ethidium bromide solutions can be disposed of by pouring through the sink. However, it is a good idea to consider using a decontamination procedure before disposal [1, 2, 4].

- ***Toxic chemicals:*** Acrylamide, TEMED, and 2-mercaptoethanol are some of the toxic chemicals used in the molecular biology laboratory, of which acrylamide is the most dangerous [1, 2, 4]. On contact with the skin, it can exert toxic effects; it has also proven to be lethal if swallowed. 2-mercaptoethanol and TEMED are two other neurotoxins that are used in procedures involved in polyacrylamide gel electrophoresis. Both have a pungent odor and hence, all of these chemicals need to be handled in a chemical fume hood. Some recommended practices, as described below, will aid an experimenter (i) in treating them all with due respect and (ii) avoid unnecessary exposure to them, thereby lowering the risk of health hazards associated with them.

1.2.2.2. MSDS (Material Safety Data Sheets)

Material Safety Data Sheet is one of the most necessary items in the laboratory. It is of utmost importance that knowledge about the characteristics of each chemical should be understood when handling by the experimenter. This information usually contains the specific general and hazardous properties of a chemical and is provided by the chemical suppliers. Hence, MSDS is a legally required technical document in a laboratory, and a laboratory should carry a **Material Safety Data Sheet** (MSDS) on file for all the laboratory chemicals that should be readily available to each experimenter. It contains chemical identity, hazard ingredients/ identity, physical-chemical characteristics, fire and explosion hazard data, reactivity data, health hazards, precautions for safe handling and use, and control measures [1].

1.2.2.3. General Safety Precautions in Handling Hazardous Chemicals in the Lab

Exposure to hazardous chemicals can occur in various ways and, therefore, should always be avoided while handling them [1].

- ***Inhalation:*** Some inorganic acids and volatile chemicals yield strong/pungent and neurotoxic vapors. The experimenter must avoid inhaling their vapor by handling these reagents inside the fume hoods and wearing masks.
- ***Skin and eye contact:*** Many chemicals, such as acids, bases, phenols, and many organic compounds, can cause severe skin irritation, skin damage, and temporary/permanent blindness upon eye contact. One must avoid direct contact with them by using proper garb like lab coats, gloves, goggles, and other necessary garb.

- ***Ingestion:*** Most of the chemicals can cause serious health issues upon accidental ingestion. The experimenters must avoid mouth pipetting of liquid reagents, should not eat or drink in the working areas of the laboratory, and should mandatorily remove gloves before leaving the lab. Washing hands thoroughly is a mandatory event for every experimenter before eating a meal.
- ***Injection:*** Every researcher is required to handle many sharp objects, such as broken glass, and discard sharp objects, such as blades, scalpels, and needles. One needs to handle them carefully and dispose them of properly in a container designated for sharp object/glass disposal.

  In a modern molecular biology laboratory, a researcher needs to handle numerous chemicals, many of which pose different kinds of hazards. Hence, one needs to apply common sense before using them.

- It is wise to consider that all chemicals pose a hazard and treat them accordingly unless mentioned otherwise about a specific chemical.
- One should label every flask/bottle/tube with its contents, its concentrations, and the date of preparation on which they were prepared and/or transferred.
- If a dangerous/corrosive/volatile material is stored, the user should label this with an appropriate warning.
- One should think carefully before carrying out the experiment, making sure that s/he do not mix or combine any inappropriate chemicals that may lead to explosion, fire, or formation of any toxic/poisonous gas.
- One must clean his/her benchtop before and after use.
- One must wash hands often and ALWAYS before leaving the lab.
- One must take off the lab coats, gloves, and other garb before leaving the lab.
- One should always remove gloves before touching phones, doorknobs, light switches, freezer handles, *etc.*
- One must ensure proper chemical waste labeling and appropriate disposal.

1.2.2.4. 'Dos and Don'ts' of Handling Different Types of Hazardous Chemicals

- ***Flammables:*** Never heat these reagents unless absolutely required, and never store them near any flame or source of a spark. In general, only open these containers in fume hoods. If you need to store more than 10 gallons of flammable liquids, you must do that in a special explosion-proof storage cabinet.
- ***Corrosives:*** Mandatorily wear **Personal Protective Equipment** (PPE) such as lab coats, goggles, and gloves when handling corrosive reagents. While preparing a reagent using one or more of these chemicals, you must add strong acids or bases to water and never follow the reverse order. Neutralize gently to avoid rapid generation of heat and gases. Strong acids and bases should be stored in separate storage hoods.
- ***Reactive chemicals:*** You must wear PPE such as lab coats, goggles, and gloves

and know the reactive properties of the chemical. Always store oxidizing chemicals away from flammable materials.

- ***Toxic chemicals:*** You must use appropriate PPE such as lab coats, goggles, and gloves. Before opening the vial and handling a specific toxic chemical, you must study its toxic properties and learn what kind of damaging effects it may have on the human body. When working with dry powder, you must wear a mask to avoid breathing the dust. Ensure the proper disposal of unused toxic reagents/materials that may come in contact with other users.

Here are some of the most common hazardous chemicals that you will encounter in the molecular biology laboratory.

Carcinogens – Formaldehyde **Mutagens** – Ethidium Bromide

Neurotoxins – Acrylamide, 2-mercaptoethanol **Teratogens** – Formamide

Nephrotoxins – Acetonitrile **Hepatotoxins** – Chloroform

Corrosives – Phenol, strong acids, and bases

1.2.3. Radiochemical Hazards and Radiation Safety

Radiochemicals are routinely used in large amounts in many experiments involved in molecular biology.[3] ^{32}P, ^{14}C, ^{35}S, and ^{3}H are the most widely used, and ^{121}I provides an example of a relatively rarely used radioisotope used in biology. Each radioisotope emits either one or more than one of the α and/or β particles and gamma radiations. Each of these emitters is associated with some amount of energy that can cause moderate to severe health hazards and are associated with cancers and other possible deadly diseases. Despite the health hazards associated with their use, radiochemicals are frequently used in the experiments involved in molecular biology principally because of several advantages offered by them. Signals obtained from the radiochemicals over the use of their non-radioactive counterparts involve exquisite sensitivity, high specificity, and much lower cost.

Since the energetic emissions emanating from all radioisotopes cause serious health hazards, safety is a major concern regarding their use in biological experiments. However, all the commonly used isotopes, such as ^{32}P, ^{14}C, ^{35}S, and ^{3}H, emit β particles of variable energy; that of ^{3}H is the weakest, and that of ^{32}P is the strongest. However, considering the amount of each kind of radioisotope needed for specific biological applications, the use of these radioisotopes in biological laboratories is really much less concerning. Typically, specific sets of safety guidelines concerning the safe use of radioisotopes ensure the protection of the health of the users from the damaging effects of radiation.

Sets of regulations and laws have been set up by different countries for the proper handling and disposal of radioisotopes. Each scientist/worker supposed to work with radioisotopes needs to be trained in the hazardous properties of radioisotopes and radiation safety protocols. Each user of radioisotope needs a license to work with the reagents, for which s/he needs to attend a course on radiochemicals and their safe handling. Various guidelines give general rules for the safe handling of radiochemicals. The most succinct advice for molecular biologists concerning the use of radiochemicals is to maintain tight containment of the radiochemicals during and after their use and safe disposal. Simple sets of safety rules concerning the use of radiochemicals are summarized below.

- Provide suitable accommodation for the radiation containment laboratory.
- Appointment of Radiation Safety Officer by the host institution.
- Prior training of the laboratory personnel in safety procedures before beginning work with radiochemicals.
- Equip the laboratory with proper radiation detection devices such as the Geiger-Muller Counter and Scintillation Counter.
- Ensure that the detection devices are properly calibrated every time.
- Spending a minimum amount of time in handling radiochemicals, which will minimize the possible exposure of the personnel to radiation.
- Maintenance of the maximum possible distance between the user and sources of radiation.
- Use of protective shielding that is appropriate for the type of radiation emitted by the source being handled (such as Perspex shield routinely used for ^{32}P, ^{14}C, ^{35}S, and ^{3}H).
- Storage of radioactive materials within lead vials (prevents any radiation from coming out as it will absorb most of the α/β particles emanating from them) in the defined work areas and freezers.
- Use of appropriate protective clothing, gloves, and goggles during the handling of the radioisotopes to protect the exposed areas of the body.
- Use of personal dosimeters to monitor the amount of exposure (if any) of the radiation to the user.
- Strict compliance with the guidelines in case of a radiological incident (such as an accidental spillage). Monitor the work area promptly using a Geiger-Muller counter and clean up the spilled area as soon as possible. Contact the radiation safety office/department if the spillage is large and beyond user control to ensure containment and prevent the spreading of contamination.
- Containment of radioactive waste in properly designated boxes and jars, which should be shielded within Perspex containers to prevent radiation leakage.
- Divide the radioactive wastes into solid and liquid wastes and keep them in appropriately labeled waste containers within shielded boxes.
- Keep the radioactive waste accumulation as low as possible.

- Monitor the area of radioactive work before the start of the experiment.
- Also, monitor the user and the work area at the end of the experiment.
- Clean and wash thoroughly, if necessary, and monitor again.

It should be noted here that complete guidelines involving radiation safety and protection are quite elaborate, and discussion of all the necessary aspects is beyond the scope of this chapter. For further reading and gathering more information concerning this issue, the reader is advised to consult a standard document such as "Design and Organization of the Radioisotope Laboratory" issued by the International Atomic Energy Agency and the references therein [3].

1.2.4. Physical Hazards and Physical Safety

Notably, many procedures and equipment routinely used in molecular biology techniques are associated with several physical hazards that may cause health issues [1, 4]. Some of these techniques are discussed below that will make a new user aware of their use and safety issues.

1.2.4.1. Ultraviolet Radiation

DNA molecules embedded in agarose gels are visualized by staining the gel with ethidium bromide followed by exposure of the gel to the UV-radiation generated in a UV trans-illuminator (see ***PROTOCOL 5.1.C.*** in ***CHAPTER 5***). Exposure of the DNA intercalated with ethidium bromide to UV radiation results in the production of a red-orange colored band in the gel, indicating the location of DNA molecules. This piece of equipment can prove to be dangerous if proper protection is not exercised. Severe skin burns and temporary/permanent loss of eyesight leading to blindness can occur if unprotected skin and eyes are exposed to UV sources directly. While viewing an agarose gel, one must wear gloves, use a protective screen, and also wear a face shield or eye goggles. This is particularly important when the screen is removed for photography. One needs to be careful that the wrists and neck are protected when handling gels on a UV trans-illuminator [4].

Other possible sources of UV radiation exposure involve the UV tube/light equipped in Laminar Flow Hood used in microbiological procedures and the UV source used in mutagenesis experiments. All laminar flow hoods are equipped with a UV source, which usually remains turned on for ten to fifteen minutes before starting any microbiological work for decontamination of undesired microbes. During the UV-light-on phase, the users are advised to close the protective Perspex shield of the hood and to stay away from the device. During the mutagenesis experiment, however, the user needs to stay close to the UV source to expose the nutrient plates seeded with desired microbes to the radiation.

In this case, the user really needs to be protected with appropriate UV-protective garb, such as gloves, laboratory coat, and face shield/eye goggles, to ensure proper safety [1].

1.2.4.2 High-voltage Electricity

Many pieces of equipment are operated on electrical power supplies with higher voltage and thus pose a threat to the safety of the worker. The power supplies used to run electrophoresis gel are extremely dangerous. It is always advisable to turn off the power supplies before and immediately after the electrophoresis procedures and unplug the electrical leads attached to the gel from the power supply when not in use. Always stay away from touching either the gel or the electrophoresis buffer during the electrophoresis run because it can cause severe electrical shock, leading to serious injury and death. Damaged electrophoresis chambers/tanks should not be used since the leaky apparatus might prove to be lethal. Therefore, care should be exercised before running a gel, and it should be ensured that both the power pack and the gel running apparatus do not pose any problem [1].

1.2.4.3. Cryogenic Hazards Associated with Procedures Involving Extremely Low Temperatures

Many procedures in modern-day molecular biology involve the use of ultra-freezers, which are used routinely for long-term storage of bacterial/microbial strains and thermosensitive biological materials (such as proteins, RNA, and even DNA). The temperature that is achieved in an ultra-freezer device ranges from -70°C to -80°C. Furthermore, many procedures, such as snap freezing of competent bacterial cells and storage of mammalian cell lines, involve rapid immersion of these samples directly from ice (0°C) into the liquid nitrogen, the temperature of which is -196°C. Extreme care should be practiced to safeguard the skin of the exposed part of the users (especially hand, wrist, *etc.*) while handling these samples to and from ultra-freezers/liquid nitrogen container. Special cryogenic gloves should be worn while transferring biological samples from and to these cryogenic sources [2].

1.2.5. Biological Hazards and Biological Safety

An important criterion in molecular biology laboratories is the use of diverse living organisms like bacteria, yeast, and various cell lines in experiments. Most of the routinely used strains of bacteria, yeast, and viruses used for laboratory purposes are pathogenically attenuated. Hence, the chance of the user being infected during their handling becomes minimal. However, it is always advisable that proper precautions should be taken while handling them. In some countries,

all the microbiological manipulations are done in laminar flow hoods to prevent contamination, while in others, the same procedures are carried out on the laboratory benches. However, in some cases, non-attenuated bacterial and/or viral strains are used for molecular experiments, and the user should be aware of the safety procedures during their handling. To deal with the handling of non-pathogenic and highly pathogenic bacterial and viral agents, typically four biosafety level laboratory practices are used that are categorized into Biosafety level-1 (BSL-1) to Biosafety Level-4 (BSL-4), where BSL 1 is the least hazardous, and BSL-4 is the most hazardous and dangerous [2]. Microbiological safety begins with the handling of different microorganisms, followed by the prevention of contamination. Various features and requirements associated with each level are briefly discussed below.

Biosafety Level 1 (BSL-1) deals with those organisms that are typically unable to cause disease in healthy individuals. Hence, minimum precautions may be exercised while handling and working with them. They can be handled on open work surfaces with minimal protection. Laboratory workers, however, must wash their hands before and after the completion of their work with these agents. Moreover, the used cultures, plates, and flasks containing used solid and liquid media should be autoclaved mandatorily before disposing of.

Biosafety Level 2 (BSL-2) is appropriate for work involving biological agents with modest potential hazard to personnel/environment. BSL-2 workers must follow the following practices along with the practices from BSL-1.

- Laboratory personnel should have specific training in handling pathogenic agents under study under the guidance of a trained scientist.
- Entry to the laboratory is kept restricted while conducting the experiments.
- Rigorous protections and precautionary measures are executed with contaminated sharp objects.
- Reactions/experimentations involving the explosions/splashes of infectious aerosols are strictly carried out in biological safety cabinets or other physical containment equipment.

Biosafety Level 3 (BSL-3) deals with organisms that cause severe and potentially lethal diseases for which treatments are still available. However, there are certain rules that need to be followed when working at this level in addition to the practices from BSL-1 and BSL-2 levels.

- A laboratory-specific biosafety manual must be drafted, which contains the specific safety precautions required to conduct the work.

- All the laboratory personnel are provided adequate medical surveillance and offered relevant immunizations (where available) to reduce the risk of an accidental or unnoticed infection.
- All the procedures involving infectious material must be done within a biological safety cabinet under an absolutely sterile and aseptic environment.
- Laboratory personnel must wear solid-front protective clothing (*i.e.*, gowns that tie in the back). This cannot be worn outside of the laboratory and must be discarded or decontaminated after each use.

Biosafety Level 4 (BSL-4) is the highest of all the levels and deals with deadly microorganisms, which can cause severe to fatal diseases for which no vaccines or treatments are available. Generally, such facilities are located in separate buildings with specially designed double doors and airflow and are kept under appropriate surveillance.

- Entry to the laboratory is highly restricted except for authorized personnel who are experienced and trained scientists.
- Work with the hazardous organisms has to be performed within biological safety cabinets.
- A positive pressure personal suit with a segregated air supply is mandatory when working with these microorganisms.
- Members should have specific and thorough training in handling extremely hazardous infectious agents.
- A specific facility operations manual is prepared and adopted.

Therefore, the experimenter needs to know at which BSL level s/he will work and adopt the recommended practices and guidelines accordingly. However, the experiments presented in this book deal mostly with the user-friendly laboratory organism *Escherichia coli*. Note that a person can get infected by inhalation, contact with eyes and skin, ingestion, and injection of the organism. Hence, the same rules apply during the handling of biological hazards as with toxic chemicals. Some general practices to maximize biological safety are mentioned below.

- Limit access to the lab at the discretion of the lab director and adequately train all lab personnel.
- Use personal protective equipment (PPE) at all times, and keep all PPE inside the lab.
- Wash hands after handling viable materials and animals, after removing gloves, and before leaving the lab.
- Always remove gloves before touching phones, doorknobs, light switches, freezer handles, *etc.*

- Avoid touching your face with your hands or gloves while carrying out any experiment.
- Keep personal items such as coats and book bags out of the lab or in a designated work area.
- Avoid mouth pipetting; use only mechanical pipetting devices.
- Minimize splashes and aerosol production.
- Disinfect work surfaces to decontaminate after a spill and even after each work session.
- Disinfect or decontaminate glassware before washing.
- Decontaminate all regulated waste before disposal using an approved method, usually autoclaving.
- Mandatorily use insect and rodent control procedures in the laboratory to prevent the spread of possible microbial contamination from one place to the other.
- Use either a laminar flow or a biosafety cabinet.

1.2.6. Disposal of Hazardous Chemicals & Biological Materials

Environmental Protection Agency oversees that the disposal of hazardous chemicals is done following the state and federal regulations [1, 2]. Chemicals need to be treated according to their toxicity level. Highly toxic chemicals are disposed of through city sewer systems at low levels in contrast to higher levels of less toxic chemicals. Biological hazards are disposed of by autoclaving in autoclavable bags made up of high melting point plastic. They are sealed and autoclaved at high temperatures and pressures to kill any living microorganisms completely (see ***UNIT 2.3*** in ***CHAPTER 2***). The following practices are routinely practiced and should be followed in any laboratory.

- Any uncontaminated, solidified agar or agarose should be discarded in the biological trash and not in the sink. They must be autoclaved before discarding them.
- Organic reagents, *e.g.*, phenol and chloroform, should be used in a fume hood, and all organic waste should be disposed of in a labeled container, not in the general trash or the sink.
- Ethidium bromide is a mutagenic substance that should be decontaminated before disposal and should be handled only with gloves. Ethidium bromide should be disposed of in a labeled container.
- Dirty glassware should be washed with hot, soapy water, rinsed well with hot water, and rinsed at least three times with distilled water.

- Broken glass pieces, sharps (blades, razors, and scalpel blades), and syringes should be disposed of in respective designated waste bins.

General Tips about the Safety and Personal Protection of the Experimenters

It is a good habit to wear disposable gloves and a Lab coat at all times.

- **Before Start Working**

- Wearing of laboratory coats.
- Wear disposable gloves and goggles when working with toxic chemicals or UV light.
- Disinfect your lab bench at the beginning using 70% ethanol.

- **During Carrying Out Experiments**

- Clean up as you proceed through experiments, and keep your work area organized.
- Do not work with UV light on.
- Read the labels on the chemical that you are using carefully; some chemicals are mutagens *e.g.*, ethidium bromide.

- **After Working**

- Wash your hands.
- Disinfect your lab bench using 70% ethanol.
- Wash your glassware and organize your working area.

1.3. KNOW YOUR LABORATORY

1.3.1. Laboratory Equipment & Reagent Orientation

Before beginning to work in a laboratory, every user is advised to become familiar with the whereabouts and locations of different equipment, chemical inventory, and other facilities present in the laboratory and the department. Typically, new personnel, after joining a new laboratory, should be guided by the laboratory manager or other trained personnel before s/he starts his/her work. A locator guide and laboratory map, along with different inventory, are often useful in such cases. Here, the most commonly used laboratory equipment and devices are discussed in brief [4].

Equipment: Every laboratory has its own sets of small and large equipment that are routinely used for the required sets of experiments in the area of interest of the Laboratory Director/Principal Investigator. Typically, each laboratory equipment is placed in a definitive and planned order based on the nature of the experiments. In many laboratories, the working benches used by individual users are located in the central region of the laboratory room, while the equipment is placed in the

peripheral benches around the room. Typically, equipment used in microbial culturing procedures, such as laminar flow hoods, incubators, and shakers, are grouped on one specific side of the laboratory for ease of approaching one from the other. Similarly, the devices used in electrophoresis, such as power pack, gel running apparatus, and UV-trans-illuminator, are placed together in a place that is close to a sink because running the gel involves washing the electrophoresis apparatus and its components and disposal of used buffer through the sink. Other laboratory equipment is also placed strategically to maximize the convenience of the experimenters. A newcomer should learn and understand the strategy used in placing each piece of equipment in the laboratory.

Micropipettes & Tip Boxes: Micropipettes are small devices routinely used in liquid handling and quantitative liquid transfer. Each laboratory has its own practice regarding maintenance and use of micropipettes ranging from p10 (capacity to handle 2-10 μL volume range) to p1000 (capacity to handle 100-1000 μL volume range) either kept on each working table or stored in a definite location on a micropipette stand. The same may apply to the location of filled, autoclaved, and empty tip boxes.

Glassware: Clean glassware and dirty glassware are placed in a designated area in the laboratory. Used glassware and dishes are generally kept in designated areas and not left in sinks, on benches, or any other place other than the designated areas. The used glassware should be submerged either in dilute solutions of phosphate-free detergent or in chromic acid. Each laboratory personnel should follow the lab procedures used to clean glassware. Always label the glassware of its contents with a label tape or marker.

Broken Glass: Used slides, cover slips, Pasteur pipettes, or any other broken glassware are disposed of only in special designated containers, while plastic pipettes are disposed of in the regular trash and never along with broken glass.

Solution/Sample Storage: All the stock and working solutions, buffers, and reagents should be clearly labeled before storing them in a refrigerator or freezer. Items should be labeled with the following information:

- Name of substance, including concentration and/or pH if applicable.
- Date prepared and stored.
- User name or initials, who prepared it.
- Items that are stored in other areas or items that are unclearly or inadequately labeled may be discarded at the discretion of the lab technician/manager/PI.

Storage of various items in the -20°C and -70°C freezer should be organized in accordance with the laboratory rules.

1.3.2. Laboratory Equipment Orientation

An important part of working in any laboratory is the appropriate use and calibration of instruments and equipment. Detailed information about the systematic procedures of operation for the specific instruments and general information about the use of laboratory equipment should be provided to a newcomer by the laboratory managers or trained technicians.

A well-equipped biology laboratory should have almost all the basic equipment to do fundamental experiments. Note that some high-end big instruments are available as general facilities from the Department/Institute, whereas basic and specialized instruments specific to the needs of the laboratory are housed in the individual laboratory. For example, a laboratory working with yeast genetics will have facilities different from the one that is working on mammalian cell lines.

Following is a list of instruments that are typically housed in a standard laboratory. Note that the list may not be comprehensive and cover every possible experiment available. The list is intended to give a general idea. In the later part of this chapter, a description of the detailed application of some of these pieces of equipment is given.

1.3.3. General Instrumentation Facilities

The following general facilities are typically housed in any biological science department:

- Containment facilities that meet the requirements of the genetic manipulation regulations that your laboratory is engaged in.
- Microbiological facilities.
- Autoclaving facilities for sterilizing media, buffers, and solutions.
- Clean areas for molecular biology work (Laminar flow cabinets are useful but not essential).
- Warm (37°C) room. Incubators can be used as an alternative, but they must be capable of incubating Petri dishes as well as cultures in test tubes and flasks from 5 mL to 2 L.
- Glassware cleaning facility.
- Darkroom facilities containing red-light sources for autoradiography, including tanks and accessories for developing and fixing X-ray films.
- Short-wavelength (302 or 366 nm) UV trans-illuminator.

- CCD detector/polaroid camera/gel documentation system for photographing agarose gels.
- Cold room for storage of large sized bottles and protein/chromatographic work.

- Sub-zero storage facilities.
- Ultra (-80°C) freezer.
- Cryogenic facilities with liquid nitrogen storage vessels.
- Radiochemical handling and disposal facilities, primarily for ^{3}H, ^{14}C, ^{35}S, and ^{32}P isotopes.
- Wet-ice machine and dry-ice storage facilities.
- Facilities for producing deionized water.

1.3.4. Departmental Equipment Facilities

Each department is equipped with several high-end instrumental facilities, which are shared by a number of research groups:

- Preparative ultracentrifuge capable of raising up to 100,000 rpm (up to 500,000Xg) rotation speed, with at least one fixed-angle and one swing-out rotor, including all the accessories. Running a sucrose or cesium chloride density gradient centrifugation can take up to 48 to 72 hours. Thus, one machine is rarely sufficient.
- Refrigerated centrifuge capable of achieving 25,000 rpm, plus three different sized rotors for accommodating 15-500 ml centrifuge buckets/tubes along with adapters and other accessories.
- Double beam UV-VIS spectrophotometer along with three different sets of cuvettes and additional Xenon lamps.
- Liquid scintillation counter with all the accessories.
- Sonicator with a variety of probes.
- Four-point balance for both large and small quantities.
- Platform shaking incubators for incubating at 45°C, 55°C, and 65°C temperatures.
- Vacuum drier (a freeze-drier is not essential).
- Vacuum oven.
- 250°C temperature oven.
- Automated DNA-sequencing machine (desirable but not essential).
- Oligonucleotide synthesis machine. This is optional: commercial synthesis of oligonucleotides is cheap and rapid, so an in-house facility is economical only if demand for oligonucleotides is high.
- Fluorescence/confocal microscope facilities.
- UV-VIS spectroflouremeter.
- Fluorescence activated cell sorter.
- CD and/or NMR spectrometer.
- Low-emperature lyophilizer.
- Electron microscopic facility.

1.3.5. Laboratory Equipment Facilities

Each research group belonging to one single individual laboratory carrying out any aspect of molecular biology experiments will require these items, sometimes in multiple numbers if possible.

- Bench-top refrigerated centrifuge, capable of achieving 20,000 rpm
- 200 Volts power supply for agarose gels, electroelution, *etc.*
- 500 Volts power supple for carrying out electro transfer for protein bands generated from SDS polyacrylamide gel electrophoresis.
- Horizontal gel apparatus of various sizes (*e.g.*, 10 X 10 cm, 15 X 10 cm, and 15 X 15 cm gels).
- Refrigerated microcentrifuge machine capable of running 2 mL and 1.5 mL microcentrifuge tubes.
- Automatic micropipettes (*e.g.*, Gilson P20, P200, P1000); each researcher will need a dedicated set of automated pipettes.
- Water baths (non-shaking), ideally three, one set at 37°C, one at 65°C, and one for various temperatures as per specific requirements.
- Micro pan balance for weighing chemicals and media ingredients.
- Vortex mixer.
- Magnetic stirrer.
- pH meter.
- Geiger-Muller counter or other types of radioactivity monitoring devices.
- Microwave oven for melting agar and preparing agarose gel.
- UV cross-linker.
- Thermal cycler for carrying PCR reaction.
- Real-time thermal cycler for carrying out quantitative PCR (qPCR).
- Hybridization oven or, as a cheap (and nasty) alternative, a bag sealer.
- Cassettes with intensifying screens for autoradiography.
- Light-box for viewing autoradiographs and stained protein gels.
- Double-distilled water supply or deionized water facility.
- 4°C refrigerator (multiple).
- -20°C freezer (multiple).
- Light microscope with phase contrast feature.
- Fume cupboard or hood for handling volatile liquid.
- Computer: for virtually all molecular biology applications, a networked PC or Macintosh is adequate.

If DNA sequencing is being performed manually using Sanger's dideoxy method, then the laboratory will also need the following devices.

- Vertical gel apparatus (*e.g.*, 21 X 40 cm).
- 3000 V power supply.
- Gel drier.

For some types of hybridization analysis, the following equipment will be needed:

- Slot/dot blotter.
- Electro transfer or vacuum blotting unit.

1.4. PRACTICAL REQUIREMENTS FOR MOLECULAR BIOLOGY RESEARCH

1.4.1. Mathematical Skills Required for the Molecular Biology Laboratory

As mentioned in the introduction (***UNIT 1.1***), the discipline of molecular biology is an interdisciplinary subject that requires analytical and quantitative skills. Therefore, an experimenter/scientist involved in the investigation in a given field of molecular biology must have elementary mathematical skills. If s/he previously has not been exposed to fundamental courses in mathematics, one must arrange to complete one course in mathematics sometime during his/her career. Some basic mathematical skills that are routinely required in various experimental techniques are briefly discussed below [1].

Exponential Numbers

The numbers that are dealt with in the laboratory are often very large or very small. Consequently, these numbers are expressed in scientific notation using exponential numbers.

These rules apply to the use of exponents as explained below:

When n is a positive integer, the expression 10^n means - multiply 10 by itself n times. Thus,

$10^1 = 10$; $10^2 = 10 \text{ X } 10 = 100$; and $10^3 = 10 \text{ X } 10 \text{ X } 10 = 1{,}000$

When n is a negative integer, the expression 10^{-n} means - multiply 1/10 by itself n times‖. Thus, $10^{-1} = 0.1$; $10^{-2} = 0.1 \text{ X } 0.1 = 0.01$; $10^{-3} = 0.1 \text{ X } 0.1 \text{ X } 0.1 = 0.001$

***Examples**: $2 \text{ X } 10^1 = 2 \text{ X } 10 = 20$*

$2.62 \text{ X } 10^2 = 2.62 \text{ X } 100 = 262$

$5.30 \text{ X } 10^{-1} = 5.30 \text{ X } 0.1 = 0.530$

$8.1 \times 10^{-2} = 8.1 \times 0.01 = 0.081$

In scientific notation, all numbers are expressed as the product of a number (between 1 and 10) and a whole number power of 10. This is also called exponential notation. To express a number in scientific notation, do the following:

- First, express the numerical quantity between 1 and 10.
- Count the places where the decimal point was moved to obtain this number. If the decimal point moved to the left, *n* is a positive integer; if the decimal moved to the right, *n* is a negative integer.

Examples: *8162 requires the decimal to be moved 3 places to the left =* 8.162×10^{3}

0.054 requires the decimal to be moved 2 places to the right = = 5.4×10^{-2}

Addition and Subtraction of Exponential Numbers

Before numbers in scientific notation can be added or subtracted, the exponents must be equal. Thus, if the exponents are not equal, then the exponents need to be equal first by adjusting the decimals followed by their addition. An example is provided below:

Example: $(5.4 \times 10^{3}) + (6.0 \times 10^{2}) =$

$(5.4 \times 10^{3}) + (0.60 \times 10^{3}) =$

$(5.4 + 0.60) \times 10^{3} = 6.0 \times 10^{3}$

Multiplying and Dividing Exponential Numbers

A major advantage of scientific notation is that it simplifies the process of multiplication and division. When numbers are multiplied, exponents are added; when numbers are divided, exponents are subtracted.

Examples: $(3 \times 10^{4})(2 \times 10^{2}) = (3 \times 2)(10^{4+2}) = 6 \times 10^{6}$

$(3 \times 10^{4})/(2 \times 10^{-2}) = (3/2)(10\,[4 - 2]) = 1.5 \times 10^{2}$

OR $\frac{(3 \times 10^{4})}{(2 \times 10^{2})} = (3/2)(10\,[4 - 2]) = 1.5 \times 10^{2}$

Determining Significant Figures

Making accurate measurements and recording them correctly are extremely

important, and the accuracy of the measurement is reflected in the number recorded. Every measurement has some uncertainty since no physical measurement is precise. The use of significant figures is a way to indicate uncertainty. The number of significant figures in quantity is typically indicated by the number of digits that are known accurately, along with the doubtful digit. The doubtful digit is always the last digit in the number. For example, in a balance, which can weigh up to + 0.01 g, a sample weighs 54.69 g. In this example, the doubtful digit is 9. When an answer given has more numbers than significant, then the last number must be rounded off. It is sometimes confusing to determine whether a zero in a number is a significant figure or not.

Generally, a Zero is a Significant Figure if:

- It lies between two non-zero digits in a number.
- It lies to the right of a number with a decimal point.
- It does not lie to the right of a number without a decimal point.
- It does not lie to the left of a number.

Examples: *For 12.40 g, the zero is significant.*

For 110 g, the zero is not significant.

For 1.004 g, the zeroes are significant.

For 0.004 g, the zeroes placed before decimal are not significant.

1.4.2. Experimental Skills

The experimental skill starts with the cleaning of glassware and plasticware, followed by sterilization. In the purification of DNA/RNA or while electrophoresing them, a thorough cleaning is a primary requisite in any experiment. Further, contamination is a frequently used term in microbiology/molecular biology to indicate the presence of an undesired microorganism/substance in a culture/preparation of a desired microorganism/biomolecule. The concept of sterilization/cleaning arises to avoid the presence of contaminant microorganisms/substances. Obviously, a beginner needs to know and understand the principals of autoclaving media versus autoclaving glassware or plasticware. Uncleanliness and sloppy work will not only result in inaccurate results but will lead to contamination and misleading results. The use of a micropipette to measure liquid can be challenging and require a lot of practice. Similarly, weighing out samples is a skill that most scientists need throughout their careers. Hence, before starting the experiment, new personnel should learn the proper and appropriate way of doing each of the

following steps [1].

1.4.2.1 Cleaning Glassware

One of the first steps towards the success of experiments lies in the cleaning of glassware and plasticware. Disastrous consequences may result from improper cleaning. Water source is a very important part of cleaning glassware. Before starting, an experimenter needs to consider what type of glassware s/he requires and what it will be used for. Generally, very soiled and greased glassware is dipped in chromic acid solution for 12-24 hours for prompt removal of dust and grease. Utmost care needs to be taken while handling chromic acid, including wearing rubber washing gloves on both hands. Glassware immersed in chromic acid is first thoroughly washed with tap water and then given a scrub, followed by a final rinse with double distilled water.

The following tips provide good practice rules involving washing glassware (or plasticware).

- ***Pre-rinse:*** All the glassware, after being used, is soaked in a detergent solution. This step of pre-rinse is necessary to prevent the contaminants and other unwanted materials from drying onto the inner surface of the glassware.
- ***Contaminant Removal:*** Preferably phosphate-free detergents and/or solvents are recommended for washing glassware. The usual procedure begins with scrubbing to remove any stubborn contaminant/debris with differently sized lab brushes that might stick to the surface. This step is followed by thorough rinsing with powerful jets of water (preferably hot /warm). Typically, a commonly used lab glass detergent is Alcon.
- ***Rinse:*** It is very important to remove the detergent and cleaning solvents that have been used from the glassware completely. Otherwise, the presence of even a trace amount of the detergent will interfere with the downstream reactions. Hence, rinsing 3 to 5 times with tap water is an essential and important step in its use in the experiments.
- ***Final Rinse:*** The final rinse is usually done with purified distilled/deionized water. This removes the dissolved salts that could be present in tap water/ some residues that might stick to the glass. Usually, the final rinse is done three times.
- ***Drying Never hand dry:*** Glassware is dried in a drying oven using heat or air-dried on a rack. Be it heat or air, they should be dried upside down so no contaminants from the air may fall in. To avoid further contamination, closed cupboards are used to store the clean, dry glassware.

Important Note: It should be kept in mind that the internal surfaces of glassware are surfaces where various reagents/buffers/biologically important materials make contact. Therefore, during cleaning and subsequent autoclaving steps, the

experimenter should take utmost care to keep this surface clean and free of any unwanted substances like detergent, acids, dirt, dust, *etc.*

1.4.2.2 Weigh it Right

It is one of the vital procedures that a scientist learns to weigh out precisely and correctly as the first thing. This step, perhaps, constitutes the most important criterion for obtaining success in experiments. Typically, samples needed in larger quantities (such as in grams) are weighed out in a so-called rough top-loading balance. In contrast, a fine four-place analytical balance is routinely used for fine chemicals in the milligram range. For every balance, keeping it clean after use and maintaining precise calibration impact all weight measurements [1]. Therefore, a little care would really help the experimenter to ensure the consistency/ reproducibility of his/her experimental outcome in the subsequent downstream events. The five typical steps to good practice of weighing are as follows.

- ***Cleaning the weighing pan:*** No matter whether the user is going to use a large or small quantity, it is important that the weighing pan should be clean before use. This is done either with a small cleaning brush provided by the manufacturer or with ethanol-soaked, lint-free tissue (*e.g.*, Kim wipes).
- ***Taring the balance with weighing paper:*** A clean oil/weighing paper is placed on the weighing pan, with the weight displayed on the display panel. The tare function of the balance is pressed to set the display to zero.
- ***Weighing out the substance precisely:*** Using a clean dried spatula, the substance to weigh is placed on the weighing paper. Note the weight displayed. Based on the initial weight, it is necessary to decide whether to add more of the substance (if the initial weigh is less than the desired weight) or to remove some substance from it (if the initial weight is more than the desired weight).
- ***Removal of the substance from the weighing machine:*** Once the correct weight is obtained, the weight should be noted, and the substance, along with the weighing paper, is removed from the pan. The substance is added to a flask, bottle, or tube for further downstream procedure.

Important Note: It should be noted that when a really tiny amount of expensive and/or costly biochemical needs to be weighed (such as a lyophilized enzyme that comes in small quantity), a common practice is to weigh out an arbitrary amount of that costly substance directly into a microfuge tube and note the weight. Once the precise weight of the arbitrarily weighed amount of the substance is determined by subtracting the weight of the microfuge tube, the actual volume of the solvent needed to obtain a solution of specific concentration is estimated, and the substance is finally dissolved into the calculated quantity of solvent right into the microfuge tube. For costly/expensive substances, the weight of the arbitrary

amount of that substance is usually determined rather than trying to weigh out a specific desired amount for two reasons. First, trying to weigh out a specific amount is difficult because it needs a trial error involving the addition and removal of the substance, which is typically associated with the loss of a substantial amount of the substance. Second, weighing directly into a microfuge tube eliminates the error that is associated with the transfer of the substance from the weighing paper to the microfuge tube.

1.4.2.3 Autoclaving

This procedure is generally carried out to kill microorganisms and their spores by heating at high temperatures under very high pressure for a stipulated amount of time. All the microbiological and molecular biological procedures need autoclaved sterile glassware, plastic ware, media, and solutions. The media in which the microorganisms are cultured should also be autoclaved and kept sterile before use. Detailed aspects of autoclaving and various crucial parameters are discussed in detail in ***UNIT 2.3*** in ***CHAPTER 2*** [1, 2].

1.4.2.4 Micro Pipetting Practice

Micropipettes are typically used for liquid handling and the transfer of relatively small amounts of liquid from one container to another. Since sometimes the volume of the liquid transferred is very low (only a few microliters), several precautions are typically practiced when handling a small amount of liquid samples.

A common proverb concerning handling a micropipette says, ***"Do not rest the micropipette devise down with a filled-tip or hold the same upside down or sideways. The liquid will not slip out if you hold it upside down but the liquid may enter the instrument through its shaft, which will result in contamination."***

The systematics of withdrawal and transfer of a liquid sample using a micropipette is discussed below.

- Always practice to set up the volume knob correctly on the micropipette. It is crucial to first take a look at the top round cap of the devise to identify its measuring range. First, it is important to pick the right pipette for the volume of the liquid sample to be dispensed. Note that the maximum value listed on the top is the largest volume the pipette can measure. For example, using a micropipette with a 100-1000 µL volume range, the largest measurable volume is 1000 µL; using a micropipette with a 20-200 volume range, it is 200 µL. Similarly, the smallest value a micropipette with a volume range of 2-20 µL can withdraw is 2 µL. In order to withdraw 0.45 mL, it is advised to select a micropipette with a

100-1000 µL volume range, to withdraw 0.15 mL, a micropipette with a 20-200 µL volume range, and to withdraw 0.015 mL, a micropipette with a volume range of 2-20 µL is required. **It is vital to practice carrying out this kind of conversion in one's mind since it will be helpful during the actual experimentation.**

- Have a graduated 1.5 mL microcentrifuge tube in a rack ready to hold the liquid you measure in the next steps. Microcentrifuge tubes are typically dubbed Eppendorf tubes. The Eppendorf represents a popular brand of laboratory plasticware.
- Place the end of the micropipette shaft on the wider hole of the microtip kept in the tip-box. It is crucial to avoid touching the tip with bare or gloved hands. Now press the end of the micropipette shaft firmly into the tip until the tip fits the shaft tightly. The 2-200 µL volume capacity (smaller) tips fit both the micropipettes with 2-20 µL and the 20-200 µL volume ranges. The tips for micropipettes with higher volume capacity (200-1000 µL) look visibly larger than the 2-200 µL capacity (smaller) tips.
- Hold the micropipette with your right hand and gently press down the plunger with your thumb or index finger (whichever feels more comfortable to you). Note that the plunger stops at two places. The first stop is designated for the withdrawal of the liquid, and the second one is fixed for ejecting the withdrawn liquid. You should practice withdrawing and ejecting the liquid several times until you feel the difference between the two stops comfortably.
- Next, depress the plunger gently until you arrive at the first stop. Dip the end of the tip just below the surface of the liquid. You should steadily touch the microtip at the inner wall of the tube just below the surface of the liquid. If you dip the tip too much into the liquid medium, the liquid will collect on the outer surface of the sidewalls of the tip and will be transferred into the collection tube during the final delivery step. This will lead to the transfer of a larger volume of liquid than desired, resulting in an error.
- Now, release the plunger very gently. It is important to release the plunger slowly. If the plunger is released too quickly, the liquid may backflow into the micropipette tip, resulting in contamination. It should be kept in mind during the handling of any viscous (thick) liquids, such as the sugar solution or glycerol if the plunger is released too quickly during the withdrawal step, the liquid will not enter the tip fast enough, and your measurement will be erroneous. Sometimes, this may happen with the less viscous liquids as well, so pipetting should always be carried out slowly and gently. One needs to be careful not to remove the tip from the liquid surface before it is filled with the desired volume and the liquid meniscus within the tip does not go up anymore. Otherwise, an air bubble may get into the tip, and less liquid than the desired volume will be transferred. Sometimes, it is possible that the plunger is released slowly, and the tip is

maintained within the liquid, yet bubbles may be formed. This means that you perhaps pressed the plunger down to the second stop instead of the first. In that case, you need to practice the stops again.

- Now, transfer the liquid by pressing the plunger slowly down to the first stop without moving the tip from the beaker. Try to avoid bubbling. Repeat the preceding step. Withdrawing and releasing the liquid a few times (dubbed pipetting up and down in the lab slang) will improve the precision of your measurement.
- Distribute the liquid into a 1.5 mL microfuge tube and ensure it is near the 0.6 mL mark (600 μL = 0.6 mL). This step is intended to re-examine pipetting to ensure that you have used the right micropipette and carried out the pipetting properly. Show the result to your instructor to judge the accuracy of your pipetting ability.
- Reject the tip in a waste container by pushing down the eject button hard. You should practice this technique several times every day since it constitutes an important ability to master. Rushing in these steps may result in the production of bubbles, which in turn leads to an error.

1.4.2.5 Working with Microcentrifuge Tubes and Labeling Them

This step involves another important skill that every experimenter in molecular biology needs to learn. This is briefly discussed below.

- While transferring the solution into the microfuge tubes, always wear gloves. Initially, after taking microfuge tubes, close them and label the lids with appropriate sample IDs. Note that marking on the lid of the microfuge tube would permit the experimenter to read and identify the sample correctly while the tube still stands on the rack. At the same time, using a permanent marker for writing/labeling would help prevent the erasing or bleeding of the writing if the surface of the lid becomes wet.
- When setting up multiple reactions like PCR or multiple digestions, take as many microfuge tubes as required and keep them in the rack. If the reaction demands the addition of a common liquid like water or buffer, then it would be wise to add it first using a single tip in all the tubes. The concept of contamination arises when another common solution has to be added. In such a case, either change tips after addition in each tube or add in each tube with the same tip, taking care not to touch the contents or even the side of the tubes. In the case of diluting acids and bases, water or any other liquid is first dispensed, followed by their addition.
- After the addition of the solution, mix the contents by either gently tapping the tube or pipetting up and down in slow motion to avoid the formation of bubbles. When working with enzymes, bubble formation has to be avoided as it can

contaminate the micropipette. In the case of setting multiple reactions, one tends to make mistakes when filling or adding a solution twice. One of the ways to avoid such mistakes is to move the microfuge tubes either one row back or forth in the rack and also close the lids after each addition.

- To check the precision of your pipetting, set the pipette to the volume that you used earlier and withdraw the liquid from the tube. If your pipetting is precise, there should not be any liquid left in the tube. When dealing with very small amounts, centrifuge the tube briefly to collect the entire amount of liquid at the bottom since small amounts tend to cling to the inner walls of the tube.

1.4.2.6 Preparation of Laboratory Reagents

The preparation of diverse reagents/solutions is a routine but extremely important task for diverse applications in molecular biology. A solution is defined as a solute (solid power or crystalline substance, usually constitutes a smaller component) dissolved in a solvent (medium in which solute is added, usually constitutes the larger component). A routine and common task for any molecular biologist is to prepare solutions almost every day, which constitutes an important step towards performing an experiment and rather the first step. If a solution is not prepared properly, it can cost a lot with respect to time and money. Therefore, to save valuable time and resources, a standard operating procedure (SOP) has been set up for the preparation of each solution to avoid mistakes. It is a good idea that all calculations used to prepare a solution are carefully noted in the laboratory notebook, including the exact mass/weight and volume of solvent used along with the date and the name of the reagent-maker and his/her initials [1]. Several conventional methods of concentration of solutions that are in common use and the methods to prepare them are discussed below.

• Percent concentrations

Percent solutions may be expressed in several ways, as listed below.

Weight per volume (wt./vol. or w/v) designates the weight (in grams) of solute per 100 mL of solution (used to describe the concentration of a solid dissolved in a liquid).

Volume per volume (v/v) specifies the volume (in mL) of solute per 100 mL of solution (used to describe the concentration of a liquid dissolved in liquid)

Weight per weight (w/w) designates the weight (in grams) of solute per 100 g solution (used to define the concentration of a solid mixed with another solid)

It is important to note that in every case, a 100 unit (either 100 mL or 100 g)

solution is used since *percent* means out of 100.

NOTE: Among the different ways of preparing solutions with percent concentrations, ***Weight per Volume*** *is the most commonly used unit of concentration, which is routinely used for small amounts of chemicals and fine biochemical reagents such as purified enzyme stocks and DNA/RNA stock solutions (for example, 0.3 μg/mL DNA).*

• Molarity

The molarity of a solution is defined as the number of moles of solute present per liter of solution. While the letter M is used as the symbol for molarity, it is also expressed as moles/liter or mol/L. Note that, by definition, one mole of every element/compound contains 6.023 X 10^{23} (Avogadro's number) numbers of atoms. Since the weights of diverse atoms are different, one mole of a given substance weighs differently (in mass units) than one mole of another substance. *Note that the mass of one mole of a given substance is equal to its atomic weight in grams.* Refer to the periodic table to find the atomic weight of routinely used elements in biochemical experiments. For example, one mole of the elements carbon and oxygen weigh 12.0 g and 16.0 g, respectively.

Example: Using a periodic table, calculate the molar mass of water (H_2O). The atomic weight of hydrogen is 1.00, and that of oxygen is 16.00. You have to count the hydrogen twice because there are two units of hydrogen and one unit of oxygen, as per the formula. Thus, the molar mass of water becomes 2.00 + 16.00 = 18.00 g/mol.

• Preparing Molar Solutions

Note that in routine research activities, we can only measure mass units and cannot directly estimate moles. To estimate the required mass of a chemical reagent required to prepare a specific volume of a solution with specific molarity, one has to transform the number of moles to mass using the chemical's molar mass as a conversion factor, as exemplified below.

Mass = molarity X *volume* X *molar mass*

Thus, how many g = moles/liter X *L X g/mole*

Do not forget to convert mL to, if necessary.

Example: *To prepare 100 mL of 1 M NaOH (FW 40.0)*

g = 40 g/mol X *1.0 mol/L X 0.1 L*

g = 4 (dissolve 4 g of NaOH in 100 mL water)

A ***molar solution*** *is one in which 1 liter of solution contains the number of grams equal to its molecular weight.*

Example: *To make up 100 ml of a 5M NaCl solution*

= 58.456 (mw of NaCl) g/mol X *moles/litre* X *0.1 litre*

= 29.29 g in 100 mL of solution

A step-by-step systematic procedure to prepare a molar solution is appended below.

- Figure out the weight (number of grams) of the chemical (often called solute) needed to make *x*M (molar) solution.
- Every solution made in the laboratory should bear a proper label.
- Weigh out the estimated amount of the chemical reagent using a calibrated balance on a weighing paper. Leave the balance clean. Close the container and return it.
- Now, take a beaker and label it using labeling tape with a sharpie. Then, add the salt/chemical that you weighed out in it. Using a graduated cylinder, measure a small amount of dH_2O (deionized water) and add it to a beaker. Since solubilization of the solute may increase the volume of the solution, it is wise to dissolve it in a small amount of solvent/water.
- To dissolve the solid completely in the solvent, put the bottle or flask containing it on a magnetic stirrer after adding a stir bar to it. One may need to add more solvent if the solution nearly reaches saturation to dissolve all the solids. The solute needs more solvent than half the final volume of water/solvent, as instructed, for complete dissolution. In such a case, the solvent is added to a maximum volume of 80% with respect to the final volume.
- Now, make up the final volume of the solution by returning the solution to the graduated cylinder and then adding more water slowly and carefully with a wash bottle. It is important to watch the meniscus (the rounded bottom surface of the water) until it reaches the final volume. The lowest part of the meniscus should line up with the final volume mark. This step is often abbreviated BTV (bring to volume). Never use a graduated beaker to bring up the final volume, as they do not measure accurately.

- Transfer the solution to a labeled bottle. **Do not put a solution into a container that is unlabeled. Always label legibly.**

Preparing Parallel Dilutions or Making "X" Solutions:

Many buffers used in molecular biology procedures are composed of the same sets of components, but often, the concentrations of each component in different buffers vary. To avoid making every buffer from scratch (which otherwise takes a lot of time), it is a good idea to make and maintain limited numbers of concentrated stock solutions of each individual component and dilute each of them in making specific buffers/reagents as needed. In routine experimental work, a 10X solution refers to a buffer that is ten-fold more concentrated than the working concentration. A 10X stock solution should be diluted by a factor of 10 (by adding 1 part of the 10X stock to 9 parts of solvent/water) to make a working (1X) solution.

Example: *To prepare 100 mL of TE buffer (10 mM Tris, 1 mM EDTA).*

Mix 1 mL of a 1 M Tris-HCL solution, 0.2 mL of 0.5 M EDTA, and 98.8 mL of sterile water.

The following formula is useful for calculating the amounts of the stock solution needed:

$C_i \text{ X } V_i = C_f \text{ X } V_f$

where $\boldsymbol{C_i}$ *= initial concentration, or concentration of stock solution;*

$\boldsymbol{V_i}$ *= initial volume, or volume of stock solution needed*

$\boldsymbol{C_f}$ *= final concentration, or concentration of the desired solution;*

$\boldsymbol{V_f}$ *= final volume, or volume of desired solution*

Note that many enzyme buffers are supplied from the vendors as concentrated solutions, *e.g.* 5X or 10X stock buffer solution (five or ten times the concentration of the working solution). These stock solutions need to be subsequently diluted such that the final concentration of the buffer in the reaction becomes 1X.

Thus, you should add 2.5 μL of a 10X Restriction Enzyme buffer, the other reagents, and water to make up the final volume of 25 μL.

Preparing Serial Dilutions

The aim of preparing serial dilutions is to prepare a specific dilution serially from previously prepared dilutions of immediately higher concentration. They are typically used in diverse applications, including the following:

- A number of chemical/microbial samples with dilutions of the same dilution factor are desired

Example: *To make a series of solution with a repeating dilution factor of 2; in other words, the concentration of each diluted solution should be half that of the previous dilution before it (such as 2 M, 1 M, 0.5 M, 0.25 M).*

- The desired final concentration of a given reagent is sometimes so small that the original volume (C1) of stock solution cannot be accurately measured as there are no micropipettes available to measure such a small quantity.

Example: *To prepare 1 mL (final volume) of 5 mM (final concentration) solution from a 10 M stock solution. To find C1, the volume of 10 M stock needed to make the final solution, the unit of mM (milli molar) has to be converted first to the unit of M (molar) so that the concentration remains the same on both sides of the equation.*

5 mM = 0.005 M, then you would use the formula

$C_1V_1 = C_2V_2$.

(10 M) X *(V_1 mL) = (0.005 M)* X *(1 mL)*

V_1 mL = (0.005 M) X *(mL)/10 M*

mL = 0.0005 mL (or 0.5 μL)

It should be noted here that although some micropipettes are commercially available, which claim to measure 0.5 μL, neither such a volume is measurable accurately nor are they available in every lab. It is, therefore, advisable to carry out a serial dilution of 1/100 or even 1/10 dilutions from a highly concentrated stock. This can be made in the following way.

The Dilution factor can be expressed as = $(V_1+V_2)/V_1$

Where, V_1 is the volume of the solution being diluted

V_2 is the volume of solvent used to dilute the solution

(Note: V_2 is the final volume of the diluted solution)

Example: *You want to do a series of 5-fold dilutions, each with an end volume of 40 mL.*

Dilution factor = (V1 + V2)/V1

5 = (V1 + 40 mL)/V1

5 V1 = (V1 + 40 mL)

4 V1 = 40 mL

V1 = 10 mL

It should be noted here the reason why V_2 becomes both the volume of solvent used in each dilution and the final volume of that dilution is as follows. Once a dilution is prepared by adding V_1 mL of solution to V_2 mL of solvent (water), V_1 mL of the previous dilution is already withdrawn to make the next one. Thus, you always end up with V_2 mL in each dilution except the very last one.

Preparing Percent Solution

Percentage (w/v) = weight (g) in 100 ml of solution;

Percentage (v/v) = volume (ml) in 100 ml of solution.

Example: *To make a 0.7% solution of agarose in TBE buffer, weigh 0.7 gm of agarose and bring up the volume to 100 ml with TAE/TBE buffer.*

Steps in Solution Preparation and Several Important Tips

- Before embarking on this job, check for instructions on the preparation of a particular solution by referring to a Laboratory Reference Manual and also check out for any specific precautions needed for handling the chemical from the label on the bottle.
- Using an analytical balance, weigh the desired amount of chemical(s) in case when the amount of the chemical is less than 0.1 g. Transfer the chemical(s) to an appropriately sized beaker/flask. Add deionized water to it along with a stir bar. Dispense less than the required amount of water. Prepare all solutions with double distilled water.
- When the chemical is completely dissolved, it is time to make up the volume by carefully and slowly adding distilled water to achieve the final volume. This is done by transferring the solution to a graduated cylinder and adding the required amount of water.
- For preparing solutions containing agar or agarose, the above rule is not followed. Instead, the required amount of agar or agarose is added directly into the final vessel.
- In the case of maintaining a solution at a specific pH, adjust the pH using a pH meter following instructions and fresh buffer solutions. If the solution needs to be sterile, autoclave it at 121°C for 20 minutes.

- Some solutions cannot be autoclaved, such as SDS. In this case, they are filter sterilized by passing through a filter with a 0.22 μm or 0.45 μm pore size.
- For bacterial cultures, within an hour or two of the preparation of the media, it should be autoclaved. They are then stored at room temperature. Before using them, it is important to check for contamination by holding the bottle at eye level and gently swirling it.
- Typically, solid media used for microbiological culture is prepared in advance, sterilized by autoclaving, and safely stored either in a cotton-plugged flask or in a screw-capped bottle. When required, the agar is melted in a microwave and poured onto sterile petri plates. If the medium requires any additional components, such as antibiotics, these are added at this point, right before the plates are poured.
- Working solutions of Tris-HCl pH=8.0 or NaCl can be prepared from their concentrated stock solutions, such as either from 1M Tris-HCl pH=8.0 or 5M NaCl, by combining autoclaved double-distilled water as a solvent in a sterile vessel.

1.4.2.7 Gel Loading

Loading DNA and RNA samples in an agarose and acrylamide gel is an important skill that every worker in the field of molecular biology requires to learn. In fact, loading DNA/RNA samples in a gel is an art that needs a steady hand. This skill is gradually developed with a lot of practice and perseverance. Detailed aspects of gel loading and various crucial parameters in gel loading are discussed in detail in ***CHAPTER 5***.

1.4.2.8 Working with Enzymes

Handling the restriction and modification enzymes used for carrying out restriction digestions and performing modification of the DNA molecule, respectively, is an important aspect of the molecular cloning method. Moreover, enzymes are also used to set up PCR reactions or ligation reactions, as well as for many other purposes. New personnel working in a molecular biology laboratory need to learn the pros and cons of handling these enzymes. Most of these enzymes are highly sensitive to heat and are extremely unstable at room temperature. Therefore, these reagents are generally stored in a -20°C freezer with 50 percent glycerol as a solvent for long-term storage. Care should be taken during the transfer of the enzymes to set up reactions. It is advisable to avoid keeping the vials containing these reagents at room temperature or even at 4°C in the freezer. Always store these vials at -20°C and take them out in a cryofreezer container (that maintains -20°C temperature) very briefly only while using. Detailed aspects of handling these enzymes and the crucial parameters involved are discussed in

detail in ***CHAPTER 6 AND 7***.

1.5. CALIBRATING LAB INSTRUMENTS

The accuracy of measurements actually depends on how the instrument is maintained. In other words, calibrated equipment will provide almost accurate measurements. Hence, calibrating the instruments in the lab is a necessary aspect of maintaining their accuracy since various physical and chemical properties of buffers and reagents, such as temperature, pH, mass, absorption, and volume, are often measured as per requirement. Factors such as movement, humidity, electrical field changes, and many others can affect the calibration of some instruments, such as pH meters, electronic balances, and micropipettes. Therefore, periodical recalibration of several pieces of equipment must be performed from time to time because the calibrated settings can change with time.

1.5.1. Calibrating a pH Meter

A pH meter is provided with an electrode that allows H^+ ions to pass through its walls but gives erroneous results for solutions that have high sodium ion concentrations as the Na^+ ions alter the electric potential determined by the electrode. There are certain limitations regarding the accuracy of a pH meter. It has been observed that most pH meters will only yield precise measurements for solutions having temperature ranges between -5°C and 60°C. High pH solutions can sometimes damage the electrode, and usually, electrodes do not provide an accurate reading above the pH of 12. Thus, it is necessary to calibrate the pH meter occasionally [1]. The method involved in the calibration of the pH meter involves the following steps.

- Choose an appropriate pH meter to calibrate. Turn the power switch of the equipment on and calibrate the instrument with the standard buffers. Typically, a standard buffer set contains standard buffers with pH 7.0, pH 10.0, and pH 4.0. For easy identification, specific inert colors are added to these buffer solutions.
- Place the electrode in a pH-7.0 buffer. Wait to see what reading is shown. If the reading does not show pH 7.0, calibrate with the knob to make the reading 7.0. Repeat the same step to calibrate the meter to pH 10.0 and pH 4.0.
- It is a crucial practice that the pH electrode should be rinsed with distilled water before dipping it into the next buffer solution. During rinsing, collect the rinsed water in a waste beaker labeled as waste. Do not rinse the pH electrode with a storage solution or buffer.
- Once calibration is done, immerse the pH electrode into an unknown solution to measure its pH. Whirl the solution gently to make sure that the solution makes good contact with the electrode and that the reading of the equipment is stable and not fluctuating before recording the final reading. Note the pH reading in

your laboratory notebook.

1.5.2. Calibrating and Using an Electronic Balance

To calibrate the electronic balances, the objects of known mass are typically used as standards. For electronic balances, which are capable of measuring up to ± 0.01g, the standard weight is usually 200 grams. These electronic balances are usually used to measure objects above 0.05 g. Once the 200-gram weight is rested on the balance scale in calibration mode, the balance identifies this weight as 200 grams as a standard and will then use this information as a reference to measure other objects of unknown mass.

- Place 50 mL of a stock solution into a 100- or 150-mL beaker.

NOTE: To avoid contamination, DO NOT pipet out any liquid from a stock solution. Pour an approximate amount of liquid from the stock solution into the beaker, and after the use, DO NOT return the used aliquots of liquid to the original container of stock solution.

- Select a specific balance and note its model number. Follow the instructions of the manufacturer to calibrate the balance. Have your instructor examine and record the live readout with the 200-gram weight.
- Put a weighing boat on the pan of the balance, followed by pressing the tare button. This will deduct the weight of the weighing boat and make sure that you obtain the weight of the substance that is kept on the boat.
- Withdraw 4 mL of orange water with a 5 mL pipette and pipette-filler/bulb and deliver the liquid onto the weighing boat. You may want to exercise drawing up and delivering the liquid back into the flask until you gather sufficient mastery on this. In case you are using blowout pipets, you need to blow out the last drop of the liquid to obtain the most precise measurement (please consult the lab equipment manual to know more about blow-out and to deliver pipettes and distinguish between them). If you use pipette filler, you need to roll the wheel up and down rapidly several times before you actually withdraw liquid. In case you use a pipette bulb, squeeze the bulb once the pipette is empty. Another good idea is to draw 5 mL of the desired liquid and then deliver only down to the 1 mL mark, which will precisely deliver 4 mL of the liquid. It is always a good idea to

  drain the rest of the liquid in the pipette into a waste beaker instead of putting it back into the bottle containing the original stock solution.

NOTE: While estimating the reading of the specific volume of liquid in a pipette, one needs to ensure that the bottom of the meniscus of the withdrawn liquid aligns with the specific marking in the pipette to which the user desires to measure and

withdraw. To prevent parallax error, the user needs to ensure that his/her eye is placed at a perfect horizontal level with the marking and meniscus. Suspend the pipette freely but steadily to keep it perpendicular to the benchtop. Avoid any droplets of any liquid on the outside/inside of the pipet before you deliver and transfer any liquid. It is also a good idea to touch the pipet tip to the inner wall of the transferring vessel during the dispensing step.

- Note the weight of the orange water and write it in the laboratory notebook. Note that 1.0 mL of water weighs 1.00 g at sea level. This information would help the experimenter to ensure the accuracy of his/her measurement.
- It is customary that every member of the team should repeat step 4, and everyone should keep adding 4 mL of orange water to the weighing boat onto the previously added orange water (in other words, do not throw liquid in the boat between successive measurements). Record the final weight of the entire volume of liquid added and divide this number by the number of people in the group to obtain the average weight of the sample. Note all the readings and calculations in the laboratory notebook and consult with your instructor.

1.6. NOTE ON USING KITS

The advancement of various techniques in molecular biology led to the development of various commercial kits that have certainly made life easier, and the time taken to perform a procedure is usually less than the usual classical laboratory method. Kits are now available to isolate DNA and RNA for their purification and extraction from the gel and for all other procedures; each one has its success story. They not only expedite the experimental plan but also produce good reproducible results. Most of the kits are user-friendly, with all the components provided in the kit along with the user manual. However, when performing an experiment using a kit, a student is actually provided with readymade solutions for a particular procedure. Most of the time, the student follows the procedure blindly according to the manual provided without having an insight and understanding of the mode of action of individual steps in the entire process. In contrast, the classical method, although laborious and requires a longer time, teaches the student about how to make solutions and also provides a good understanding of the rationale of the action of each individual step. This will equip the student with the ability to troubleshoot better if an experiment fails. Hence, it is always advisable for a beginner to learn the basic underlying principles of laboratory procedures using the traditional way. Therefore, a beginner should stay away from the kit and cling to the traditional/conventional methods as this will provide him/her an opportunity to learn to make solutions, adjust pH, autoclave solutions and cultures, *etc.* At the end of the day, these lessons will help the student in achieving success in his/her experimental work.

1.7. RESEARCH STRATEGIES FOR MOLECULAR BIOLOGY

The ultimate goal of a molecular biology project might deal with a protein product of a gene or its regulation or interaction with other proteins. Whatever the goal of a given project, each project begins with the isolation of a particular gene or a segment of DNA involved in controlling a function. The study of the role of a gene involved in a particular function of interest is usually accomplished either by cloning the gene or by PCR amplification. Cloning of the gene is accomplished by enzymatic digestion followed by ligation of the fragment into a vector, whereas PCR or polymerase chain reaction constitutes amplification of a specific segment of DNA using a thermostable DNA polymerase (such as Taq Polymerase). The choice between these two processes depends on the availability of starting material. It is important to decide which method should be used and adopted depending on the goal of the project. Hence, careful planning is required before beginning a project. If cloning is used, PCR will be used later for screening colonies. The correct clone will then be maintained in the *E. coli* host. If PCR is used instead to amplify a segment of DNA, then this amplified product will be used to clone a particular vector. This section will describe an outline of the cloning and PCR methods [4].

1.7.1. Gene Cloning in Outline

The procedure using a segment of exogenous DNA or gene (often called insert) is incorporated in a plasmid (often dubbed cloning vehicle/vector) followed by its successful introduction and continued propagation in a bacterial host (*E. coli* is routinely used) is referred to as cloning (see ***UNIT 6.3*** in ***CHAPTER 6*** for detailed description on cloning). The essential steps by which a gene is cloned are listed below.

- The specific segment of DNA or a gene (dubbed insert), which might previously exist in another vector, is either fished out by PCR amplification or excised by digestion by a set of desired restriction enzyme(s) from the previous plasmid. A specific target segment can also be prepared from a genomic DNA sample by selective amplification of the desired segment using polymerase chain reaction (see ***UNIT 6.4.3*** in ***CHAPTER 6*** for a detailed description of PCR). The fragment, whether prepared by restriction enzyme digestion or by polymerase chain reaction, is then inserted into linearized plasmid DNA to yield recombinant DNA molecules.
- This resultant vector + insert DNA is then inserted into host *E. coli,* yeast, fungal cells, or even intact animal and plant cells to allow the recombinant molecules to multiply in the host cells using the host machinery of DNA replication. *E.coli,* being the simplest organism, is generally used for all kinds of

manipulation or for incorporating genetic alterations (any sort of changes) in the favorite gene, such as single base substitution, insertion, deletion, *etc.*

- The copies of recombinant DNA molecules multiply as the host cells divide, and these newly generated DNA molecules are transmitted to the progeny bacterial host cells.
- Continued replication of the host cells results in the multiplication of these clones, producing millions of copies of the desired gene, each of which consists of a copy of a single desired DNA molecule.

One of the objectives of cloning consists of the detailed investigation and study of a given gene of interest in question. In addition, using this powerful method, a new gene can be discovered by a thorough screening of a genomic DNA library consisting of different clones.

1.7.2. PCR in Outline

Polymerase chain reaction (PCR) is a powerful process of amplification of a specific target DNA sequence *in vitro* to produce a large amount of this DNA sequence. This reaction is carried out by mixing a set of reagents followed by cycling them at three different temperatures repeatedly in a thermal cycler (see ***UNIT 6.4.2.*** in ***CHAPTER 6*** for details). This equipment is equipped with a metallic platform harboring multiple slots that allows incubation of multiple reaction tubes containing PCR reaction at three specific temperatures (94°C for denaturation of template DNA, 50-60°C for annealing of the primers, and 72°C for extension) for 30-60 seconds each for 30-35 cycles that is dictated by a user-driven program. There are certain basic steps in this procedure, which are appended below.

- A specific DNA from the organism of choice (often called the target/template DNA) is first prepared from the source organism of interest and then denatured at 94°C.
- Two oligodeoxynucleotides (called primer sets) complementary to upstream and downstream flanking sequences located at two termini of the gene or the segment of DNA to be amplified are previously designed, synthesized, and eventually added to the reaction. These primers are then allowed to anneal with the denatured DNA at their target/complementary sites when the incubation temperature is lowered to around 50-60°C (called annealing temperature) based on an intrinsic property of each primer called melting temperature (T_m). This step is called the annealing step.
- A special DNA polymerase (Taq Polymerase) isolated from the thermophilic bacterium *Thermus aquaticus* is added to the reaction containing the template DNA, a supply of four substrate deoxyribonucleotides followed by raising the

incubation temperature to 72°C (at which the thermostable DNA Polymerase is most active) is used. This is the DNA synthesis or the extension step in which a large number of target DNA is synthesized from the extension of 3′-OH of each primer.

- This cycle of the alteration in temperatures, *i.e.* denaturation to annealing and extension, is repeated 30-40 times such that during each cycle, the number of newly synthesized DNA molecules doubles. This exponential amplification process finally yields a large amount of product DNA sequence of a predicted size, which can be eventually detected by gel-electrophoresis (see ***UNIT 5.2*** in ***CHAPTER 5*** for detailed description of gel electrophoresis).

1.7.3. The Choice Between Cloning and PCR

Notably, PCR and cloning both end up with somewhat similar results. However, the major distinction between these two processes involves the time required to complete each one. Cloning is time-consuming and involves the isolation of DNA until the identification of the right clone in a library. In contrast, PCR is a rather quick method that involves setting up a reaction with the required oligonucleotide primers. However, cloning still remains the first choice over PCR due to certain difficulties [4].

- Performing a PCR reaction actually requires information on the DNA sequence of the gene in question. This is a prerequisite to designing primers or oligonucleotides complementary to the sequence of the flanking gene segment. If the project deals with an unknown segment of the DNA whose sequence is unknown, the oligonucleotide primers cannot be designed, and consequently, carrying out a PCR reaction is impossible.
- Amplification of a long stretch of DNA is difficult using PCR. Typically, sequence lengths ranging from 3 kb to 10 kb of DNA can be easily amplified by PCR. An experiment involving a longer stretch of DNA under study will be difficult to amplify, and in that case, the choice of PCR will not be suitable.

Thus, if you are working with a relatively shorter sequence of DNA, such as 3 kb DNA, whose sequence is known, PCR should be the method of choice. However, if the DNA sequence is unknown or if the length of the DNA is relatively long, one should opt for cloning.

Basic Techniques Needed for Cloning and PCR

It is advisable to first learn the basic techniques of handling bacteria and the preparation of DNA and RNA before going ahead with cloning and PCR. Whatever procedure is adopted, one needs to know certain basic techniques of the laboratory [4].

Handling Bacteria (Chapter 2)

The ability to grow pure bacterial cultures without contaminating them is one of the basic techniques a molecular biologist must learn. This is because all manipulation in the inserted DNA fragment can easily be performed inside bacteria as it is the simplest organism and easy to handle. This is the reason why a molecular biologist wanting to clone a piece of DNA in another organism, like yeast, baculovirus, or mammalian cells, first clones it into a bacterium. Hence, all cloning experiments involve the use of *E. coli* as the host organism. Thus, it is vital to learn the techniques for handling this superorganism [1, 4].

Sterility is an essential step in microbiology. A student, thus, has to adapt to the techniques to grow pure cultures of a host bacterium without contaminating them. In addition, he also needs to learn how the sterility of the working surfaces is maintained by taking proper precautions involving the use of biosafety cabinets/laminar flow hoods and wearing proper clothing and gloves. At all times, one must use sterile materials like pipette tips, microfuge tubes, and glassware and wear gloves even when not handling bacteria. Maintaining sterility is essential to prevent any sort of contamination from work areas or from the secretion from the skin that may degrade DNA, RNA, and even restriction enzymes.

Preparation of DNA (CHAPTERS 3 AND 4)

Isolation of DNA (both plasmid and genomic DNA samples) is the first task given to a beginner. The underlying principle lies in preparing unfragmented, pure DNA without contamination from cellular RNAs and proteins. Whatever organism is used, the basic principle is to lyse the cells and remove the lipids, proteins, and carbohydrates. There are various techniques for different organisms. The objective is to prepare a pure variety of DNA, which can later be fragmented with restriction enzymes in a defined manner or be used for PCR reaction. DNA preparation is also required to purify recombinant DNA molecules to further perform different manipulations in the gene under study. Vector molecules can be easily maintained in bacteria and are now available commercially.

Separating DNA by gel electrophoresis (CHAPTER 5)

Gel electrophoresis is a widely used procedure that is routinely utilized to separate DNA fragments of different sizes based on their mass difference. Agarose is the gel material used and is commercially available with different specifications. Separation of DNA fragments of different sizes is dictated by multiple parameters *e.g.*, the ratio size net charge over the mass of the separating molecule, the strength of the electric field applied, and various other factors. It is a key

technique in modern-day molecular biology, which is regularly used to verify the integrity and purity of a given DNA sample, estimate the size of an unknown DNA sample, and evaluate the digestion of DNA with one or a combination of restriction enzymes. At the same time, the technique is also useful in the purification of a particular DNA fragment from a mixture of DNA fragments of varied sizes. If the fragment size is very small in the order of several hundreds of nucleotides, then polyacrylamide gels are used instead. They are poured in vertical trays, are difficult to handle, and require skill.

Purifying DNA Molecules from Electrophoresis Gels (CHAPTER 5)

Towards the construction of recombinant DNA molecules, the gene that needs to be inserted in the vector is prepared by various means. Whatever the method used, the insert DNA is checked and verified in an agarose or polyacrylamide gel and then needs to be purified. In this procedure, the band is excised from the gel and subsequently purified. However, during the excision of the agarose slice containing the desired DNA band, the electrophoresis buffer and the agarose-containing ions and other chemicals may contaminate the DNA sample, which in turn may affect further downstream enzymatic reactions. Various methods have evolved to circumvent these problems. Hence, the choice of a particular method will depend on the nature of a specific experiment. However, commercially available kits have made these processes much easier.

Construction of Recombinant DNA Molecules (CHAPTER 6)

The construction of recombinant DNA molecules is the first step in gene cloning. Once the DNA segments harboring the sources of the vector (such as the mother plasmid) and the insert (such as genomic DNA) are prepared, restriction endonucleases and modification enzymes are employed. Restriction enzymes, often called molecular scissors, are site-specific endonucleases that essentially make specific cleavage at the distinct sites in the plasmid or genomic DNA. They can create two types of termini that consist of either blunt ends or single-stranded sticky ends. They might have a single or numerous cleavage site(s) on a given DNA. A circular vector molecule with a single restriction site for a particular enzyme, when cut or ‘restricted’, makes the molecule linear. This is extremely important to create linear vector molecules, which can then be used to join with the insert DNA. DNA ligases can join two linear fragments that have been previously prepared by restriction digestion of these DNA molecules. Two molecules with similar ends are compatible to be joined. In other words, two blunt-ended molecules or two single-stranded sticky-ended molecules created with the same restriction enzyme can be joined by the ligase. Compatible ligatable ends can also be created when two different restriction enzymes are used to

generate two different ends. A blunt end can be converted to a staggered end or vice versa. This is accomplished by a set of enzymes called modifying enzymes, which will be dealt with later in ***CHAPTER 6.*** Restriction enzymes are also used to check PCR products to see if the amplified DNA has the recognition site for a given restriction enzyme. In addition to traditional cloning procedures, a brief introduction to Gateway cloning technology is also presented to familiarize the students with this emerging technology of molecular cloning.

Introduction of Recombinant Molecules into Host Cells and Recombinant Selection (CHAPTER 7)

The ligation of the insert DNA and the vector will produce a recombinant DNA molecule, which needs to be introduced into a host *E.coli* by a process known as transformation. There are a number of transformation procedures available. These procedures allow the recombinant molecule to make copies of itself as the host cell divides, resulting in colonies upon plating on agar media. A specific proportion of the entire host bacterial cells present in the culture will receive the recombinant DNA (harboring the insert DNA ligated to the vector), and another fraction of host cells will accept only the self-ligated vector molecules that do not contain insert. The presence of the insert in the colonies, therefore, needs to be identified or screened. The insert DNA segment borne by the cloning vector is typically used in the detection of recombinant colonies (*i.e.*, colonies that harbor insert DNA ligated to the vector). Insertional inactivation, *i.e.*, the inactivation of the previously inserted gene(s) in the multiple cloning site of the vector molecule *via* the insertion of the insert DNA, typically leads either to a phenotypic change (*e.g.*, using antibiotic resistance/sensitivity) (see ***UNIT 7.2.1***) or to the production of colored colonies (*e.g.*, owing to the presence of a chromogenic substrate) (see ***UNIT 7.2.2***). At this stage, it is possible to identify the correct recombinant clone had the cloned gene conferred an identifiable phenotype on the host cell or construction done in such a way that the recombinant molecules contain the correct gene. In reality, the selection of recombinant clones actually involves another set of experiments due to the presence of false positives. This usually involves the use of PCR or restriction enzyme-based analysis.

Cloning Vectors (CHAPTER 3)

Cloning vectors play a vital role in the production of recombinant molecules. They are available in various sizes, and the selection of the vector is very important for a particular cloning experiment. The choice of a vector will depend on the genes present in it and the mode of selection for recombinants. While some plasmid vectors are engineered to insert relatively small fragments (5kb or less), the others were developed as specialized vehicles to receive DNA fragments over

a certain size.

There are diverse classes of plasmid vectors, but all of them were developed either from naturally occurring plasmids or from genomes of bacteriophage λ or M13. These vectors (plasmids) are all self-replicating molecules containing an origin of replication, and they use the replication machinery of the host for their replication. These plasmids/vectors, therefore, provide a way for sustaining and propagating within their host bacterial cells, thereby offering the essential criteria required for the procurement of a recombinant clone.

Apart from cloning, there are plasmid vectors that are available to check the expression of the cloned gene and are referred to as expression vectors. These vectors are also equipped with special features. Wide varieties of vectors are available commercially now, and one needs to choose them carefully before designing an experiment. It is always advisable to begin with the simplest vector if it suits the requirement, and if successful, then one should use the same vector for future experiments. One should be ready to change the vector if a problem is encountered with recombinant selection or handling.

CONCLUSION

In the preceding sections, a thorough account of various aspects of working in a molecular biology laboratory is presented that each student must know and learn. These involve the safety issues in the laboratory that provide protection to the experimenter from various kinds of potential dangers/hazards associated with various chemical, physical, and biological factors. Moreover, basic descriptions of various essential equipment and various skills needed to perform successful experiments involved in molecular biology are also presented. Finally, the chapter concludes with the presentation of the essential experimental strategies employed in molecular cloning procedure. The fundamental knowledge and essential information presented in this chapter will help new students become successful and experienced researchers.

FURTHER READING

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CHAPTER 2

Microbiological Techniques for Molecular Biology

Abstract: This chapter provides the experimenter with an overview of the microbiological procedures and techniques indispensable for molecular cloning. We presented detailed descriptions of all the essential techniques associated with handling the laboratory's favorite microorganism, the gram-negative bacteria *Escherichia coli*. The chapter begins with the step-by-step procedures of different aseptic techniques, description, compositions, and preparation procedures of different microbial growth media, which is followed by exhaustive procedures of inoculating and culturing *E. coli* in those growth media and methods associated with monitoring their growth. We also discuss different storage methods used to preserve *Escherichia coli* on long-term and short-term basis that are routinely carried out in cloning procedures. These rules that the experimenter needs to adhere to are outlined to achieve successful results.

Keywords: Colonies, Liquid media, Plating, Spreading, Serial dilution, Solid.

2.1. INTRODUCTION

Countless microbes populate our ambiance, including the water we drink, the air we breathe, and the food we eat. While many of them are harmful and cause deadly diseases, some of them are truly beneficial in helping us digest our food and conduct many other lifesaving processes. In modern-day laboratories, numerous model and medically/industrially important microbes, including various non-pathogenic bacteria, bacteriophages, and yeasts, are used for various purposes. A majority of their applications involve facilitation to manipulate nucleic acids (DNA/RNA) *in vivo* to introduce various alterations/changes and to study different metabolic processes, genetics, physiological functions, and molecular interactions in microbes. While handling our desired microbe, it is important to ensure that it is not contaminated with other undesired microbes, which are widespread in the surrounding atmosphere. In the present chapter, the basic microbiological techniques and practices will be discussed to ensure the appropriate and safe use of living microorganisms. These procedures are vital for the following reasons:

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- To prevent contamination of workers and equipment in the laboratory.
- To avoid contamination of the cultures of desired microorganisms from other undesired microbes.
- To avert accidental release of the microorganisms outside the laboratory.

Procedures typically practiced to prevent unwanted consequences, as discussed above, are collectively termed aseptic techniques [4]. The most common aseptic technique routinely followed to ensure the appropriate use of the microorganisms in the laboratory, avert health hazards of the users, and prevent accidental release of microbes in society is called sterilization. Sterilization involves any process that efficiently kills or eliminates transmissible microbes including bacteria, viruses, fungi, and prions, from any surface, equipment, foods, medications, biological culture medium, *etc.* [4]. Fundamental procedures of sterility are discussed in ***UNIT 2.3*** below:

2.2. CATEGORIES OF BASIC MICROBIOLOGICAL TECHNIQUES

The microbiology techniques are categorized as follows:

- **Culturing and aseptic techniques**
- **Bacteria enumeration**

2.3. Aseptic Techniques

Advancement in our knowledge about microorganisms, their habitats, hosts, and biology over the last century led to the development of sets of laboratory rules for the safe handling of microorganisms. Culturing the desired microorganisms free from other unwanted microbial species in the environment is one of the fundamental challenges in a microbiology laboratory. Consequently, the success of the experiments relies on the technique adapted to culturing, isolating genetically pure culture or a clone derived from a single cell. Hence, extreme care needs to be followed during the handling of the microorganisms to prevent unwanted contamination of experimental microbial culture with another undesired microbe. **Aseptic techniques** enable laboratory workers to successfully culture a microbe, free from contamination with other undesired microbes. **Aseptic techniques** involve methods that prevent the introduction of unwanted microbial organisms (typically called **contaminants**), and they are typically performed under sterile conditions [1, 2, 4, 5]. While culturing for a particular organism, it is necessary to ensure that the desired organism is selectively introduced into the culture medium and that other environmental organisms do not contaminate it. Contamination by fomites (any inanimate object/substance capable of transporting pathogens from one medium or individual to another) can also be prevented by

the use of aseptic techniques [4]. Routinely used aseptic techniques are appended below:

2.3.1. Sterilization

The complete elimination of all contaminating microbes from laboratory equipment, materials, and culture media is referred to as sterilization [4]. Viruses, bacteria, fungi, spores, and other vegetative cells are killed in this process. Our environment is full of different kinds of microbes. To prevent microbes on surfaces from becoming airborne and entering the cultures, the first step towards this end is to keep the doors and the windows of the laboratories closed at all times. The process of sterilization used in the laboratories involves both physical and chemical methods when dealing with cultures and plates. For example, the method to sterilize nichrome loops used for inoculation is by flaming in a Bunsen burner or incinerator before and after use [4]. Heating the lip of the flask or tube before pouring culture media onto another plate and during the transfer of materials from one tube to another is a mandatory step to prevent contamination. It should be noted that accidental exposure of a sterile object to a non-sterile ambiance destroys the sterility of the sterile substance. To ensure sterility, therefore, the lids/stoppers of a sterile bottle/flask should remain held in the hand (and not placed on other surfaces such as countertops) during the transfer of sterile liquids from one tube to another to maintain sterility of both the flask/bottle and its content [3, 4]. Typically, sterilization methods are categorized into two groups:

- ***Physical Methods*:** This category includes sterilizations using

- Thermal (Heat) sterilization
- Ultraviolet radiation (*e.g.*, UV, infrared, gamma radiation, and X-ray)

- ***Chemical Methods (Disinfection)***

Generally, phenol and many other related compounds, dyes, soaps, detergents, alcohol, gaseous compounds, and heavy metals and their compounds are used to destroy microorganisms.

2.3.1.1. Physical Methods

- **2.3.1.1.A** ***Heat sterilization*** is the most reliable method of sterilization that completely destroys enzymes and other essential cellular constituents of other undesired contaminating microbes [4 - 6]. The process is more effective in a hydrated state because the moist heat effectively kills the tough microbial spores, which typically escape killing by dry heating. (Table **1**). Moist heat sterilization, therefore, requires much lower heat input under conditions of high

humidity that kill microbes efficiently. In this state, hydrolysis and denaturation of proteins of the undesired microbes take place, thereby completely killing them. In contrast, much higher heat input is required under a dry state as oxidative changes of various cellular components take place [4 - 6].

Table 1. A tabular comparison between various processes of sterilization.

Method of Sterilization	Mode of Action	Advantages	Disadvantages	Applications
Heat Sterilization	Kills bacterial endotoxins. Moist heat kills bacterial endospores	Most widely used. Cheap and reliable method of sterilization	Applicable only to thermostable materials and substances	Dry heat is appropriate for sterilizing glassware. Moist heat is the most reliable method for decontamination of laboratory waste and the sterilization of laboratory glassware, media, and reagents.
Radiation Sterilization	Damage to nucleic acids *via* ionization, adduct formation	Useful for Industrial Applications	Not recommended for sterilization of growth media, buffers, and reagents since high radiation leads to radiolysis of water. This can cause loss of chemical properties of these reagents	Generally applied to articles in the dry state, such as petri plates, plastic culture dishes, cell culture dishes, and pipettes in the industry
Filtration Sterilization	Does not destroy but removes the microorganisms	Used mainly for the Sterilization of liquids that are thermolabile.	Cannot distinguish between viable and non Viable particles.	Vitamins, antibiotics, and thermolabile growth factors.
Chemical Sterilization	Kills microorganisms *via* the lethal action of various chemicals	Quick and effective method of killing microorganisms	Many of these reagent are corrosive to skin and reactive to test chemicals and reagents	Surface sterilization

(i) Dry heat Sterilization

Thermostable products like flasks and glass pipettes can be sterilized by exposing them at a temperature of 160-180°C for at least 2 hours or more depending on the temperature and the sizes and/or volumes of the glassware. Dry heat is typically used for sterilizing glassware and metallic instruments using its power of good penetrability and non-corrosive nature. Notably, dry heat destroys bacterial endo-

toxins (or pyrogens). The following methods are routinely used to employ dry heat in the laboratory.

- Incineration
- Red heat
- Flaming
- Hot air oven

Dry heat sterilization of small to large volumes of empty glassware such as culture bottles/flasks/tubes/glass pipettes is usually carried out in a hot air oven. Inoculation loops are usually sterilized by exposing them to dry heat *via* flaming before inoculating microbial cells into the culture media. The lips of the tube or flask containing the culture are also flamed before transferring their sterile contents into another sterile container [2, 4].

(ii) Moist Heat Sterilization (Sterilization by Autoclaving)

An autoclave is a machine/device that uses high-temperature steam and pressure to efficiently kill microorganisms such as bacteria, viruses, fungi, and spores on items that are placed inside its pressure vessel. It is often used as a steam sterilizer in research laboratories, healthcare, food, and pharmaceutical industries. Autoclaving is the most reliable method for the sterilization of laboratory glassware, culture media, reagents, and buffers and decontamination of laboratory microbial waste using pressurized steam to destroy microorganisms and is, therefore, most widely used (Fig. **1**). The rationale of sterilization by moist heat relies on the principle of elevation of the boiling point of water in the presence of high pressure. The high pressure created by the accumulation of steam in a closed vessel leads to the elevation of the boiling point of water (or steam). This high heat is capable of killing all the microbes, including viruses, fungi, and spores present under such conditions. Moist heat thus generated causes the death of the microorganisms by coagulation of the proteins. The optimal composition of steam within an autoclave is 3% liquid and 97% gas. Any change in the percentage of moisture increases or decreases sterilization time. In practice, sterilization time is calculated according to optimum steam conditions and steam's ability to transfer energy to the non-sterile load prior to sterilization [2, 4].

PROTOCOL 2.1: PROCEDURE FOR RUNNING AN AUTOCLAVE

Typically, autoclaves are operated at 121°C/250°F at a pressure of 15-20 psi (Note that this temperature is generated by steam generated from 15-20 psi pressure) for 15-20 minutes. Importantly, the presence of a finite quantity of air is able to depress the final temperature of autoclaving from 121°C, which affects sterilization from microorganisms because many microbes/spores are capable of

escaping from the steam action at this lower temperature, which eventually causes inefficient autoclaving. Hence, it is important to ensure that all of the air inside the autoclave is displaced completely before finally sealing the vessel. The stages of operation of autoclaves include complete air removal, steam admission/pressure generation/temperature elevation, and sterilization cycle (including heating up, holding/exposure, and cooling stages). It is important to note that the moist heat generated from pressurized steam is extremely efficient in killing the microbial spores, which are not proficiently killed by the dry heat of the same temperature (121°C/250°F) typically generated in a dry heat oven [3].

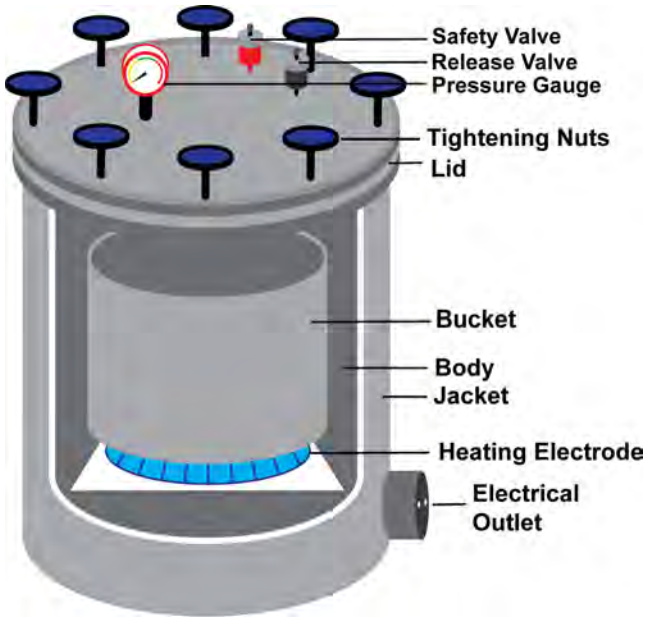

Fig. (1). Schematic diagram showing different parts of a laboratory autoclave.

In general, an autoclave is operated at a temperature of 121°C for at least 15-30 minutes by using saturated steam under at least 15 psi of pressure. The following steps are routinely followed while sterilizing reagents/media in an autoclave.

Materials

Liquid Medium

Solid Medium

Glassware

Plasticware

Equipment

Vertical Autoclave

Procedure

- Before beginning to use the autoclave, it should be checked for any items left from the previous cycle.
- A sufficient amount of water is then put inside the chamber.
- The materials to be sterilized are placed inside the chamber.
- Lid is then closed, and the screws are tightened to ensure an airtight condition, and the electric heater is switched on.
- The safety valves are adjusted to maintain the required pressure in the chamber.
- Once the water inside the chamber boils, the air-water mixture is allowed to escape through the discharge tube/release valve to let all the air inside be displaced. Complete displacement can be ensured once the water bubbles cease to come out of the pipe.
- The drainage pipe/release valve is then closed, and the steam inside is allowed to reach the desired levels (15 lbs in most cases).
- Once the pressure is reached, the whistle blows to remove excess pressure from the chamber.
- After the whistle, the autoclave is run for a holding period, which is 15-20 minutes in most cases.
- Finally, once the exposure time is over, turn off the main switch of the autoclave heater.
- Allow sufficient time to cool down the device, which will lower the steam pressure as well, which can be monitored in the pressure gauge.
- When the pressure comes back to the normal atmospheric pressure, slowly open the screws. Remember that the device is still very hot. Wear a pair of asbestos gloves to operate the autoclave after the running cycle.
- After opening the lid, allow 1 to 2 minutes for the steam accumulated inside to come out completely. This will protect you from being severely burnt by the accumulated steam (remember that the burning capacity of steam is much higher than that of the boiling water).

In order to check whether the autoclaving process has been conducted properly, autoclave tapes are used on the containers to be sterilized, which change color when the appropriate temperature is reached and the holding period is maintained for the correct time. Note that this method of sterilization can only be applied to thermostable products. Note that completely sealed bags or bottles typically explode in the autoclave, and therefore caps of all bottles must be kept loose before the autoclaving procedure. Autoclavable nylon bags are available for

autoclaving plastic tubes such as pipette tips and microfuge tubes. Alternatively, these materials may also be autoclaved by putting them in a beaker that is wrapped with aluminum foil.

Important Notes and Tips

- It should be noted that once the microbial growth media, including both solid (for plates, slants, and top agar) and liquid (rich and minimal broth for flaks and tubes) media, are prepared, they must be autoclaved immediately.
- It is the best practice to prepare media in multiple small aliquots (in either tubes or flasks/bottles) for safe storage while using only one at a time.
- One must remember that when sterilizing microbial growth medium by autoclaving, overheating must be avoided. Overheating may cause the pH of the media to change, and caramelization of the media containing carbohydrates causes a change in the color of the media that adversely affects the growth of bacteria. Nutrient agar with acidic pH becomes hydrolyzed under this condition. This method of sterilization is used for all ordinary media, buffers, fluid reagents, already used/discarded culture media, glassware, and other apparatus like bacterial filters and rubber caps.
- Importantly, moist heat sterilization by autoclaving cannot be used for sterilization of any thermolabile substances, such as solutions of vitamins and antibiotics, *etc*., which are typically sterilized by filtration (see below).

2.3.1.1.B.Radiation Sterilization

Sterilization can also be accomplished by diverse kinds of radiation, such as gamma rays and electrons. These radiations cause ionization and free radical production within microbial cells, which increases cellular toxicity, leading to their death. Exposure to far-range ultraviolet (UV) radiation carrying relatively higher energy causes excitation of the electrons present in various biomolecules, especially nucleic acids, contaminating microbial cells and causing massive DNA damage and eventual death. Industrial scale articles (articles of large size or numerous in number, *etc*.), which are otherwise inconvenient to sterilize in the dry state either by hot air oven or by autoclaving, are routinely subjected to radiation sterilization. Electromagnetic radiation (*e.g.*, gamma rays and UV light) and particulate radiation (*e.g.*, accelerated electrons) are used for sterilization from microbial contamination. For example, gigantic numbers of Petri plates, cell culture dishes, 96/6-well culture plates, and plasticware in the industry are routinely sterilized using Gamma irradiation. UV light, with its much lower energy and poor penetrability, is used for sterilizing air, the surface of aseptic work areas, and treatment of manufacturing-grade water [2, 4] (Table **1**).

2.3.1.1.C. Mechanical Sterilization

Filtration Sterilization

Application of Filtration for Sterilization of Liquids

In modern biological research, many substances are routinely used, which undergo thermal decomposition. For example, vitamins, growth factors, and antibiotics that need to be used under sterile conditions (as many of them are supplemented to microbial growth media for specialized applications) are extremely thermolabile. Consequently, owing to their heat sensitivity, they cannot be sterilized either by a dry or moist heating procedure, as mentioned above. These reagents are routinely sterilized by filtration.

Remarkably, unlike other procedures, in this procedure, microorganisms are not destroyed but are removed from these working solutions aimed to be sterilized. Filtration involves passing the liquid reagents to be sterilized through the sterile (achieved by prior autoclaving) filter device containing the filter membrane with appropriate pore size (typically 0.22 microns) under a positive pressure that is generated either using a syringe (for 1-10 ml quantity) or a vacuum pump (for 20-200 ml quantity). The sterile filtrate is collected in a sterile tube or larger container, depending on the volume of the filtrate. Filtration is capable of preventing the passage of both viable and non-viable particles of more than 0.22-micron diameter. This process is used for liquids and gases to check their clarification and sterilization [2, 4] (Table **1**). There are two types of filters used in filtration sterilization of the liquids.

- *Depth filters* are made of diatomaceous earth, unglazed porcelain filters, sintered glass, or asbestos packed in such a way as to form twisted channels of minute dimensions.
- *Membrane filters* are made of cellulose acetate, cellulose nitrate, polycarbonate, polyvinylidene fluoride, or some other synthetic material. Basically, they are porous membranes about 0.1 mm thick. By positive or negative pressure or by centrifugation, fluids are made to traverse membranes that selectively trap all the microbial cells present on the surface of the membrane while allowing the sterile filtrate to pass through and be collected in a sterile container placed downstream.

Application of Filtration for Sterilization of Gases

- *HEPA* (High-Efficiency Particulate Air) filters present in laminar flow hoods can remove particles that are greater than 0.3 microns in diameter. These filters can remove up to 99.97% of particles present in the air. To remove larger particles, air is first passed through pre-filters and then passed through HEPA

filters [4].

2.3.1.2. Chemical Method (Disinfection)

This method is employed principally for surface sterilization. Disinfection aims to eliminate microorganisms, which may cause contamination of glass wares, microbial growth media, and other important reagents, thereby spoiling important experiments. Disinfection uses various chemical agents like Lysol, phenol, chlorine, alcohol, and heavy metals and their compounds to kill microbes or inhibit their growth from inanimate objects or surfaces [4]. Killing or inhibiting the growth depends on the contaminating microorganisms and concentration of the disinfectants used. Disinfectants are applied to inanimate objects to prevent the growth of the organisms. Vegetative microbes and viruses are typically eliminated by the use of disinfectants. Note that bacterial endospores are not very easy to eliminate by this method. A common disinfectant in microbiological laboratories is ethanol, which is routinely applied on working benches to keep a check on microbial contamination. A side–by-side comparison between various methods of sterilization is appended below (Table **1**).

2.3.2. General Rules To Follow In A Microbiology Laboratory

The following rules must be followed to reduce microbial contamination from ubiquitous microflora.

- Always wear a laboratory coat or an apron while working in the laboratory. This will protect the clothing of the experimenter from contamination with undesired microbes.
- Carefully wipe out the laboratory bench tops with a disinfectant like lysol (1:500), phenol (1:100), spirit, or 90% ethanol before and after each experiment.
- Never move media, equipment, and especially bacterial cultures outside the laboratory.
- Do not place contaminated inoculating loops, needles, and pipettes on the bench tops.
- Loops and needles must be sterilized by incineration.
- Pipettes and cultures should be disposed of in designated receptacles following thorough autoclaving.
- All microbial cultures should be handled as potential pathogens.
- Wash hands with liquid detergent/soap before entering and before leaving the laboratory.
- In case of accidental spillage of the culture, promptly cover spilled cultures and broken culture tubes/flasks with filter/tissue paper. Saturate the place of spillage with disinfectant for at least 15 minutes, followed by thorough cleaning of the area.

- Never pipette any broth cultures or chemical reagents by mouth. Pipetting is to be carried out with the aid of a mechanical/electronic pipetting device.
- Aseptic techniques must be rigorously observed at all times.
- Familiarize yourself in advance with the exercise to be performed.

2.4. MICROBIAL CULTURING TECHNIQUES

One of the primary microbiology techniques that is used in a molecular biology laboratory is the routine culturing of microbes and their isolation. The main objective of handling the micro-organisms is to support the growth of the microorganism. Different microorganisms require different culture media, which supply their nutritional requirements. Here, we will mainly deal with the culturing of ***Escherichia coli***, an all-time favourite organism for molecular biologists. However, before getting deeper into the culturing techniques, it is imperative to introduce the different kinds of microbial growth media.

Microbial culture is a method that allows the multiplication of bacterial/microbial cells in or on a culture medium under controlled laboratory conditions [5]. The exact conditions required for optimal replication will depend on the target bacterial species. To be cultured successfully, bacteria/microbes require nutrients in the culture medium.

2.4.1. Microbial Growth Media

A culture medium is a solid or liquid preparation containing all the necessary nutrients required by microorganisms for their growth under controlled conditions. It is routinely used to grow, transport, and store microorganisms. Based on the nature of the physical state of the growth medium, all microbial culture media used in the laboratory can be classified into two main categories:

(i) Solid Culture Media

As the name suggests, apart from nutrients and salts, this culture media is composed of a solidifying agent (called agar) that forms a semi-solid gel-like matrix with a well-defined surface [2, 4]. Chemically, agar is composed of a complex polysaccharide from the red alga *Gelidium*. At room temperature, it remains in the solid state (typically powdered form), and when it is added to the liquid broth along with other components (see below), the broth appears as a thick and opaque suspension. However, when this agar-containing broth is subjected to sterilization by autoclaving, it undergoes liquefaction at the boiling temperature of water. Following sterilization, when the agar-containing medium is stored at room temperature, initially, the medium remains in a semi-liquid state and does not solidify until the medium cools to 42°C. Right before solidification, the semi-

liquid medium is poured onto the sterile petri plates, whose storage room temperature is set at 18-20 hours. Prolonged storage allows initial solidification followed by further drying of the semi-solid medium that is essential for manipulating the microbial cells on the agar surface using an inoculating loop, spreader, or sterile toothpicks. At this point, the solid medium is used for any microbial application. A solid medium is used mainly for culturing bacteria, bacteriophage, and fungi [4, 6].

(ii) Liquid Culture Media

Liquid culture medium always remains in a liquid state at room temperature and flows freely in the containers when tilted. This type of media is used to culture bacteria and other microorganisms. Its growth is monitored by the change in the media from clear to colloidal suspension. Liquid media are called *broths* [4, 6]. Based on the composition of the medium, two types of media are available for growing *E.coli* and other microorganisms:

2.4.1.A. Minimal media or minimal broth (first formulated by Julien Davis) is a cultural media with minimal necessities, as mentioned in Table **2**, generally without the presence of amino acids to support the growth of wild-type microorganisms (*e.g.*, wild-type *Escherichia coli*). Minimal media added with only a single selected agent (either an amino acid, vitamins, nucleic acid, sugar, or any other substance) is labeled as supplementary minimal media, which is used in culture-specific lines of auxotrophic mutants [1, 3]. Note that auxotrophic mutants are microorganisms that cannot synthesize one of the essential components, such as amino acids, nucleotides, vitamins, etc., due to a specific gene mutation leading to a metabolic defect that impairs their ability to make that specific substance. These mutants can only grow in a supplementary minimal media that contains a specific metabolite that the auxotrophic organism cannot make. A wild-type microorganism, *e.g.*, *E. coli,* can synthesize all essential metabolites from the simplest ingredients and, therefore, can survive and grow in a minimal medium.

PROTOCOL 2.2: PROCEDURE TO PREPARE MINIMAL MEDIUM

Minimal media is a defined medium with the composition varying on the microorganism being cultured. Typically, this medium contains a carbon source such as sugar/succinate, various inorganic salts (salts of essential elements like magnesium, nitrogen, phosphorous, and sulfur), and water. Typically, this kind of media contains a carbon compound such as glucose (which serves both as a carbon and energy source) and salts, which supply nitrogen, phosphorus, and trace metals. The pH of the minimal agar medium is about 7.0 ± 0.2 at 25ºC. Wild-type microorganisms can synthesize all other kinds of complex biomolecules from these simplest ingredients for their survival.

Materials

Dextrose

Ammonium sulphate

Dipotassium phosphate

Monopotassium phosphate

Sodium citrate

Magnesium sulphate

Agar

dH_2O

1 M HCl

1 N NaOH

Equipment

Glass beaker, conical flasks, cotton plugs, pH meter/paper, pipettes and tips, Petri dish, weighing balance and paper boats, magnetic stirrer, stirring bar, and autoclave.

Procedure

- Weigh the appropriate amount of each ingredient, as mentioned above, separately based on the final volume of the media to be prepared. Consult Table **2** below:
- Suspend the ingredients (except agar) in a glass beaker containing about 90/450/900 mL of dH_2O.

Table 2. Composition of the minimal medium.

Ingredients	For 100mL	For 500mL	For 1000ml
Dextrose	0.1 gm	0.5 gm	1 gm
Ammonium sulphate	0.1 gm	0.5 gm	1 gm
Dipotassium phosphate	0.7 gm	3.5 gm	7 gm
Monopotassium phosphate	0.2 gm	1 gm	2 gm
Sodium citrate	0.05 gm	0.25 gm	0.5 gm

(Table 2) cont.....

Ingredients	For 100mL	For 500mL	For 1000ml
Magnesium sulfate	0.01 gm	0.05 gm	0.1 gm
Agar (Optional)	1.5 gm	7.5 gm	15 gm
Distilled water	Up to 100 ml	Up to 500 ml	Up to 1000 ml

In addition to the above reagents, one needs 1M HCl and 1M NaOH to maintain the pH of the medium.

- Dissolve the components in the beaker using a magnetic stirrer (Mild heating may be applied to dissolve the medium completely).
- Adjust the pH of the medium to the desired value.
- Adjust the broth to a final volume of 100 mL/500 mL/1 L using dH_2O.
- Transfer the broth to a conical flask or aliquot into smaller volumes.
- Now add an appropriate amount of the agar (if a solid medium is to be prepared) based on the final volume of the media (*i.e.*, 15 gm agar for 1L of the media).
- Close the mouth of the flask with a cotton plug. Seal it further with a piece of square paper/aluminum foil and a rubber band.
- Autoclave for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.
- Mix well and pour into sterile Petri plates.

Storage

Prepared broth media can be stored at 2-8°C for about 6 months to 2 years and when the products are not allowed to freeze.

Caution

Opening and closing of media should be done in aseptic conditions only.

Adding agar before adjusting pH might result in mild hydrolysis.

2.4.1.B. A rich medium is defined as a microbial growth medium that provides the cells with plenty of amino acids, carbohydrates, nucleotide precursors, vitamins, and other metabolites, as mentioned in Table **3**. Both the wild-type and metabolically defective *E. coli* and other microorganisms grow more rapidly in this medium. There are various different kinds of rich media compositions, and recipes are available. We will discuss the most commonly used composition.

Table 3. Composition of the LB medium.

Reagent	For 100 mL	For 500 mL	For 1000 mL
Agar	1.5 g	7.5 g	15 g

(Table 3) cont.....

Reagent	For 100 mL	For 500 mL	For 1000 mL
NaCl	1 g	5 g	10 g
Tryptone	1 g	5 g	10 g
Yeast extract	0.5 g	2.5 g	5 g

2.4.1.B.1. Luria-Bertani Medium

This is the most widely used rich medium in most of the applications in molecular biology protocols. Giuseppe Bertani formulated LB broth in 1951 in an attempt to optimize *Shigella* growth and plaque formation. Although LB media is known as "Luria Broth", "Luria-Bertani" medium, or "Lennox Broth", it was initially named "Lysogeny Broth". LB broth's agar form should be designated LA but is now referred to as LBA/LA. There are many formulations available for LB medium. In this book, we provide the most used formula (based on CSH protocols) [2, 5, 6].

Applications

LB is a rich medium used as a general-purpose bacterial culture medium, especially for Enterobacteriaceae members (*E. coli* is one of them). Lysogeny broth is also used for coliphage plaque assays. Cold Spring Harbour Protocol recommends pH to be 7.0. However, depending on the applications, the pH of this medium can vary between 7.0 and 8.0. The pH can be adjusted either with 1N NaOH or 1N HCl as needed.

PROTOCOL 2.3: PROCEDURE TO PREPARE LB MEDIUM

Materials

Agar

NaCl

Tryptone

Yeast Extract

1 M HCl

1 N NaOH

Equipment

Glass beaker, conical flasks, cotton plugs, pH meter/paper, pipettes and tips, Petri dish, weighing balance and paper boats, magnetic stirrer, stirring bar, and autoclave.

Procedure

- Determine the desired volume of the medium to be prepared and weigh out the appropriate amounts of different reagents according to Table **3**.
- Add the reagents to a glass beaker and make up the volume to 90% of that planned with dH2O (*e.g.*, 900 mL if you have planned for 1L broth). DO NOT add agar (if preparing LB medium with agar) at this point.
- Dissolve the components in the beaker using a magnetic stirrer.
- Adjust the pH to the desired value (see the discussion about the pH above for details).
- Adjust the broth to the final volume using dH_2O.
- Transfer the broth to a conical flask or aliquot into smaller volumes.
- Add agar if planned to make LB medium with agar. Adding agar after distributing into smaller volumes will ensure equal distribution.
- Close the mouth of the flask with a cotton plug. Seal it further with a piece of square paper/aluminum foil and a rubber band.
- Autoclave for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.

Storage

Store broth at room temperature.

Caution

Opening and closing of media should be done in aseptic conditions only.

Adding agar before adjusting pH might result in mild hydrolysis.

2.4.1.B.2. 2XYT Medium

2xYT is a nutritionally rich liquid microbial growth medium used for the propagation of recombinant strains of *Escherichia coli* and M13 bacteriophage or other filamentous single-stranded DNA bacteriophages [6].

Applications

This medium contains a mainly enzymatic digest of casein and yeast extract. Casein enzymatic hydrolysate acts as a source of nitrogenous compounds like amino acids and peptides. Yeast extract supplies B-complex vitamins and

minerals. Sodium chloride maintains the isotonic condition of the medium. (Table **4**). These components provide the necessary growth factors for rapid growth and replication of bacteriophage (without weakening the host cells) and *Escherichia coli* (allowing the bacteria to recover from the stress of transformation). The pH of 2X YT medium is about pH 6.8 ± 0.2 at 25°C. pH can be adjusted using either 1N NaOH or 1N HCl.

Table 4. Composition of the 2X YT Medium.

Ingredients	For 100 mL	For 500 mL	For 1000 ml
Tryptone	1.6 gm	8 gm	16 gm
Yeast extract	1 gm	5 gm	10 gm
NaCl	0.5 gm	2.5gm	5 gm
Distilled water	Upto 100 ml	Upto 500 ml	Upto 1000 ml

PROTOCOL 2.4: PROCEDURE TO PREPARE 2X YT MEDIUM

Materials

Tryptone

Yeast Extract

NaCl

dH_2O

1 M HCl

1 N NaOH

Equipment

Glass beaker, conical flasks, cotton plugs, pH meter/paper, pipettes and tips, Petri dish, weighing balance and paper boats, magnetic stirrer, stirring bar, and autoclave.

Procedure

- Determine the desired volume of the medium to be prepared and weigh out the appropriate amounts of different reagents according to Table **4**.
- Suspend the ingredients in a glass beaker containing about 90/450/900 mL of distilled water.

- Dissolve the components in the beaker using a magnetic stirrer. (Heat may be applied to dissolve the medium completely).
- Adjust the pH of the medium to the desired value.
- Transfer the broth to a conical flask or aliquot into smaller volumes.
- Close the mouth of the flask with a cotton plug. Seal it further with a piece of square paper and a rubber band.
- Autoclave for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.
- Mix well and pour into sterile Petri plates.
- Allow the medium to cool before adding, such as antibiotics (if needed).
- Alternatively, the commercially available 2x YT agar media powders can be used. Weigh the mixture of content as prescribed by the manufacturer.

Storage

Prepared Broth Media should be Stored at 2-8°C. Refrigerate if required; do not allow the products to freeze.

Caution

Many supplements, like antibiotics to the medium, are heat-sensitive. These ingredients, therefore, cannot be autoclaved. As mentioned above, if antibiotics are to be added to the medium, they are filter-sterilized prior to use and then added to the medium accordingly when the medium is either cold (for liquid medium) or when the temperature of the medium is around 42°C-50°C (for agar containing medium).

2.4.1.B.3. Terrific Broth

Terrific broth, developed by Tartoff and Hobbs, is a buffered and enriched medium used to cultivate recombinant strains of *E. coli*. Terrific broth (TB) contains more tryptone and yeast extract than the standard LB medium (refer to Table **5**), which is used for almost all applications involving liquid bacterial culturing steps such as protein expression and cloning.

Table 5. Composition of the terrific broth.

Ingredients	Molarity	For 100mL	For 500mL	For 1000mL
Tryptone	-	1.2 gm	6 gm	12 gm
Yeast extract	-	2.4 gm	12 gm	24 gm
K_2HPO_4	72 mM (174.18 MW)	1.25 gm	6.25 gm	12.5 gm
KH_2PO_4	17 mM (136.09 MW)	0.23 gm	1.15 gm	2.3 gm
Glycerol	-	0.4 ml	2 ml	4 ml

(Table 5) cont.....

Ingredients	Molarity	For 100mL	For 500mL	For 1000mL
Agar	-	1.5 gm	7.5 gm	15 gm
Distilled water	-	Up to 100 ml	Up to 500 ml	Up to 1000 ml

The terrific broth is a nutrient-rich growth medium that supports the growth of *E.coli* until it reaches very high cell density. It maintains growth in the logarithmic phase, resulting in larger yields of plasmid DNA and heterologous proteins. Tryptone and yeast extract act as the sources of carbon, nitrogen, vitamins, and amino acids required for the growth of bacteria. The potassium phosphate buffer system acts as the source of potassium, which also prevents cell death and eventually prevents the drop in the pH of the culture media. Glycerol is added to the medium instead of glucose as a carbon source. Glycerol is not reduced to acetic acid. Glucose acts as a repressor in various protein expression systems, whereas glycerol promotes growth [5, 6].

Applications

It gives a higher plasmid yield per volume compared with LB media. TB can obtain higher yields of recombinant protein expression in *E. coli*. However, some proteins require slow *E. coli* growth for proper folding. For such cases, it would be better to use M9 media. The pH of the medium should be adjusted to 7.2 ± 0.2 at 25°C either with 1N HCl or 1N NaOH.

PROTOCOL 5: PROCEDURE TO PREPARE TB MEDIUM

Materials

Tryptone

Yeast Extract

K_2HPO_4

KH_2PO_4

dH_2O

1 M HCl

1 N NaOH

Equipment

Glass beaker, conical flasks, cotton plugs, pH meter/paper, pipettes and tips, Petri

dish, weighing balance and paper boats, magnetic stirrer, stirring bar, and autoclave.

Procedure

- Determine the desired volume of the medium to be prepared and weigh out the appropriate amounts of different reagents according to Table **5**.
- For 1 liter of medium, about 50.8 gm of dehydrated media is added to 900 mL of distilled water.
- Add 4 mL of glycerol and mix well, and the final volume is adjusted to 1000 ml. Boil for 1 minute and dissolve the contents properly with regular agitation. If all ingredients are adequately dissolved, you can skip this step.
- Adjust the pH to 7.2.
- Dispense the desired volume in proper containers.
- Add agar powder (1-1.5%) if planned to make solid media.
- Sterilize them by autoclaving at 121°C for 15 to 20 minutes.
- Add appropriate antibiotics to the media before solidifying to allow the selection of transformants.

Storage

It is hygroscopic in nature and moisture-sensitive. After preparation, the medium is yellowish-brown in color and is stored at 2-8°C in the refrigerator. It can be stored for about four weeks.

2.4.1.B.4. SOC Broth

SOC (super optimal broth with catabolite repression) is a variant of SOB media. SOC is a nutrient-rich culture media developed by Douglas Hanahan in 1983. SOC is typically used for bacterial transformation. Since it is a nutrient-rich medium, it helps bacteria to recover faster after heat shock or electroporation [5, 6] (Table **6**).

Table 6. Composition of the SOC Broth.

Reagent	MW.	Working Concentration	Composition for 1L
Tryptone	NA	2%	20 g
Yeast extract	NA	0.50%	5 g
NaCl	58.44	10 mM	0.5 g
KCl	74.55	2.5 mM	0.18 g

Applications

SOC medium is optimized for growing transformed *E. coli*. The SOC medium pH is adjusted to 6.8 - 7.0 with either 1N NaOH or 1N HCl. Approximately 1-2 mL of 1N NaOH is required for adjusting 1L of the medium.

PROTOCOL 6: PROCEDURE TO PREPARE SOC MEDIUM

Materials

Tryptone

Yeast Extracts

NaCl

KCl

1 M HCl

1M NaOH

Glucose

Equipment

Glass beaker, conical flasks, cotton plugs, pH meter/paper, pipettes and tips, Petri dish, weighing balance and paper boats, magnetic stirrer, stirring bar, and autoclave.

Procedure

- Prepare stock solutions of 1M Glucose and 2M $MgCl_2$ separately following the recipe given in the Appendix (see ***SECTION A1***) and filter sterilize them by using a 0.22-micron membrane filter under a laminar airflow chamber. Store them at 4°C for long-term use.
- Determine the volume to be made and weigh the remaining reagents required for the preparation of SOC, except $MgCl_2$ and glucose. Table **6** indicates the required mass to be weighed.
- Add the reagents to a glass beaker and add de-ionized water increase the volume to 90% of the planned volume. For *e.g.*, if you plan to make 1 L of the media, add 900 mL of water. The remaining volume is adjusted after the adjustment of pH and other components (if any).
  Note: While making up the final volume, reserve 2.5% of the total volume for adding 1M glucose and 2M $MgCl_2$ at the end.
- Dissolve the components in the beaker using a magnetic stirrer.
- Adjust the pH to the desired value (see the pH section above for details).

- Adjust the broth to the final volume using de-ionized water.
- Transfer the broth to a conical flask or aliquot into smaller volumes.
- Close the mouth of the flask with a cotton plug. Seal it further with a square piece of paper and a rubber band.
- Autoclave for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.
- After autoclaving, cool down the medium to room temperature under a laminar airflow chamber.
- 11 Add the required volume of 2M $MgCl_2$ and 1M glucose (for example, for making 1 L medium, add 5 mL of 2M $MgCl_2$ and 25 mL of 1M glucose) stock solutions right before use and mix thoroughly before use.

Preparation of SOC Agar media

If you plan to prepare solid media, add agar (1.5%; *i.e.*, 3.75 g/250 mL media) after step no. 5 in the above procedure. You can add $MgCl_2$ and glucose just before the medium gets solidified (~60°C). If the medium is already solidified, bring it to 60°C using a water bath to add components.

Storage

SOC medium can be stored at room temperature and is stable for several months.

Caution

Do not add agar at this point if you are preparing a solid medium.

Before use, be sure to add the proper amounts of sterile glucose (20 mM final) and magnesium chloride (10 mM final).

2.4.2. Inoculation

This technique involves the introduction of the microorganism into a culture medium in an aseptic condition using a device called an inoculation loop. This loop consists of a platinum or nichrome wire, one end of which is coiled to form a loop. The other end of the wire is inserted into a handle for holding the device. The loop portion of the device is used to transfer a finite amount of microbial cells from one stock medium to a working medium. Before touching microbial cells, the loop is heated to red-hot condition thoroughly to sterilize it. This hot loop is then left at room temperature for a few minutes to sufficiently cool down. The sterile loop is then used to transfer a finite amount of cells from stock culture (called the **inoculum)** into a culture medium. This process is called **inoculation**, which is followed by incubation of the inoculated culture at an appropriate temperature (mostly at 37°C) with vigorous shaking in a shaking incubator to allow the growth of the inoculated microorganisms.

2.4.3. Isolation

Typically, for all the genetic and molecular biological applications, isolation of a pure culture is a prerogative step. Despite the exercise of all the precautions in the laboratory, a microbial culture may become contaminated with other unwanted microbes. Consequently, sometimes the microbial culture becomes impure or mixed (containing more than one type of microbes), especially upon storage for a long period. Purification of a particular desired microbial strain from a mixture of other undesired microbes involves **isolation** from a mixed culture [1]. This is done by various processes. One such process involves culturing the microbes on a selective culture media, which allows the selective growth of only the desired bacterial strain. A pure culture of the microbial strain is achieved after repeating the same purification procedure several times to eliminate all possible contamination by other microbes. Culture plates with discrete/isolated individual colonies are a signature of pure culture.

2.4.3.1. Common Isolation Techniques

The most common isolation methods to isolate microbes from mixed cultures are as follows:

- Streak Plate method.
- Spread Plate method.

Streak Plate Method

This is the most commonly used isolation method. To prepare a pure culture of a desired bacterial strain or to obtain individual bacterial colonies from a concentrated suspension culture, an inoculation loop is first dipped into the liquid culture suspension, followed by streaking onto the plane surface of the solid culture medium in a plate (Fig. **2**). The plate that is streaked is known as the streak plate [4 - 6].

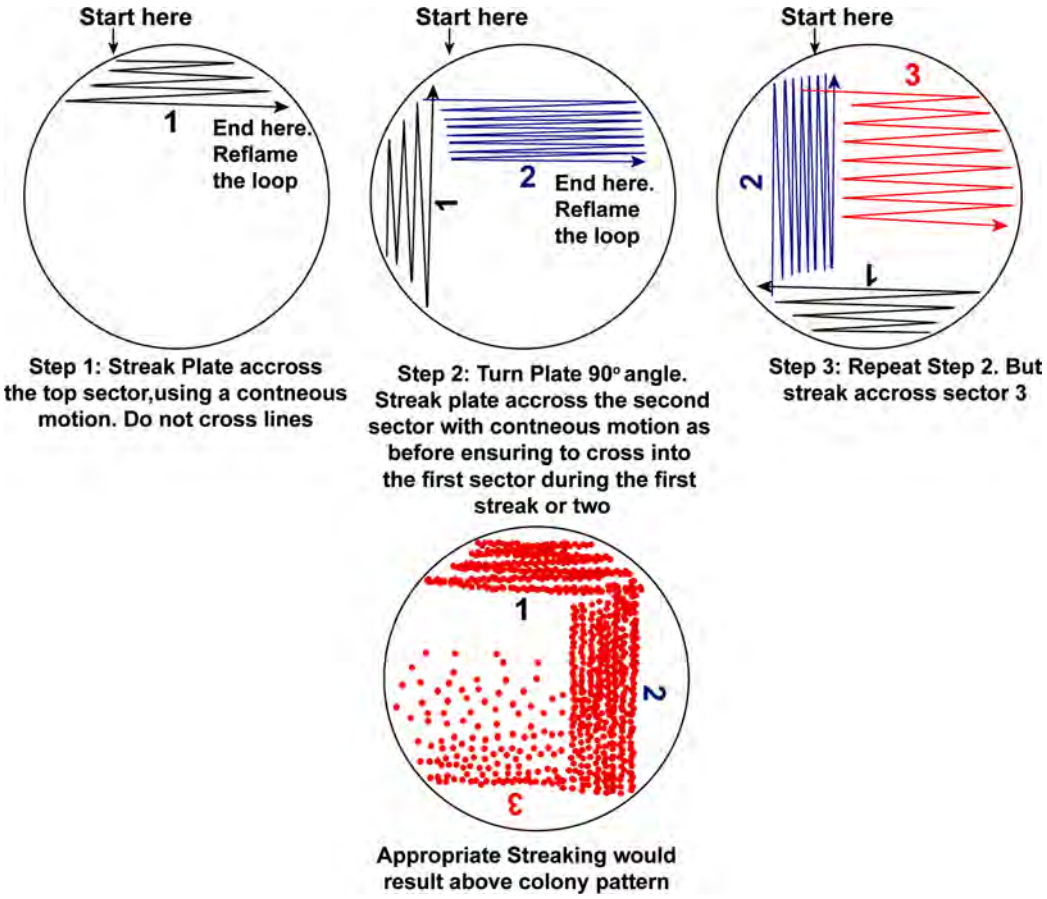

Fig. (2). An illustration of the streak plate technique.

PROTOCOL 2.7. PROCEDURE FOR STREAKING CULTURE OF *ESCHERICHIA COLI* ON SOLID MEDIA TO ACHIEVE SINGLE COLONIES

Materials

Overnight culture of *E. coli* in liquid LB broth or nutrient broth.

Nutrient agar medium in Petri plate (solid medium).

Equipment

Inoculation loop, Bunsen burner, laminar flow hood, incubator.

Procedure

- Pour a sterile nutrient agar medium into a sterile petri dish.
- Stand the poured plates at room temperature overnight to allow the medium to solidify properly.
- A sterile inoculation loop (by flaming, as mentioned above) is dipped into a bacterial culture grown overnight and then transferred onto the solid medium in the form of lines, as shown in Fig. (**2**). These lines are referred to as streaks.
- Streaks are made back and forth across the agar surface until about one-third of the diameter of the plate has been covered.
- Then, re-sterilize the loop by flaming it and allow it to stand at room temperature for a few minutes to cool down.
- Touch one end of the first streak with the sterile loop and then streak for the second time at right angles to the first streak (Fig. **2**). This causes the bacteria to be dragged out from the first streak and effectively undergo dilution.
- Flame the loop and streak again for the third time at the right angles to the second streak and parallel to the first.
- Cover the plate with the lid and incubate the streaked plate upside down in the incubator at 37°C for 16-18 hours in an incubator.

Spread Plate Method

The spread plate technique is the method of isolation and enumeration of microorganisms in a mixed culture and distributing it evenly. This method is extremely effective in obtaining individual colonies of the microorganisms from a mixture of more than one microbe. This technique involves the uniform spreading of a small amount of bacterial culture suspended in a solution over an agar plate using a sterilized L-shaped glass rod (called spreader). The plate needs to be dry and at room temperature so that the agar can absorb the bacteria more readily. A successful spread plate will have a countable number of isolated bacterial colonies evenly distributed on the plate [3, 5, 6] (Fig. **3**).

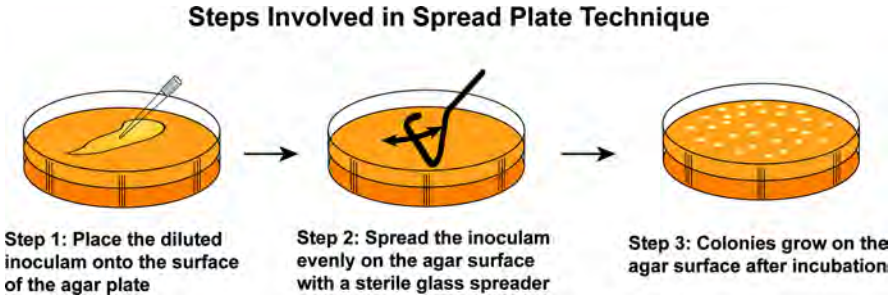

Fig. (3). An illustration of the spread plate technique.

PROTOCOL 2.8. PROCEDURE FOR SPREADING THE CULTURE OF *ESCHERICHIA COLI* ON SOLID MEDIA TO ACHIEVE SINGLE COLONIES.

Materials

Overnight culture of *E. coli* in liquid LB broth or nutrient broth.

Nutrient agar medium in Petri plate (solid medium).

A series of 5 mL liquid LB medium in a 10 mL culture tube.

Equipment

Glass spreader, Bunsen burner, micropipettes, microtips, laminar flow hood, incubator.

Procedure

This method begins with an overnight culture of the microorganism as the starting material.

- Prepare an appropriate dilution of 10^{-6} or 10^{-7} from the overnight culture *via* serial dilution method as described in ***UNIT 2.4.4.2A*** below:
- Pipette out 0.1-0.2 ml aliquot from the appropriate desired dilution culture (10^{-6} or 10^{-7}) onto the center of the agar plate.
- Dip the L-shaped glass spreader into alcohol.
- Flame the glass spreader over a Bunsen burner.
- Allow the spreader to stand at room temperature for a few minutes for proper cooling.
- Spread the liquid culture aliquot evenly over the surface of the agar using the sterile glass spreader, carefully rotating the Petri dish underneath at the same time.
- Incubate the plate at 37°C for 24 hours upside down for the colonies to appear.
- Now calculate the CFU value of the sample. Once you count the colonies, multiply by the appropriate dilution factor to determine the number of CFU/mL in the original sample.

Culturing of *Escherichia coli*

Escherichia coli is a rod-shaped gram-negative bacterium that harbors a circular chromosome about 3 million base pairs (bp) long. Its ability to grow on minimal medium and rich medium, ease of genetic manipulations, and easy handling and

maintenance have made it a perfect choice to use this creature as one of the user-friendly favorite model organisms in molecular biology procedures.

2.4.4.1. Growth on Liquid Media

2.4.4.1. A. Growing an Overnight Culture

Overnight cultures (ON) are small and freshly saturated cultures of *E. coli.* The main requirements for growing overnight cultures involve a plate containing freshly grown bacterial colonies and a culture tube containing sterile minimal or LB/YT/SOC media [1, 6].

PROTOCOL 2.9: PROCEDURE TO GROW AN OVERNIGHT CULTURE OF *E. COLI*

Materials

Freshly grown bacterial (*E. coli*) colonies on a LB plate or LB slant.

A culture tube containing sterile LB/YT/SOC media.

Equipment

One inoculation loop, a sterile pipette and tips, Bunsen burner, laminar flow hood, and incubator.

Procedure

- Turn on the laminar flow hood and the UV lamp under closed conditions for 10-15 minutes.
- Turn off the UV light and turn on the visible fluorescent lamp.
- Sterilize the working area by wiping the countertop of the Laminar flow hood with an ethanol-moist paper towel.
- Sterilize your hand by thoroughly rubbing both your hands with ethanol.
- Take a LB/YT plate containing freshly grown bacterial culture with well-isolated single colonies. This plate should be prepared a day in advance either by streak-plate or spread plate methods as described above.
- Turn on the Bunsen burner.
- Hold the inoculation loop with your right hand between your thumb and first forefinger like a pen. Sterilize the loop by flaming it and holding it at room temperature for cooling down.
- Hold the overnight grown LB plate with a single colony with your left hand. Carefully pick bacterial cells from a well-isolated single colony. Care should be

taken while transferring to minimize skin contact and also with contaminated air.
- Put the plate back and hold the culture tube with your left hand.
- Remove the cotton plug of the culture tube using the rest of the fingers or palm of your right hand (remember you are still holding the inoculation loop with your right hand using your thumb and first forefinger).
- Flame the lip of the culture tube with your left hand.
- Dip the loop with bacterial cells in the sterile liquid LB/YT/SOC media and shake/agitate the loop while it is still dipped in the medium for complete transfer of the cells from the loop.
- Remove the loop, flame the lip of the culture tube, and put the cotton plug back into the mouth of the culture tube.
- Disperse the transferred cell mound within the inoculated culture by shaking the tube gently.
- Place the inoculated medium in the incubator shaker with gentle shaking at 60 rpm at 37°C. It usually takes 6-8 hours to reach saturated condition with a density of 1–2 x 10^9 cells/ml.

Useful Tips: It is always advisable to flame the lip of the tube while opening and closing the cap.

2.4.4.1.1B. Growing Larger Cultures

Sometimes, it is necessary to grow *E. coli* cells in much larger volumes for preparing plasmid or genomic DNA, total cellular RNA, or a recombinant protein on a much larger scale. Overnight cultures (ON) typically serve as starting materials or inoculum for growing larger cultures of *E. coli*.

PROTOCOL 2.10: PROCEDURE TO GROW LARGE CULTURE OF *E. COLI*

Materials

Freshly grown overnight cultures of *E. coli* in LB/YT/SOC plate or LB slant.

A culture flask containing sterile 25-500 mL of LB/YT/SOC media.

Equipment

A sterile pipette and tips, Bunsen burner, laminar flow hood, and shaking incubator.

Procedure

- Turn on the laminar flow hood and the UV lamp under closed conditions for 10-15 minutes.
- Turn off the UV light and turn on the visible fluorescent lamp.
- Sterilize the working area by wiping the countertop of the Laminar flow hood with an ethanol-moist paper towel.
- Sterilize your hand by thoroughly rubbing both your hands with ethanol.
- Take a LB/YT plate containing freshly grown bacterial culture with well-isolated single colonies. This plate should be prepared a day in advance either by streak-plate or spread plate methods as described above.
- Turn on the Bunsen burner.
- Take the overnight grown culture. Hold the culture tube with your left hand and remove the cotton plug with your right hand.
- Thoroughly flame the lip of the tube.
- Using an automated pipette and tip, remove an appropriate volume of overnight inoculum.
- Put the culture tube back and take the culture flask with the sterile medium with your left hand. Remove its cap/cotton plug and flame the lip of the flask thoroughly.
  (Note: the volume of inoculum that is withdrawn from overnight culture should be $1/100^{th}$ of the volume of the larger culture. For example, use 1 mL of the overnight culture as inoculum for 100 mL of the media in the larger culture).
- Transfer the withdrawn inoculum to the larger volume of sterile medium in the flask.
- Flame the lip of the flask thoroughly and put the cotton plug/cap back. Gently shake the flask to disperse the inoculated cells uniformly to the medium.
- Transfer the inoculated flask to an appropriately sized slot in the incubator shaker and grow the culture at 37°C with vigorous agitation (~300 rpm) to ensure proper aeration.

Useful Tips: In the case of temperature sensitive strains, the culture should be grown either at 25°C or 30°C.

To ensure proper aeration during the growth, an Erlenmeyer flask or baffled flask whose volume is at least 5 times more than the liquid culture should be used.

2.4.4.2. Growth on Solid Media

In multiple types of experimental procedures (*e.g.*, isolation of a pure bacterial strain, isolation and purification of a desired recombinant clone), it is necessary to isolate an individual bacterial cell physically from the other surrounding cells of either the same or different species. While bacterial cells grow in the liquid

medium in a flask under aerated shaking conditions, they are subject to continuous Brownian motion and, therefore, undergo continuous mixing. Thus, it is impossible to isolate a single bacterial cell from the others from a mixture of bacterial cells grown in a liquid culture. In contrast, while numerous bacterial cells are spread on the surface of a solid medium, they do not undergo any Brownian motion, and each cell tends to stick to the solid surface at a particular point. In other words, the bacterial cell, during its growth on the surface of a solid medium, does not move around and grows under restricted movement. This unique feature of the growth of bacterial cells on the liquid versus solid medium is the key to designing the strategy of their isolation and purification scheme by growing them on a solid medium. Another important consequence of the growth of bacterial cells on the solid medium is colony formation. A colony is a mound of cells (containing several million cells) resulting from the restricted growth of a single cell spotted at a fixed location on the agar surface during the streaking/spreading procedure. Thus, theoretically speaking, if one hundred different bacterial cells (either the same or different species) are spotted at various isolated locations on the surface of a solid medium, these cells will produce one hundred separated colonies following their growth. Thus, growth on the solid medium is an important procedure to isolate single and pure clones of a given bacterial strain. Below, we discuss various experimental procedures associated with the growth of bacteria on a solid medium.

2.4.4.2A. Tittering and Isolating Bacterial Colonies by Serial Dilutions

Each cell in a population is descended from its progenitor (founder) cell, and hence, growing bacterial cells from a single colony (which are genetically identical) into a liquid medium ensures that each cell in the culture has the same genetic makeup. Thus, before carrying out a liquid culture of genetically identical bacterial cells, it is obligatory to generate freshly grown bacterial cells grown on a solid medium in the form of well-isolated colonies. To generate such plate from a liquid culture, serial dilutions are typically done to titer that culture (Fig. **4**). The main goal of this procedure is to reduce the concentration of growing cells in a culture (usually ranging from 10^7 to 10^9 cells/mL) to three to four orders of magnitude so that the concentration of the resulting suspension becomes 10^3 to 10^4 cells/mL. Plating of a small aliquot of this diluted cell suspension usually leads to the formation of about several hundred colonies on one plate, which can be counted easily and reliably [6]. A detailed protocol for carrying out serial dilution is provided below:

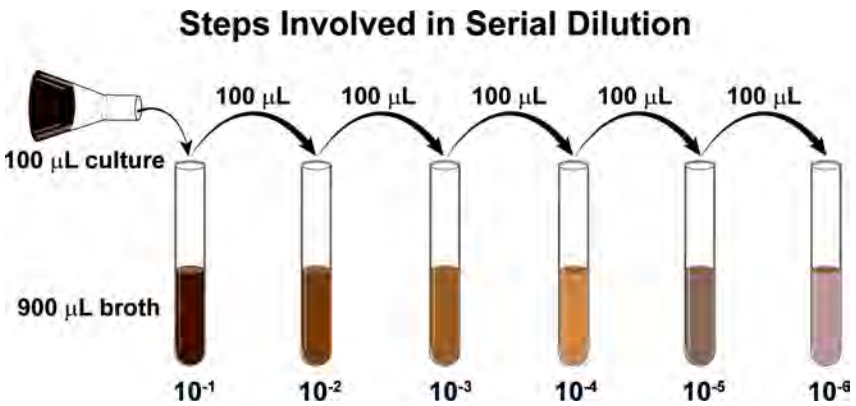

Fig. (4). Schematic diagram showing the steps involved in serial dilutions.

PROTOCOL 2.11: PROCEDURE TO CARRY OUT SERIAL DILUTIONS AND PLATING OF AN OVERNIGHT *E. COLI* LIQUID CULTURE

Materials

Freshly grown overnight liquid cultures of *E. coli* in LB/YT/SOC medium.

10 to 15 sterile plastic or glass tubes for dilution.

Sterile water or LB medium as diluent.

LB agar plates.

Equipment

A sterile pipette, microtips, laminar flow hood, glass spreader, Bunsen burner, and incubator.

Procedure

- Take five sterile tubes and label them as -1, -2, -3, -4, -5, and -6.
- Dispense 900 µL sterile water or LB medium in each of them.
- Pipette out 100 µL of freshly grown bacterial culture and add this culture to the 900 µL water/LB medium marked 10^{-1}. Mix thoroughly by vortexing. This is ten times (10^{-1} times) dilution of the original culture (Fig. **4**).
- Pipette out 100 µL of ten times (10^{-1} times) diluted culture and add this culture to the 900 µL water/LB medium marked 10^{-2}. Mix thoroughly by vortexing. This is one hundred times (10^{-2} times) dilution of the original culture.
- Pipette out 100 µL of hundred times (10^{-2} times) diluted culture and add this culture to the 900 µL water/LB medium marked 10^{-3}. Mix thoroughly by

vortexing. This is one thousand times (10^{-2} times) dilution of the original culture (Fig. **4**).

- Repeat this dilution procedure two more times to achieve 10^{-4}, 10^{-5}, and 10^{-6} times dilution in tubes marked 10^{-4}, 10^{-5}, and 10^{-6}, respectively. Mix the diluted culture thoroughly by vortexing each time.
- Pipette out100 µL of liquid from each of the dilution tubes and spread them on a solid medium in individual petri plates marked 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} (see ***PROTOCOL 2.7*** above for details).
- Incubate the plates at 37°C without shaking overnight or until well-separated single colonies appear on the dilution plates. The number of viable bacteria in the original culture is calculated from the number of colonies formed on the dilution plates using the following formula.

$$\text{Cell concentration in original culture} = \frac{\text{No. of colonies (CFU) on an agar plate}}{\text{Total dilution Factor X volume plated (in mL)}}$$

2.4.4.2B. Replica Plating

Replica plating is a technique that is used to test the ability of the growth of one or more microbial strains (such as a series of bacterial mutant cells) on different nutritional conditions (Fig. **5**). In this technique, the original plate containing test bacterial colonies is referred to as the master plate [6].

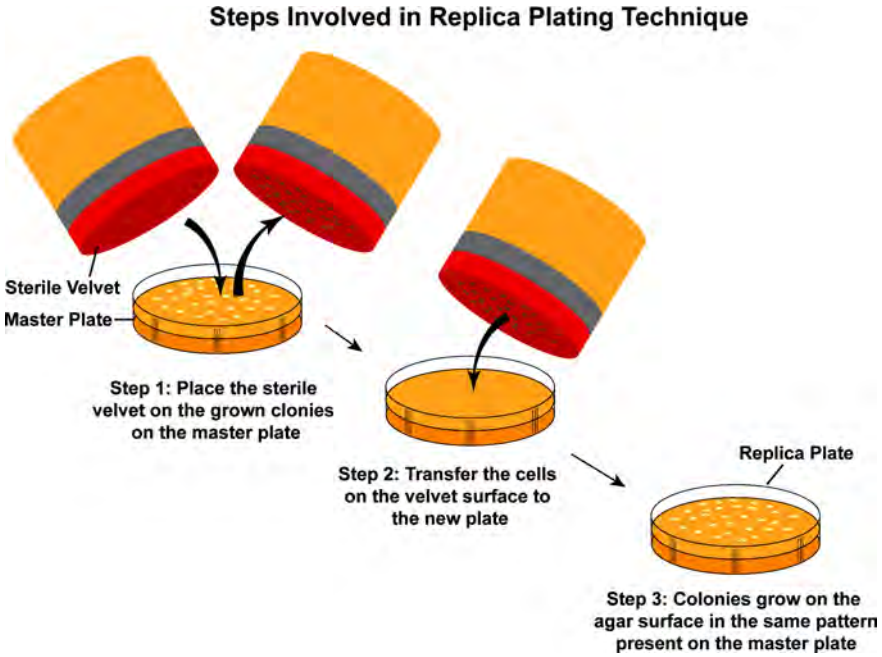

Fig. (5). Schematic diagram showing the steps involved in replica plating.

PROTOCOL 2.12: PROCEDURE TO CARRY OUT REPLICA PLATING OF *E. COLI* COLONIES GROWN ON LB AGAR

Materials

Freshly grown colonies of *E. coli* in LB agar plate.

Equipment

Replica block, sterile velvet, metal ring, and incubator.

Procedure

- Cut a few squares of velvet with enough surface area to cover the base of the wooden block. Typically, a diameter of 14 cm is suggested.
- Prepare the replica block by securing the sterile velvet to the block with the help of a metal ring.
- Lay the master plate on the fitted velvet on the replica block in such a way that the colonies touch the velvet and eventually are transferred on the velvet surface, as shown in Fig. (**5**).
- Move the master plate and rest a new plate on the velvet so that all the colonies from the velvet surface are again transferred to this new plate (replica plate), as shown in Fig. (**5**).
- Incubate the replica plate at 37°C without shaking overnight or until well-separated single colonies appear on the dilution plates.

Note:

- It is critical to mark the orientation of both the master and replica plates and place them in the same orientations on the velvet to maintain their relative coordinates while performing steps 3 and 4. In this way, the original pattern of colonies on the master plate can be maintained on the replica plate, and the colonies on the two plates can be compared.
- The velvets can be washed, autoclaved, and reused.
- If velvets are not available, pieces of sterile filter paper or disposable replica plates can be used ("Repli-Plate" Colony Transfer Pads, American Laboratories #59901).
- The replica block is a wooden or metal cylinder that fits snugly inside a petri plate (see Fig. **5**).
- Alternatively, a master plate is prepared using toothpicks and the grid, which is composed of well-separated colonies. In this case, the master plate is a fresh plate onto which 50 to 100 colonies have been gridded. With the help of the rep-

lica block, the colonies are transferred from one to another. This technique has many applications to recombinant DNA work (see Fig. **2** in ***CHAPTER 7***)

2.4.5. Monitoring the Growth: Bacteria Enumeration

Counting or enumeration of bacterial cells at various phases of their growth in a liquid broth is an important technique, and a systematic analysis of bacterial cell count as a function of time which portrays its rate of growth. This plot is called a growth curve (see below), and a plot of cell number *vs.* time signifies various phases of growth [2, 4 - 6]. Exact knowledge of various phases and their time length is important for many procedures which are routinely used in molecular cloning procedure. For example, the successful introduction of the plasmid or ligated DNA into a bacterial host, which is an essential part of molecular cloning, requires a good knowledge of the specific part of the growth phase of the host bacterial cell at which the DNA must be introduced. Below, two different protocols to enumerate bacterial cells while they are growing in a liquid broth are provided, followed by a brief procedure to carry out a bacterial growth curve.

2.4.5A. Enumeration with a Count Slide

The equipment utilized to count the number of cells in a culture is known as a count slide (also called a hemocytometer) (Fig. **6**). This procedure uses a clean count slide on which a clean cover slip is placed [6].

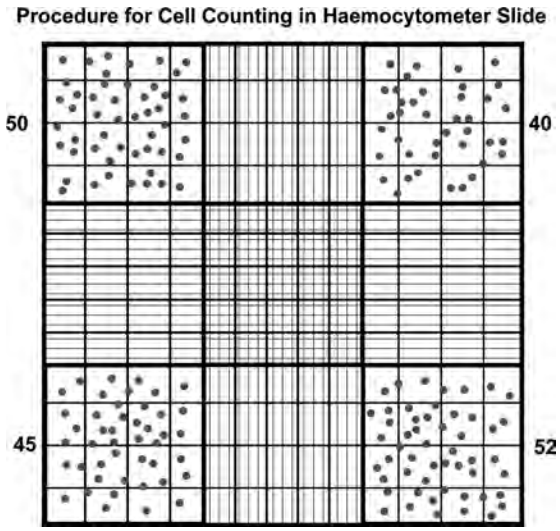

Fig. (6). Schematic diagram showing the organization of the four corner chambers containing 16 squares each and the center chamber having 25 squares. The grey dots represent the distribution of the cells in each of the sixteen squares for illustration purposes.

PROTOCOL 2.13: PROCEDURE TO DETERMINE TOTAL (VIABLE AND DEAD) CELL CONCENTRATIONS OF *E. COLI* CELLS GROWING IN LB BROTH

Materials

Freshly growing liquid broth culture of *E. coli* in LB/YT/SOC broth (in tube or flask).

Equipment

Glass hemocytometer slide, coverslip, ethanol, sterile pipettes and tips, sterile culture tubes, laminar flow hood, phase contrast microscope.

Procedure

- Clean the glass hemocytometer and coverslip with alcohol before use.
- Moisten the coverslip with water and affix it to the hemocytometer.
- Gently swirl the tube/flask to ensure the cells are evenly distributed and the culture is homogenized.
- Withdraw 0.1 mL of broth using a sterile micropipette/tip and place in a culture tube containing 0.9 mL sterile water to achieve 10^{-1} dilution. Mix gently.
- Repeat step 4 twice to achieve 10^{-2} and 10^{-3} dilutions.
- Using a micropipette, take 100 µL of cell suspension from every dilution and apply it to the hemocytometer. Fill both chambers very gently underneath the coverslip, allowing the cell suspension to be drawn out by capillary action.
- Using a phase-contrast microscope, focus on the grid lines of the hemocytometer with a 40X objective (400X magnification).
- With the help of a hand tally counter, count the cells in one set of 16 squares (as shown in Fig. **6**). Let's assume that the count in this square is 50. When counting, employ a system whereby cells are only counted when they are set within a square or on the right-hand or bottom boundary line.
- Move the hemocytometer to the next set of 16 corner squares and carry on counting until all four sets of 16 corners are counted. Let's assume that the count in squares 2, 3, and 4 are 40, 45, and 52, respectively (as shown in Fig. **6**).
- Take the average cell count from each of the sets of 16 corner squares and multiply by 10,000 (10^4). Multiply by dilution factor to correct for the amount of dilution made from the original suspension culture.
- The final value is the number of total (viable and dead) cells/mL in the original cell suspension.

Example: If the cell counts for each of the 16 squares were 50, 40, 45, or 52, the average cell count would be:

(50 + 40 + 45 +52) ÷ 4 = 46.75

46.75 x 10,000 (10^4) = 467,500

467,500 x 5 = 2, 337, 500 cells/mL in original cell suspension.

2.4.5B. Enumeration of Viable Cells by Growing Bacteria on a Solid Medium

Notably, the procedure described above, although gives us an account of the bacterial count, it does not distinguish between the live and dead bacterial cells. In other words, the count that is obtained using this procedure reflects the number of both live and dead bacterial cells. In order to find the count of viable cells in a growing culture, the following procedure is useful.

PROTOCOL 2.14: PROCEDURE TO DETERMINE VIABLE CELL CONCENTRATION OF *E. COLI* CELLS GROWING IN LB BROTH

Materials

Freshly growing liquid broth culture of *E. coli* in LB/YT/SOC broth (in tube or flask).

Equipment

Sterile pipettes and tips, sterile culture tubes, LB agar plates, L-shaped glass spreader, laminar flow hood, phase contrast microscope.

Procedure

- Gently swirl the tube/flask to ensure the cells are evenly distributed.
- Withdraw 0.1 mL of broth using a sterile micropipette/tip and place in a culture tube containing 0.9 mL sterile water to achieve 10^{-1} dilution. Mix gently.
- Repeat step 4 twice to achieve 10^{-2} and 10^{-3} dilutions.
- Using a micropipette, take 100 µL of cell suspension from every dilution and spread evenly on the surface of three LB agar plates as described above.
- Incubate all the plates at 37°C without shaking overnight or until well-separated single colonies appear on the dilution plates.
- Count the colonies (CFU) that appeared on each plate. Take the average CFU counts from each of the three plates for each dilution and multiply by the dilution factor to correct for the amount of dilution made from the original suspension culture.
- The final value is the number of viable cells/mL in the original cell suspension.

Example: If the cell counts for each of the were 350, 371, and 320 in three different plates after a dilution of 10^{-5}, the average cell count would be:

(350 + 371 + 320) ÷ 3 = 347

347 x 100000 (10^5) = 3.47 X 10^7 cells/mL in original cell suspension.

2.4.5C. Enumeration with a Spectrophotometer

A spectrophotometer is a device that determines the absorbance of chromophores. Interestingly, the progressive growth of bacterial cells in liquid culture increases the concentration of cells in that culture, which results in the increasing turbidity of the growing culture. If a visible light wave of a higher wavelength (590-600 nm) is passed through such a turbid bacterial culture, the light waves are actively scattered by the bacterial cells. Within a range of values, the amount of light scattering by the bacterial cells is directly proportional to the number of bacterial cells present in the culture. This physical property of growing bacterial cells is harnessed to determine their concentration by measuring the amount of 600 nm light scattered by the culture. At 600 nm wavelength, the level of absorbance (OD_{600}) will depend on the distance between the cuvette and the detector, which varies among spectrophotometers, often by a factor of 2. In order to avoid confusion, it is always wise to crosscheck the number of cells by the count slide method or by tittering for viable colonies (as discussed above). A 10-fold dilution is recommended for a visibly turbid culture. Generally, it is considered that 0.1 OD_{600} unit is roughly equivalent to 1×10^8 cells/ml for a culture grown in a rich medium. The number of cells/ml is calculated from whichever suspension (the undiluted or the diluted) has an OD_{600} <1 [5].

To determine the arbitrary cell count of a growing culture, a small aliquot is withdrawn aseptically from a growing culture and subjected to 1:10 or 1:20 dilution. The OD_{600} diluted culture is determined by spectrophotometer and noted. Note that, as always, the reading should be restricted within the linear range of the spectrophotometer (the range where the linearity of Lambert-Beer Law holds good). For most of the brands of spectrophotometers, this range is 0.1 to 1.2. So, if the OD_{600} of a culture is found to be 1.6, it should be diluted further to obtain an OD_{600} that lies within the above-recommended range.

2.4.5D. The Bacterial Growth Curve

The growth curve of a bacterium consists of four phases, as shown in Fig. (**7**). When the bacterial cells are inoculated to the fresh medium, the bacteria do not divide for quite some time, and this initial phase is called the ***lag phase.*** It is believed that during this time, bacterial cells adapt to the new environment

(culture medium). After this period, the bacteria begin to grow exponentially, and this phase is referred to as the ***log phase***. In this phase, the bacterial cells divide rapidly, and the doubling time of a bacterium is typically 20-30 minutes in the rich LB medium. This dividing phase is sometimes subdivided into ***early-log***, ***middle-log***, and ***late-log phases***. After the ***late-log phase***, a point is reached where the nutrients or oxygen start to become depleted from the medium and waste products (such as acids) from the cells accumulate, and the cell density increases to–2 × 10^9 cells/ml, which is accompanied by the cessation of growth and is called ***saturation***. After a while, owing to the massive depletion of nutrients and extensive accumulation of waste product, the bacterial cells start to die, and this phase is known as the ***death phase*** [5, 6]. A detailed protocol to determine the growth curve is given below:

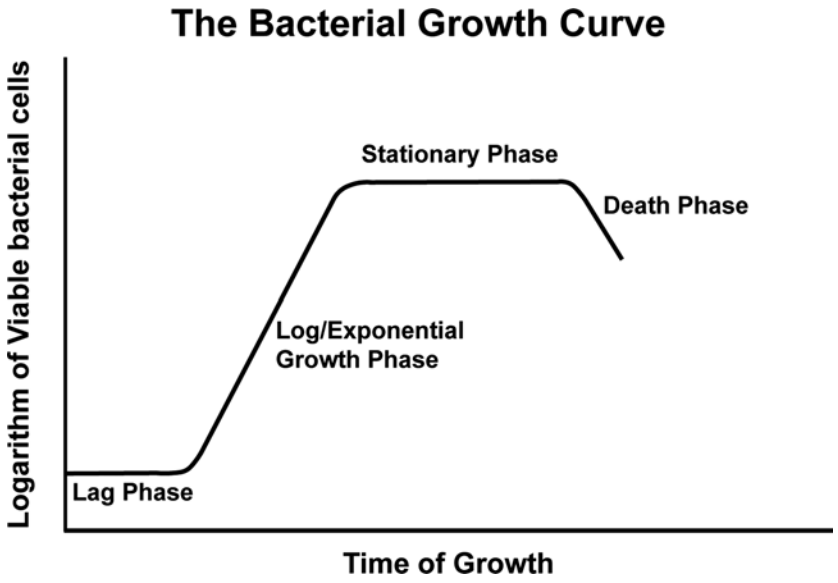

Fig. (7). Schematic diagram showing different phases of a bacterial growth curve.

PROTOCOL 2.15: PROCEDURE TO DETERMINE THE GROWTH CURVE OF *E. COLI* CELLS GROWING IN LB BROTH

Materials

Freshly grown liquid overnight culture of *E. coli* in LB/YT/SOC agar plate.

Equipment

Sterile pipettes and tips, sterile culture tubes, 200 mL sterile LB medium in 1-liter flask, laminar flow hood, UV-VIS spectrophotometer/colorimeter.

Procedure

- Inoculate 200 μL of freshly grown overnight culture containing a specific strain of *E. coli* into the 200 mL sterile growth (LB) medium in the 1-liter conical (preferably baffled) flask.
- Incubate the flask in a shaker incubator at 37°C with vigorous shaking (250-300 rpm speed).
- Withdraw aliquots at every 30-minute interval and determine the concentration either by determining the OD_{600} of the culture spectrophotometrically or by determining the viable count (as described above) of the cells following appropriate dilution (if needed).
- Continue this process for at least 10-12 hours and either record the OD_{600} value or subject the culture for viable count determination.
- If the viable count is determined in the experiment, incubate all the plates in an incubator at 37°C without shaking.
- Count the number of CFUs that appear on the plate the following morning.
- Plot either the OD_{600} value (if done spectrophotometrically) or the average CFU value (if viable counts are determined) as a function of time to generate the growth curve (Fig. **7**).

2.4.6. Preservation of Stock Cultures

It is extremely important to preserve bacterial strains for the long term that are procured either from companies or from other research laboratories as gifts. The rationale for long-term preservation of bacterial strains involves preventing growth by lyophilization or freezing at ultra-low temperature (typically in ultra-freezers where temperature ranges from -70°C to -80°C) in the presence of cryoprotectants (such as glycerol) [6]. It is always recommended to maintain duplicate cultures in different locations to avoid the dangers of losing the preserved strain due to the failure of the ultra-freezer.

Although bacterial cells are maintained by sub-culturing on plates for some time, it is not advisable to leave these cells on plates for a very long period since the organism is susceptible to genetic change under these conditions due to various reasons. A permanent glycerol stock is prepared and then left in a -70°C/-80°C freezer. Under this condition, the cryopreserved strain is stable and can be stored for two to three decades. Whenever required, a small aliquot from this frozen stock can be regenerated in an LB medium and can be used for all kinds of experiments. Therefore, once a new bacterial strain is procured, a newly constructed or a bacterial cell containing mother or recombinant plasmid is generated, a permanent glycerol stock must be prepared as soon as the bacterial cell or plasmid construct has been confirmed. This stock must be placed in the general laboratory stock collection with the appropriate documentation and

location information. This procedure applies not only to *E. coli* but also to any organism for which a frozen stock is prepared and maintained. Notably, all plasmid constructs, including construction intermediates, must be maintained in bacterial host cells, not as naked DNA stocks. For each construct, at least two stocks should be made. To prepare a glycerol stock for *E. coli* cells, combine 1.4 mL of a freshly grown overnight culture with 0.6 mL of sterile 50% glycerol. Mix well by vigorous vortexing. Transfer to two different cryovials labeled with the strain name, the date, and the user initials/name. Immediately place a dry ice/ethanol bath into a box in the -70°C ultra-low freezer. Note the location and enter data into the laboratory strain book.

2.4.6A. Preservation of Short-Term Cultures

Short-term cultures include maintaining cells on agar plates with proper antibiotics if required. These plates are stored in 4°C and may be used for a month [2, 6]. The main disadvantage of this method lies in contamination of the stored plates and desiccation over time. Both are partially avoided by wrapping or sealing the plates with parafilm. Liquid cultures can be stored for a few days. However, for those strains that need antibiotics owing to the harboring plasmid, this procedure is not recommended as long storage of the culture causes depletion of the antibiotic and consequent loss of the plasmid.

2.4.6B. Stab and Slant Cultures

Stab cultures are grown in tubes containing nutrient medium with 0.6% agar, which is one of the effective methods to keep bacterial strains for relatively longer periods [3]. A brief protocol for making the stab and slant cultures is given below:

PROTOCOL 2.16: PROCEDURE FOR THE PREPARATION OF STAB CULTURE OF *E. COLI* FOR PRESERVATION

Materials

Freshly grown liquid overnight culture of a desired strain of *E. coli* in LB/YT/SOC (plus antibiotic if applicable) agar stab/slant vials.

Equipment

4 X 1 cm sterile tube with a water-tight cap, Inoculation loop/needle, and sterile culture tubes.

Procedure

- Take one 4 X 1 cm sterile tube with a water-tight cap (The tubes and the caps were previously autoclaved separately for 15 minutes at 121°C).
- Fill two-thirds of the volume of the tube with sterile (autoclaved) nutrient agar medium at 50°C and leave the tube with a loose cap at room temperature for 1-2 hours to allow the agar medium to solidify.
- The tubes are stored at 45°C for another 1-2 hours to remove moisture.
- Dip the inoculation needle into the fresh overnight culture.
- Stab the loop containing the viable bacterial cells from the culture straight down the middle of the agar several times at different locations.
- Incubated the tube overnight at 37°C with the loose cap.
- The colonies should appear disc- or lens-shaped suspended inside the agar matrix.
- Following growth, the tube is tightly capped and sealed with candle wax or melted parafilm if a more effective air-tight seal is required.
- Store the tube by mounting it in polystyrene sheets in cupboards or drawers away from direct sunlight. They stay good even after 10 years.

Note: It is advisable to check whether the culture has grown in the stab region.

2.4.6C. Preservation of Cultures with Glycerol or DMSO

Glycerol and DMSO are cryoprotectants that reduce damage from ice crystals (13). The use of cryoprotectants helps in the preservation of cultures at ultra-low temperatures by preventing damage to the cell membranes and keeping the cells alive [2, 4 - 6]. Typically, 15-25% (V/V) glycerol (final concentration) is added to the culture and then stored at - 20°C or at -80°C in an ultra-low freezer or sometimes at -196°C in liquid nitrogen. This is the simplest way to preserve bacterial cells for long-term use. Cell survival depends on the freezing rate, therefore, efforts should be made to control this step optimally for the bacterial strains used. Nutrient plates are stored by the addition of 15-25% (v/v) glycerol along with the nutrient medium. DMSO at *5-7%* (v/v) can also be used as a cryoprotectant [5, 6]. Note that the -20°C temperature is not very effective because of the formation of eutectic mixtures exposing cells to high salt concentrations. A brief protocol for preserving bacterial cultures in glycerol is provided below:

PROTOCOL 2.17: PROCEDURE FOR THE PREPARATION OF GLYCEROL STOCK OF *E. COLI* FOR LONG-TERM PRESERVATION

Materials

Freshly grown liquid overnight culture of desired strain *E. coli* in LB/YT/SOC (plus antibiotic if applicable), 50% (W/V) sterile glycerol.

Equipment

Sterile pipettes and tips, sterile cryovials, vortex, laminar flow hood, ultra-low freezer.

Procedure

- From the freshly grown overnight bacterial culture, add 500 µL of the overnight culture to 500 µL of 50% glycerol in a 2 mL cryovial and gently mix by vortexing.
- Freeze the glycerol stock tube at -80°C. The stock is now stable for years as long as it is kept and maintained at -80°C. Subsequent freeze and thaw cycles reduce shelf life.
- To recover bacteria from your glycerol stock, open the tube and use a sterile loop, toothpick, or pipette tip to scrape some of the frozen bacteria off of the top. Do not let the entire glycerol stock unthaw! Streak the bacteria onto an LB agar plate.
- Grow your desired bacterial strain overnight at the appropriate temperature. The next day, you will be able to start an overnight culture for bacterial cells harboring the plasmid DNA prep the following day (see ***CHAPTER 3***).

Note:

- Make the 50% glycerol solution by weighing 50 gm of 100% glycerol in a sterile graduated cylinder. Check the volume of 50 gm of glycerol. Make the volume to 100 mL with dH_2O. Sterilize the solution by autoclaving at 121°C for 15 minutes in a liquid cycle.
- Snap top tubes are not recommended for making glycerol stock as they can open unexpectedly at -80°C.

CONCLUSION

This chapter presents a thorough account of all the techniques required to work with bacteria *E. coli,*which is an inseparable part of molecular cloning. A vivid description of the aseptic techniques and methods of culturing the microorganism are provided here, along with the protocols for preparing different culture media.

A newcomer will benefit from the elaborate diagrammatic representation of different methods of isolation of single colonies from cultures, as mentioned in this chapter. Importantly, the bacterial growth curve will enlighten a beginner scientist with the knowledge of the different phases of bacterial growth. Lastly, protocols to preserve the bacterial strains for further experiments complete the information on microbiological work.

FURTHER READING

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CHAPTER 3

Isolation and Purification of Plasmid Dna

Abstract: This chapter introduces experimenters to the handling and the use of plasmids as cloning vehicles. The chapter begins with the fundamental biology and classifications of different plasmids, followed by various protocols to isolate plasmid DNA from *E. coli* by alkaline lysis and boiling methods from small, medium, and large cultures. Various critical parameters and notes to be considered while performing each step are also included at different places, which is essential for successful isolation. Finally, purification, storage of plasmid DNA, and recipes for reagents and solutions sum up this chapter.

Keywords: Copy number, Extraction, Harvesting, Miniprep, Mediprep, Maxiprep, Plasmids.

3.1. INTRODUCTION TO PLASMID VECTORS

Plasmids are one of the most important tools in recombinant DNA technology. They are covalently closed, circular, double-stranded, and autonomously replicating DNA molecules. Typically, every plasmid DNA possesses a specific DNA sequence (dubbed origin of DNA replication) that supports their replication inside bacterial cells independent of the genomic/chromosomal DNA of the *E. coli* host. Plasmid is stably inherited in an extrachromosomal state within the host bacterium due to the presence of the sequence referred to as replication origin. They are universally used in modern biological research to study gene structure and function and to analyze the protein products of functional genes. Their occurrence in high abundance, variation in structure, size, mode of replication, number of copies per bacterial cell, the ability to propagate in different bacteria, the ability of transferability between bacterial species, and the ability to carry different traits cause them to occupy an important place in the field of molecular biology.

The plasmid size can vary from small to large, ranging from a few kilobases to some hundreds of kilobases. Apart from circular plasmids, gram-positive and gram-negative bacteria have been reported to harbor linear plasmids. Most of the plasmids are not required for the survival of the host bacterial cells in which they reside. However, under certain conditions, they are essential for the survival of the

host bacterium under specific environments. For example, antibiotics in the growth medium, such as ampicillin, tetracycline, kanamycin, *etc.*, impose an obligation on the host bacterium to maintain the resident plasmid, which harbors resistance genes for any of these antibiotics. This mutualism between the host bacteria and the resident plasmid allows the survival and growth of the host bacterium in the presence of a specific antibiotic [1, 3, 4].

Replication of plasmids relies not only on the host-encoded proteins but also on the plasmid-encoded functions. The independence in the replication of plasmids depends on the presence of specific conserved sequences acting as the origin of the replication process (dubbed *ori*). Smaller plasmids typically use the DNA replicative enzymes/factors encoded by the host cells, whereas larger plasmids, in contrast, carry specific genes that code for special enzymes necessary for their own replication. Interestingly, under certain conditions, some plasmids may integrate into the host bacterial chromosome. They are known as episomes or integrative plasmids. At this stage, they replicate along with the bacterial chromosomes [1, 3, 4].

Plasmid copy number relies on the number of plasmids in a bacterial cell. If the bacterial cell cycle is synchronized with plasmid replication, then a low copy number of plasmid molecules per bacterial cell arises (dubbed stringent replication). On the other hand, the plasmid can be triggered to carry out its own replication independent of the host cell cycle, which in turn results in hundreds of copies of plasmid molecules per cell (called relaxed replication). The copy number of a given plasmid is an important parameter in many kinds of procedures involving molecular cloning. Typically, copy numbers of plasmids are governed by their replicons. A replicon of a given plasmid is a genetic unit that includes its replication origin and associated control elements. The origin of replication of most of the plasmids consists of several hundred base pairs, which define the sites of action of the host and plasmid-encoded replicative enzymes. Most often, a plasmid replicon is defined as the minimal sequence of DNA that is sufficient to support the autonomous replication of the entire circular DNA molecule as well as to maintain its requisite copy number. Notably, all the earlier generation plasmids harbor a replicon derived from a plasmid pMB1 (used to exist only in 15-20 copies per cell), which was engineered subsequently to create replicons that yield very high copy numbers in the modern-day plasmids used in molecular cloning. Recombinant DNA technology makes use of these plasmid vectors, which are now available in large varieties and are sold by a number of companies. Several special application plasmids were also developed as low-copy number plasmids, which are used in specialized applications, including cloning and expression of unstable and lethal genes, constructing bacterial artificial chromosomes (BACs) used to clone large segments of foreign DNA as plasmids in *E. coli*.

3.1.1. Plasmids as Cloning Vehicles

Plasmids are routinely used as a vehicle to introduce foreign exogenous DNA fragments up to 20-25 kb in size into a bacterial host to clone it. Once the fragment is ferried inside the host as part of the plasmid molecule, it undergoes multiple rounds of replication to produce millions of copies of itself (see ***CHAPTER 6, UNIT 6.2*** for details). To be used as a cloning vehicle, a plasmid must harbor three essential features that include a replicon (also known as replicator), a selectable marker (usually encodes an antibiotic resistance gene), and a cloning site (carries cleavage sites of multiple restriction enzymes) [1, 3, 4] (Fig. **1**).

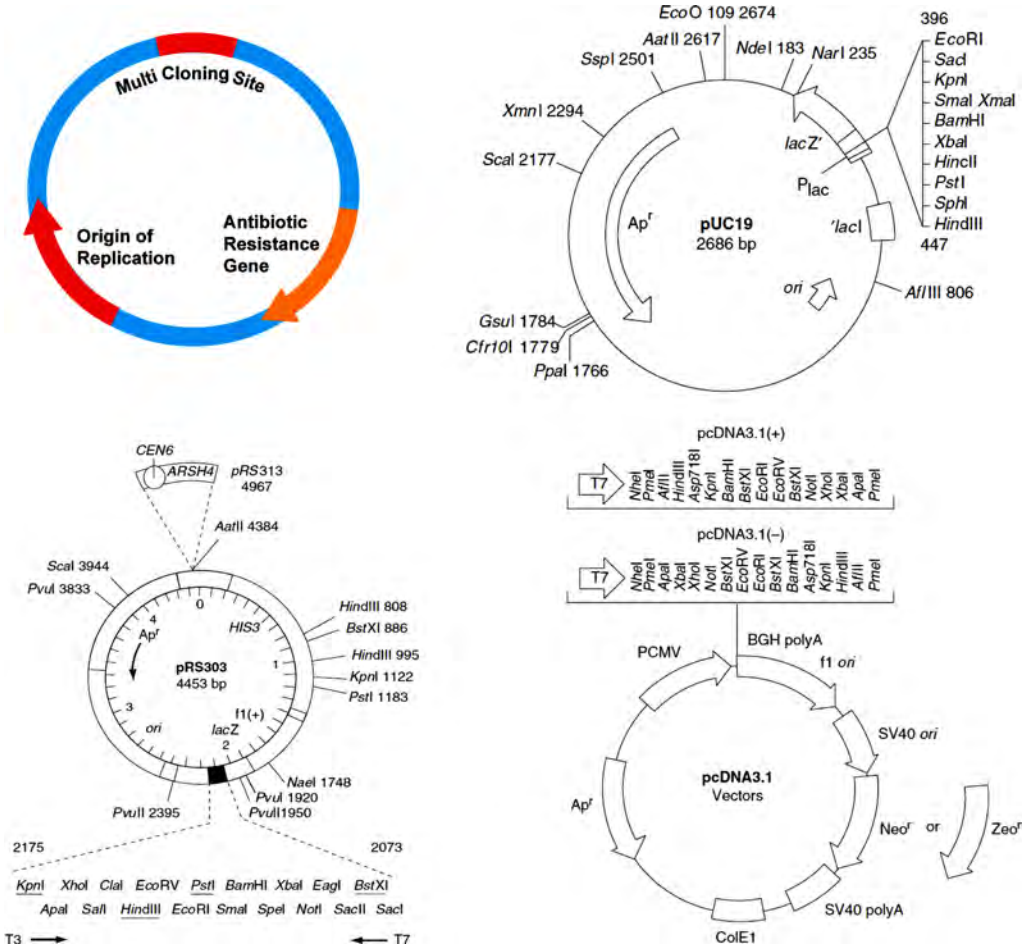

Fig. (1). Schematic representation of a plasmid and the map of three commonly used plasmids.

Origin of Replication

The replicon/replicator dictates the autonomous replication of the plasmid DNA and, hence, subsequently determines its copy-numbers. Essentially, all the plasmid vectors that were engineered to serve as cloning vehicles harbor two kinds of replicators. One class of replicators originally derived from a plasmid pMB1 maintains a very low copy number of the harboring plasmid per cell per generation. These plasmids are known as stringent or low-copy plasmids. Low-copy-number plasmids become important when working with a cloned DNA sequence whose protein product it encodes either kills the host or makes it very sick. In this case, the copy number of the plasmid needs to be kept low to control the gene dosage of the cloned sequence. The second class of replicators maintains very high copy numbers of the plasmid, which are known as relaxed or high copy plasmids (see next ***UNIT 3.1.2***). These relaxed control plasmids are more widely used in molecular cloning because preparing a large quantity of these plasmids in pure form is very easy and convenient. As mentioned above, replicators of most of the modern-day plasmids were derived either from ColE1 or pMB1. Notably, the popular pBluescript series of plasmids contain a ColE1 origin (Fig. **1**), whereas the other popularly used plasmid series called pUC plasmids are derived from pMB1 [1, 3, 4].

Selectable Marker

The selectable marker consists of a gene encoding resistance to specific antibiotics necessary for the growth, survival, and maintenance of the host bacterium carrying plasmid in the presence of the antibiotics. Typically, genes encoding the resistance to antibiotics such as ampicillin, tetracycline, kanamycin, and chloramphenicol are the most commonly used bacterial selectable markers for plasmid vectors. After the ligation procedure, when host bacterial cells are transformed with plasmid DNA carrying an insert (dubbed recombinant DNA) (see ***UNIT 6.7***) and then plated on ampicillin-containing LB plates (see recipes in ***UNIT 7.2.5***), those cells that take up the plasmid are the ones that will selectively grow on the medium. Note that the antibiotic-resistance phenotype conferred is dominant over the antibiotic-sensitive phenotype of host cells that do not possess the plasmid vector. Notably, two sets of selectable markers are generally used for shuttle vectors (vectors used in *E. coli* and yeast/mammalian systems). In these vectors, one set of selectable markers is introduced for the selection of *E. coli* (such as bacterial antibiotic resistance genes). Yet another set of selectable markers distinct from those used in *E. coli* (such as genes encoding nutritional markers for yeast vectors or encoding antibiotics that kill mammalian cells) are generally used for selection in yeast and in cultured mammalian cells, respectively [1, 3, 4] (Fig. **1**).

Cloning Site

The cloning site (also known as Multicloning site or Polylinker site) defines a specific segment in the plasmid DNA into which a piece of desired foreign DNA is inserted. The cloning site harbors the cleavage sites of multiple (nearly twenty enzymes) restriction endonucleases in tandem that operate independently of replication of the plasmid or its ability to confer the selectable phenotype on its host. It should be noted here that the cleavage sites of the restriction enzymes present in the cloning site are always unique so that when the plasmid is treated with one or two of such enzymes, the vector always becomes linear in conformation from the circular topology (Fig. **1**). The piece of foreign exogenous DNA is also preferably cleaved with the same set(s) of restriction enzyme(s) so that the two free ends of the vector and insert DNAs become compatible for ligation. The two free ends of the insert DNA are subsequently ligated/glued to that of the linearized plasmid molecule to form the recombinant DNA molecule. The presence of large numbers of cleavage sites in multiple cloning sites (i) ensures that the appropriate enzyme(s) sites will always be available for cloning most DNA fragments, (ii) provides sites for diverse restriction endonuclease acting as unique reference restriction sites permitting rapid restriction mapping analysis of the insert, and (ii) allows for a great deal of flexibility when manipulating the cloned DNA. The recombinant plasmid consisting of the vector and insert molecules together is then incorporated into the host bacterium (see ***UNIT 6.7***). As the host cell grows, the recombinant plasmid undergoes replication and propagation within the host cell, thereby producing millions of copies (clones) of the recombinant molecule. Plasmids, therefore, facilitate the process of cloning by acting as a vehicle through which an exogenous foreign DNA can be incorporated into a bacterial host and stably multiplied [1, 4].

Furthermore, the sequences within the multicloning site are designed to permit the efficient manipulation/analysis of insert DNA. Multicloning sites of various plasmids are designed to be flanked by sequences for which there are commercially available complementary oligonucleotides (M13 reverse, −20, and −40 primers) that can be used for priming polymerase chain reactions (PCR) or DNA sequencing reactions. Such primers provide useful tools for amplification or sequencing any DNA fragment inserted into the polylinker.

In addition to the cloning procedure, other kinds of genetic manipulations in a foreign gene cloned in the plasmid, *e.g.*, base substitution, addition, or deletion, are also routinely carried out using various established protocols. Subsequent effects (if any) of these genetic alterations on the functionality of the cloned genes are subsequently analyzed following the incorporation of these altered alleles. Collective knowledge from these experiments unveils our understanding of how

the gene functions under specified environmental cues and how that function is regulated at the molecular level. Hence, the isolation of various plasmid molecules in a pure form constitutes the essential fundamental step in molecular cloning. Diverse methods of plasmid DNA isolation are currently available to meet one's needs. Among these methods, the most widely used ones are described below:

3.1.2. Types of Plasmids

The number of molecules of a plasmid found in a single bacterial cell is termed a copy number. It ranges from one to more than 100 per cell, but this number is specific for a given plasmid residing in bacterial cells. Plasmids are classified as relaxed and stringent plasmids on the basis of the number of copies of resident plasmids per cell per generation [1, 4].

Relaxed Plasmids

These plasmids are normally maintained at multiple copies per cell, usually more than 20 per bacterial cell per generation. Typically, their copy numbers vary from 20-200 per cell per generation. High-copy-number plasmids tend to be under relaxed control of replication. The initiation of the DNA replication of these relaxed plasmids typically relies on a process that is controlled by plasmid-encoded functions, and their replication does not require the unstable host replication initiation proteins that must be synthesized at the start of the bacterial cell cycle. Consequently, it has been demonstrated that when protein synthesis of the host bacteria is inhibited by treating the cells with chloramphenicol or spectinomycin, the plasmid copy number dramatically increases. These antibiotics inhibit the host protein synthesis, and thereby, the initiation proteins, which are required for host chromosomal replication, are absent. Moreover, before each round of chromosomal replication event, these initiation proteins need to be freshly synthesized as they are unstable and hence cannot be stored. The inhibition of the host DNA replication permits the complete usage of the major components, such as the host pool of other replication proteins, substrates (dNTP-pool), *etc.*, in the replication of the relaxed plasmids, thereby permitting a huge enhancement of their copy numbers.

Stringent Plasmids

These plasmids have a limited number of copies per cell, usually less than 20. These plasmids are usually under stringent control of replication. Their replication is coordinated with that of the bacterial chromosome. The initiation of their DNA replication relies on the otherwise unstable proteins, which need to be freshly synthesized before each bacterial cell cycle. As the copy number remains very

low, their random segregation does not ensure that each daughter cell receives a copy of the plasmid. However, most low-copy-number plasmids harbor genes that ensure their maintenance within the bacterial population.

Notably, plasmids with larger copy numbers are more useful for gene cloning experiments and are widely used in most of the routinely used cloning procedures in modern biology.

Apart from the above classification, currently available plasmids can also be categorized on the basis of the nature and specific application of the plasmid. They are listed below:

- Plasmid vectors for the production of single-stranded DNA
- Plasmid vectors for cloning large insert
- Plasmid vectors for expression of large quantities of recombinant proteins
- Plasmid vectors for reporter gene fusion
- Plasmid vectors for yeast
- Plasmid vectors for cultured mammalian cells

A map of several commonly used plasmids is presented in Fig. (**1**).

SPECIAL INFORMATION PANEL: AMPICILLIN

• Ampicillin is an antibiotic that is bacteriocidal and kills only growing gram-negative organisms like *E. coli*and*Haemophilus influenza.* Otherwise, it is relatively nontoxic to eukaryotes [1, 3, 4].

• This is an amino penicillin that affects the synthesis of the peptidoglycan layer, which occurs when the bacteria grow logarithmically. The cell wall is composed of long glycan chains of amino sugars cross-linked by peptide chains of D-amino acids.

• Peptidoglycan cross-link reaction, catalyzed by a transpeptidase, is inhibited by this ampicillin. It binds to the transpeptidase and prevents its activity, hence inhibiting cell wall formation [1, 3,5].

• Bacteria exert their resistance to this antibiotic by synthesizing β-lactamase present in its periplasm. This periplasmic enzyme catalyzes the hydrolysis of the cyclic amide bond of the β-lactam ring of the penicillin before it enters the cell, resulting in the detoxification of ampicillin.

• During division, the formation of the rod-like structure of the septum is inhibited by penicillin. Penicillin inhibits the activity of the enzymes called penicillin-binding proteins or PBPs, which are required for septum formation.

3.2. ISOLATION OF PLASMID DNA

Various methods have been developed to isolate plasmid DNA from the bacteria in a purified form. The major challenge in the isolation of plasmid DNA in its pure form from its host bacterium involves the strong possibility that the plasmid DNA may become contaminated with the resident chromosomal DNA. If such contamination occurs, then it is very difficult to separate the plasmid DNA in its pure form by eliminating the host chromosomal DNA. Almost all of the current methods, therefore, employ procedural steps that take advantage of the difference in sizes of the plasmid DNA and the host chromosomal DNA. Consequently, the key to successful isolation of plasmid DNA in most of these procedures involves the differential separation of the plasmid (much smaller) from the bacterial genome (much larger) that is accomplished by partial lysis of the host bacterial cell. Partial lysis allows the preferential release of the smaller plasmid molecules and arrests most of the larger chromosomal DNA molecules within the partially lysed host cells. This differential release ensures that the resulting preparation of the plasmid DNA is majorly free from bacterial chromosomal DNA (see below) [1, 3, 4].

The second challenging issue involving plasmid DNA isolation involves the potential vulnerability of the plasmid DNA molecules to the cellular deoxyribonuclease (DNase), leading to their destruction. Note that the action of these nucleases is normally checked by the cell's regulatory mechanisms while the bacterial cells remain alive, and they typically do not attack the resident plasmid DNA. However, when the host cells are lysed during the isolation procedure, these control mechanisms become destroyed, leading to reckless behavior of the resident nucleases that may potentially destroy the plasmid DNA. All isolation procedures, therefore, employ a novel strategy that promptly inactivates these nucleases. Note that all cell harvesting/extraction buffers contain EDTA (Ethylene Diamine Tetra Acetic acid), which is a potent chelating agent for the divalent Mg^{2+} ions that are used by these nucleases as their co-factor. EDTA promptly chelates the divalent Mg^{2+}/Mn^{2+} cations and readily inactivates the nucleases to protect the resident DNA [1, 3, 4].

Three following steps are involved in isolation protocols/methods of plasmid DNA preparation:

- **1.** ***Growth of the bacterial culture***

The starting material for the isolation of plasmid DNA is the host bacterial cell that harbors the desired plasmid. A single colony of this host bacterium with the resident plasmid is used to inoculate in the liquid media containing appropriate antibiotics and is grown until the late log phase. Plasmids are always purified

from these cultures. High-copy number plasmids (*e.g.*, pUC series), mostly used in the labs, generate a high yield of plasmid DNA from cultures that have been grown the late log phase in the LB medium in the presence of ampicillin.

- **2.** ***Harvesting and lysis of bacteria***

Bacteria harboring plasmids are recovered from the culture by centrifugation and are subjected to lysis by any one of a large number of methods, including treatment with ionic or non-ionic organic solvents, alkali, or heat. The choice of the method is dictated by (a) the size of the plasmid, (b) the strain of host *E. coli*, and (c) the downstream application in which the plasmid DNA will be subsequently used.

- **3.** ***Purification of plasmid DNA***

The salting out method is generally used to purify DNA. When the plasmid is required in the ultra-pure form, CsCl-ethidium bromide gradient equilibrium centrifugation is commonly used.

3.2.1. Isolation and Purification of Plasmid DNA By Alkaline Lysis

Plasmid DNA isolation by the alkaline lysis method is one of the most commonly used methods. This method has been used for more than forty years with success. Plasmids can be isolated from most of the bacterial host strains from a culture size of 1 mL to 500 mL using this technique. Each of these steps is described below, along with their rationale and modes of action of various reagents.

3.2.1.1. Experimental Rationale

1. Growth of Host Bacteria Harboring Plasmid Followed by its Harvesting

Bacterial cells harboring the plasmid are grown to saturation in an LB medium in the presence of an appropriate antibiotic (typically ampicillin). This step enables the production of millions of copies of the resident plasmid within the host cells during its overnight growth. The host bacterial cells are then harvested by centrifugation, and the supernatant is removed either by inverting or aspiration (Fig. **2**) and then resuspended in **Solution-I** (Glucose-Tris-HCl-EDTA buffer) medium in the presence of RNase A. Tris-HCl in this solution maintains the pH of the medium to slight alkaline level, promoting DNA to denature partially. Glucose maintains the osmolarity of the medium to prevent the premature lysis of cells, and disodium EDTA protects the integrity of the plasmid DNA by chelating Mg^{2+} ions (Note that Mg^{2+} ions are cofactor of DNase enzymes and unavailability of

Mg^{2+} ions thus prevents the biochemical destruction of the DNA). RNase A destroys RNA present in the cellular contents when the membrane is lysed.

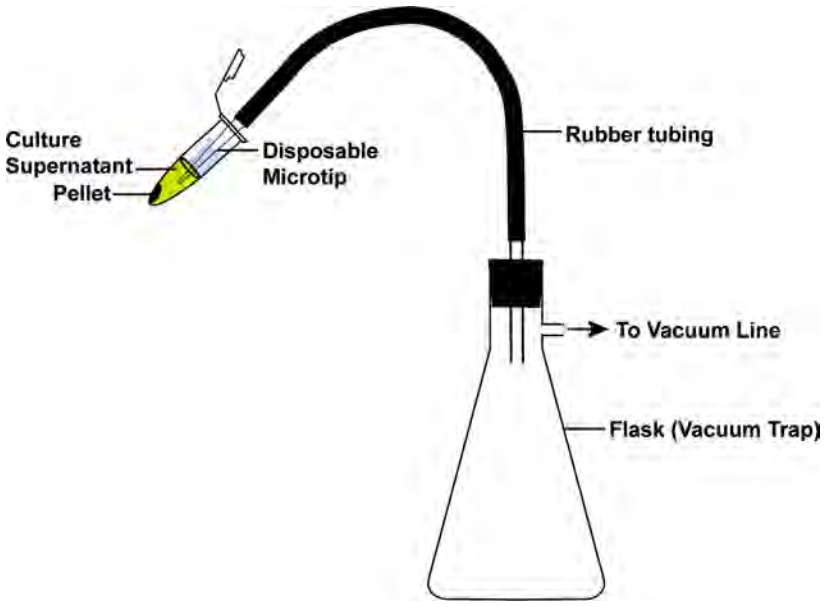

Fig. (2). Aspiration of supernatants using a disposable micro tip connected to a vacuum line *via* a rubber tubing.

2. Partial Lysis of the Bacterial Cell Followed by Denaturation of the Plasmid and Chromosomal DNA

The host cell is then subjected to partial lysis, which is typically accomplished by the ionic detergent sodium dodecyl sulfate (SDS) in the presence of sodium hydroxide (NaOH). SDS lyses the cell membrane by solubilizing the lipid bilayer. Furthermore, it also denatures cellular proteins and disrupts protein-lipid interactions. NaOH in the medium facilitates the denaturation of both chromosomal and plasmid DNA. Bacterial chromosomal DNA remains attached to the cell membrane at many places. Owing to the rupturing of the cell membrane, the genomic DNA suffers breakage into smaller pieces. Typically, this process is allowed to continue for a limited period to restrict cell lysis to a partial level, which selectively allows nearly complete release of the intact plasmid molecules into the medium, while chromosomal DNA, being much larger in size, remains mostly inside the host cell. The selective release of plasmid DNA during the partial lysis is thus a key step in obtaining pure plasmid DNA free from chromosomal DNA. In addition, exposure of the bacterial extract to strong anionic detergent at an alkaline pH denatures both the plasmid and chromosomal DNA molecules by completely disrupting the base pairing of the DNA molecules.

Plasmid DNA is a covalently closed circular molecule; its two strands remain intertwined as they are unable to separate from each other even in the alkaline atmosphere. When neutral conditions (pH of the medium) return, the two strands of the plasmid DNA fall into place (renature) in contrast to the two strands of the chromosomal DNA, which fall apart as they are long, linear, and not covalently closed. Therefore, the intensity and duration of this alkaline exposure are the critical steps in the isolation procedure. Neutralizing the alkaline pH to the neutral pH has sharply contrasting impacts on covalently closed circular plasmid and broken linear DNA (see next section).

3. Selective Renaturation of the Plasmid DNA by Neutralization of the Medium

The lysate/extract containing mostly the denatured plasmid DNA along with other contaminants is neutralized in this step by the addition of acidic potassium acetate (pH-5.5). The addition of potassium acetate leads to the restoration of the normal condition of the medium by lowering its pH to the neutral one, which promotes the renaturation of plasmid DNA but not of chromosomal DNA. As discussed above, plasmid DNA molecules are much smaller in size and have covalently closed circles; their two strands remain topologically intertwined even after denaturation. This intertwined state of the plasmid DNA strands makes its renaturation easy and efficient during the neutralization process. Bacterial chromosomal DNA, in contrast, is much larger and has a high complexity, which is broken into smaller pieces during the lysis step. Consequently, their renaturation during the neutralization process is extremely inefficient. It is thought that broken pieces of chromosomal DNA strands take much longer time to find their complementary strands during renaturation since they are not intertwined like plasmid DNA. Consequently, as the single strands of broken chromosomal DNA are intermingled and without proper alignment, their renaturation is not achieved under this condition. In a parallel process, denatured proteins, broken chromosomal DNA, and cellular debris become entangled to form a larger complex coated with sodium/potassium dodecyl sulfate (KDS). When sodium ions are replaced with potassium ions, a thick white insoluble precipitate(KDS) of the complex from the solution is observed. Plasmid DNA, being circular and covalently closed, renatures efficiently and remains in solution.

4. Concentration and Recovery of the Plasmid DNA

The reannealed plasmid DNA that remains soluble in the medium is separated from the white flocculent precipitate by centrifugation, in which the plasmid DNA partitions in the supernatant. The supernatant is then collected in a fresh tube, and the diluted DNA is further concentrated by precipitation with ethanol or isopropanol using a method known as salting out. Alcohol (ethanol or

isopropanol), in the presence of a relatively high concentration of salt (such as ammonium acetate, lithium chloride, sodium chloride, or potassium/sodium acetate), facilitates the precipitation of the DNA. The counter ions present in the salt mask the negative charge of DNA that eventually allows the DNA to precipitate out of the solution. The precipitate of plasmid DNA formed in the presence of isopropanol is subsequently isolated and purified by centrifugation in the form of a pellet. Plasmid DNA pellet is converted into a soluble form by dissolving it in a TE buffer.

PROTOCOL 3.1: ISOLATION AND PURIFICATION OF PLASMID DNA BY ALKALINE LYSIS METHOD: MINIPREPARATION

This protocol is used for the isolation and purification of plasmid DNA in small-scale (2-5 mL) bacterial cultures by treatment with SDS and alkali. DNA yield resulting from this procedure is sufficient enough to be screened by electrophoresis or be subjected to restriction endonuclease digestion and other routine applications of molecular cloning.

Materials

Host bacteria bearing plasmid previously grown in LB plate supplemented with an antibiotic.

LB Medium.

Buffers/Reagents/Solutions

Antibiotic for plasmid selection: Ampicillin (stock 50-100 mg/mL).

Alkaline lysis solution I: 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Alkaline lysis solution II: 0.2 N NaOH, 1% (w/v) SDS (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Alkaline lysis solution III: 5 M potassium acetate (60 mL), glacial acetic acid (11.6 mL) (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Isopropanol

70% and 95-100% ethanol

TE (pH 8.0) buffer

DNase-free RNase (stock 1 mg/ml)

Phenol Chloroform Isoamyl Alcohol Mix (25:24:1)

3M Sodium Acetate

Equipment

Incubator shaker, Laminar flow hood, microcentrifuge, water bath (37°C), micropipettes, and microtips.

Glassware/Plastic Ware

Microcentrifuge tubes and tips.

Procedure

- On an LB agar plate containing ampicillin (or an appropriate antibiotic), streak the bacterial strain (harboring the plasmid). Incubate the plate in inverted condition at 37°C overnight to get a well-isolated single colony.

NOTE: Use aseptic techniques during streaking and inoculation.

- Transfer a well-isolated single colony into a 5 mL LB medium containing ampicillin (or an appropriate antibiotic). Grow at 37°C with vigorous shaking for 14-16 hrs to get a thick and saturated culture.

NOTE: Save the unused portion of the culture until the procedure is complete.

- Transfer 2-5 mL overnight grown culture into a fresh sterile microcentrifuge tube and harvest the cells by spinning in a centrifuge for 5 minutes at 8000 rpm at 4°C.
- Discard the supernatant either by aspiration (see Fig. **2**) or by decanting, followed by inverting the tube on a paper towel to remove the excess medium.
- Resuspend the pellet in 100 μL of alkaline lysis **solution I**. Mix the contents by tapping the tube or vortexing the pellet using a vortex mixer. Make sure that the suspension disperses uniformly and no cell clumps remain (Refer to Table **1**).

NOTE: Complete resuspension of the bacteria is required. This is done so that all or most of the cells can be exposed to lysis reagents. No visible clumps of bacteria should remain after proper resuspension of the pellet in solution I. Otherwise, it will result in a low yield of the plasmid.

NOTE II: The suspension should appear hazy and viscous after the addition of solution I.

Table 1. A Comparison among Small-, Medium- and Large-scale Preparation of Plasmid DNA by Alkaline Lysis Method.

-	Starting culture volume	Solution I (X vol.)	Solution II (2X vol.)	Solution III (1.5X vol.)	Isopropanol 0.6 vol.	Volume of 70% Ethanol	Volume of TE
Minimum Scale	2-10 mL	100 µL	200 µL	150 µL	270 µL	200 µL	20 µL
Medium Scale	30 mL-50 mL	2 mL	4 mL	3 mL	5.40 mL	1 mL	50 µL
Maximum Scale	250 mL-500 mL	4 mL +1 mL lysozyme (25 mg/mL)	10 mL	7.5 mL	14 mL	5 mL	1000 µL

- Add 200 µL of freshly prepared **solution II.** Close the tube tightly and very gently mix the contents by inverting the tube 4-5 times very slowly. Incubate at room temperature (RT) for 5 min. **(Refer to** Table **1**).

NOTE I: DO NOT VORTEX at this stage. Also, make sure that the entire suspension comes in contact with the entire inner surface of the tube during mixing.

Note II: The addition of Solution II to the cell resuspension turns from opaque to transparent. Vigorous stirring or vortexing of the lysate should be strictly avoided at this step since the bacterial chromosome can shear to form small free chromosomal fragments in the supernatant. In the next step, these chromosomal fragments will co-purify with the plasmid DNA as a contaminant. Because plasmids are supercoiled, both DNA strands remain entangled even after denaturation. Therefore, gentle but thorough mixing of the solution by inverting the tube 4-6 times should be done. This step is perhaps the most crucial step in plasmid isolation.

Note III: Lysis for 5 minutes is enough for maximum release of plasmid DNA from the cell with minimum release of chromosomal DNA. This time is crucial as it also reduces the exposure of the plasmid to denaturing conditions.

- 150 µL of ice-cold **solution III** is added, and the contents are mixed by sharply inverting the tube 4-5 times. Store on ice for 5-10 mins. **(Refer to** Table **1**).

NOTE I: Immediately after the addition of ice-cold solution III, a white fluffy flocculent precipitate should appear. Precipitation is enhanced by using a chilled neutralization buffer and by incubating the tube on ice.

NOTE II: It is important that the solution be thoroughly but gently mixed to ensure complete precipitation.

- Spin the tube containing lysate in a centrifuge at 10,000 rpm for 15 min at 4°C.

NOTE: Tight white pellet should be observed on the wall of the tube.

- Transfer the supernatant into a fresh tube gently without disturbing the pellet.

NOTE: Be careful during the transfer of supernatant as the pellet can suddenly become loose and slide into the tube.

- 0.5 mL of isopropanol is then added to the supernatant. Invert the tube to mix. Incubate for 30 mins to an hour at room temperature.

NOTE: A slight turbidity will be seen in the suspension.

- Spin the tube in a centrifuge at 10,000 rpm for 15 min at room temperature.

NOTE I: The centrifugation step, if done at 4°C, would have caused the SDS to be precipitated along with the nucleic acids. At 25°C, i.e., room temperature, precipitation of SDS is prevented, which is desired.

NOTE II: While placing the tubes in the microcentrifuge rotor, the hinges are always faced outwards. This causes the precipitate to always be collected as a pellet on the inside surface at the hinge side, which is the furthest region from the center of rotation. Arranging all the sample microfuge tubes in the same way (placing their hinges outwards) will make it easy for the user to know where to find the pellets of nucleic acids, which are very often formed as tiny pellets. Marking the expected regions of pellet formation with a lab marker prior to the centrifugation step is also a good idea.

- Carefully aspirate off the supernatant using an aspirator as described above. Remove the last traces of liquid by storing the microfuge tubes on a paper towel in an inverted position for 1 minute.
- Add 500 μL of 70% ethanol. Mix and wash thoroughly by inverting the tube several times. Vortexing at this stage is also carried out if heavy salt contamination is suspected. If the pellet becomes loose after washing, centrifuge at 10,000 rpm for 5 minutes at 4°C in the same way as described above.
- Drain off the 70% ethanol by aspiration and keep the tube on a paper towel in an inverted position.
- Dry the DNA pellet by standing the tube at room temperature or at 37°C for 5-10 minutes with its mouth open.

NOTE: Do not over dry the pellet as it will sometimes be very difficult to dissolve it using TE buffer in the next step.

- Add 50 µL of 1X TE to the pellet when it turns transparent, and resuspend by tapping the tube.
- Prepare a 1% Agarose gel (See ***CHAPTER 5***).
- Prepare the sample for loading in the gel. Mix 5µL of the DNA sample with an appropriate volume of gel-loading dye on a parafilm strip. Then, load and run the gel at 100V for 30-40 minutes (See ***CHAPTER 5***).
- Visualize the crude preparation with the UV gel documentation system (See ***CHAPTER 5***).

Expected Observations and Result

Typically, more than one band of plasmid DNA is usually visualized following the gel electrophoresis, signifying the existence of more than one topological isomer of the plasmids. They include covalently closed supercoiled, nicked (break in the phosphodiester linkage in only one of the strands of the plasmid DNA), and linear (break in the phosphodiester linkage at the same position in both strands of the plasmid DNA) forms. Among these forms, the native form of the plasmid is represented by the supercoiled conformation, whereas the other forms are generated due to mechanical agitations at various steps during the isolation procedure. Supercoiled forms migrate at the fastest speed, and nicked forms of the plasmid migrate at the slowest speed. Linear forms of plasmid DNA are found to migrate with an intermediate mobility. Following electrophoresis, therefore, one to three bands of the plasmid DNA sample of variable intensities are typically visible, which depends on how much nicked and linear forms are present in the plasmid DNA preparation. In addition, total cellular RNA is also present in the sample, which migrates much faster as a big blotch than the plasmid bands in the gel. The RNA contamination is removed by treating the crude DNA sample with Dnase-free RNase treatment as described below:

Purification of DNA

- Add 5 µL of Dnase-free RNase (to achieve a final concentration of 100 µg/mL) from a stock solution of 1 mg/mL. Incubate at 37°C for 30 minutes to an hour, followed by 20 minutes at 55°C.
- Following RNase treatment, add 150 µL of TE buffer to the sample to increase the volume of the sample to 200 µL. Add an equal volume of Tris-saturated phenol: chloroform: isoamyl alcohol (25:24:1). Vortex the tube several times vigorously for complete mixing.

NOTE: This step removes the contaminating proteins from plasmid DNA preparation. When phenol-chloroform-isoamyl alcohol mixture is vigorously mixed with the aqueous DNA sample (a process typically dubbed extraction) and allowed to stand at room temperature, typically two phases form – an upper aqueous (lower specific gravity) and the lower organic phase (higher specific gravity). During the mixing step, phenol denatures proteins and solubilizes denatured proteins and lipids, which are dissolved in chloroform. During the denaturation process, the proteins make complexes with phenol, resulting in the formation of a phenol-protein complex, which partitions to the (lower) organic phase as well as in the interphase between aqueous and organic phases. DNA at pH 8.0 is selectively partitioned to the aqueous (upper) phase. Phenol gives a fuzzy interface, which is sharpened by the presence of chloroform. Isoamyl alcohol reduces foaming during the entire procedure.

- Spin the tube at full speed in a tabletop centrifuge for 5 minutes at room temperature.
- The DNA is present in the upper aqueous phase, which is withdrawn very carefully with a pipette tip into a fresh tube. Be careful not to contaminate the aqueous phase with the interface that appears as a white layer containing denatured proteins.

NOTE: It is usually a good idea to deliberately leave a little bit of the aqueous phase just above the white interphase during the transfer of the aqueous phase from the first-time extraction with phenol-chloroform-isoamyl alcohol. This will result in a protein-free preparation of the plasmid DNA, though it will incur some DNA loss.

- Repeat the above process.
- Add an equal volume of chloroform:isoamyl alcohol (24:1) to the aqueous phase and extract the DNA as above.

NOTE: Chloroform will dissolve any traces of phenol from the aqueous layer.

- Precipitate DNA by adding $1/10^{th}$ volume of 3M sodium acetate pH 5.3 (20-25 μL) and 2.5 volumes of absolute ethanol (500 μL). Mix well and incubate at -80°C for half an hour or at -20°C overnight.
- Collect the precipitated nucleic acid by centrifugation at maximum speed in a microfuge at 4°C for 20 minutes.
- Remove the supernatant by decanting or aspiration. Remove all the liquids by inverting the tube on a paper towel for one minute.
- Wash the pellet by adding 0.2 ml of 70% EtOH (to remove salts). Vortex the solution.

- Recover the DNA pellet by centrifuging the tube in a microfuge at 4°C for 2 minutes. Remove the supernatant as before.
- Dry the pellet at room temperature for 5-20 minutes.
- Dissolve the pellet in 20 µL TE.

Critical Parameters

- A fixed set of volumes of the solutions I, II, and III in plasmid DNA isolation are optimized for different volumes of culture in LB medium and are routinely used. Inefficient lysis may occur if any of the parameters are deviated from the regular protocol. Otherwise, one has to compromise on the quality of plasmid preparation.
- Larger culture volumes are used for large-scale isolation of low-copy plasmids. It may be beneficial to increase the lysis buffer volume in order to increase the efficiency of alkaline lysis, thereby increasing the DNA yield.

Precaution

- Gloves should be worn during the entire isolation procedure.
- The temperature and incubation period should be maintained strictly.
- Ethidium bromide (EtBr) is carcinogenic. Hence, extreme care needs to be taken while handling this chemical. Gloves should be worn all the time.
- Dispose of the electrophoresis buffer in a proper designated area, and do not contaminate elsewhere.
- Temperature and incubation time need to be adhered to according to the protocol.
- Alkaline lysis solution- II (SDS- NaOH) is prepared fresh and mixed gently.

For a thorough troubleshooting guide, please consult Table **2** below:

Table 2. Troubleshooting in isolation of plasmids by alkaline lysis method.

Problem	Likely Causes	Cures
Isolated DNA is resistant to restriction endonuclease digestion.	Care was not exercised during the removal of excess growth medium during the harvesting step. Some host *E. coli* strains (HB101 and its derivative strains) shed cell wall components that were carried over to the final plasmid DNA preparation that inhibits the activity of the restriction enzymes.	Extract the DNA one more time with phenol: chloroform: isoamyl alcohol followed by recovery by ethanol precipitation and washing by 70% ethanol. Finally, resuspend the DNA in TE and carry out the digestion process again. During isolation, the bacterial cell pellet is dissolved in ice-cold STE (0.25X of the original bacterial culture) and centrifuged. Remove any last traces of STE by aspiration (consult **Step 4 of *PROTOCOL 3.1***), resuspend the bacterial pellet in alkaline lysis solution I, and isolate the plasmid following the standard procedure.

(Table 2) cont.....

Problem	Likely Causes	Cures
Plasmid DNA is contaminated with chromosomal DNA.	Bacterial cells were over lysed. Care was not taken to restrict the lysis step to 5 minutes for small-scale or 10 minutes for large-scale preparation.	Repeat the isolation procedure again, but during the isolation procedure, strictly restrict the time of lysis to no longer than 5 minutes (for small-scale) or 10 minutes (for large-scale).
Very little or no DNA is visible on the gel.	A pellet of the nucleic acid was mistakenly discarded during the isopropanol precipitation or ethanol wash step.	Remove the ethanol carefully by aspiration right after the centrifugation step. If the pellet of the nucleic acid appears loose, repeat the centrifugation to tighten the pellet.
Plasmid DNA is visible in the gel before the restriction digestion but converted into a smear after the digestion procedure.	The plasmid DNA was contaminated by bacterial DNase (*endA*), which was subsequently activated by the Mg^{2+} ion present in the buffer of the restriction enzyme. In this case, the most likely source of bacterial DNase could be the contaminated TE that was used for dissolving the plasmid DNA. Care should be taken while handling TE to prevent it from getting contaminated.	Sterilize each batch of TE by autoclaving and dispense into 1-5 mL aliquots into sterile microfuge tubes. Use a fresh aliquot for each preparation. If the plasmid DNA contains bacterial DNase as a contaminant, subject the DNA preparation to phenol:chloroform:isoamyl alcohol followed by recovery by ethanol precipitation and resuspend in fresh TE.

PROTOCOL 3.2: ISOLATION AND PURIFICATION OF PLASMID DNA BY ALKALINE LYSIS METHOD: MIDI-PREPARATION

This protocol is used for the isolation and purification of plasmid DNA from (20-50 mL) bacterial cultures. DNA yielding from using this protocol is enough for screening by electrophoresis, restriction endonuclease digestion analysis, and other routine applications of molecular cloning.

Materials

Host bacteria bearing plasmid previously grown in LB plate supplemented with an antibiotic.

LB Medium.

Buffers/Reagents/Solutions

Antibiotic for plasmid selection: Ampicillin (stock 50-100 mg/mL).

Alkaline lysis solution I: 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Alkaline lysis solution II: 0.2 N NaOH, 1% (w/v) SDS (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Alkaline lysis solution III: 5 M potassium acetate (60 mL), glacial acetic acid (11.6 mL) (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Isopropanol.

70% and 95-100% ethanol,

TE (pH 8.0) buffer,

DNase-free RNase (stock 10 mg/ml),

Phenol Chloroform Isoamyl Alcohol Mix (25:24:1),

3M Sodium Acetate.

Equipment

Incubator shaker, Laminar flow hood, microcentrifuge, water bath (37°C), micropipettes.

Glassware/Plastic Ware

250 mL conical flask, glass or plastic pipettes, oak ridge centrifuge tube, microcentrifuge tubes and tips.

Procedure

- On an LB agar plate containing ampicillin (or an appropriate antibiotic), streak the bacterial strain (harboring the plasmid). Incubate the plate in inverted condition at 37°C overnight to get a well-isolated single colony.

NOTE: Use aseptic techniques during streaking and inoculation.

- Transfer a well-isolated single colony into a 5 mL LB medium containing ampicillin (or an appropriate antibiotic). Store at 37°C with vigorous shaking for 14-16 hrs to get a thick and saturated culture.

NOTE I: Save the unused portion of the culture until the procedure is complete.

NOTE II: The culture should be appropriately aerated. For proper aeration, the volume of the flask should be at least five times greater than the volume of the culture. The flask should be cotton plugged to allow passage of air, and the culture should be vigorously shaken at 37°C.

- Transfer 40-45 mL overnight grown culture into a sterile 50 mL oakridge centrifuge tube and centrifuge at 2000g (4000 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C.
- Discard the supernatant by aspiration (see Fig. **2**) or decanting, followed by removing the excess medium in the tube by inversion on a piece of paper towel, leaving the bacterial pellet as dry as possible.

NOTE: It is important that the last traces of the medium be removed from the bacterial pellet. If the medium is not completely removed, the plasmid DNA yielded from such a procedure will inhibit the activity of the restriction endonuclease from digesting it. This is because the remains of the cell wall in the last traces of the medium will be carried over as contaminants throughout the procedure, finally affecting the purity of the yielded DNA. This, in turn, will eventually interfere with the activity of the restriction endonuclease.

- Add 2 mL of alkaline lysis **solution I** to this pellet and mix the contents by tapping the tube. Alternatively, vortex the suspension to disperse the pellet uniformly. (Refer to Table **1**).

NOTE: The presence of cell clumps in the suspension will ultimately affect the plasmid yield. This is because the number of cells that get exposed to lysis solution will get reduced if not completely resuspended. Hence, no visible clumps of bacteria should remain after proper resuspension of the pellet in solution I. Otherwise, a low yield of the plasmid will result if the pellet is not completely dispersed.

NOTE II: The suspension should appear hazy and viscous after the addition of solution I.

- Add 4 mL of freshly prepared **solution II.** Mix gently with a rolling motion, taking care not to shake the contents violently. Incubate for 10 minutes at room temperature. (Refer to Table **1**).

NOTE I: DO NOT VORTEX at this stage. Also, make sure that the entire suspension comes in contact with the entire inner surface of the tube during mixing.

NOTE II: The addition of Solution II to the cell resuspension makes it turn from opaque to transparent. Vigorous stirring or vortexing of the lysate should be strictly avoided at this step since bacterial chromosomes will shear into fragments and contaminate the plasmid DNA preparation, which in turn will be difficult to eliminate. Because plasmids are supercoiled, both DNA strands remain entangled

even after denaturation. Hence, this step is considered the most crucial step in plasmid isolation, where gentle mixing of the solution is essential.

NOTE III: Release of the maximal amount of plasmid DNA from the cell takes only 5 minutes, whereas the release of chromosomal DNA is minimized. This, in turn, reduces the exposure of the plasmid to denaturing conditions. Hence the incubation time of 5 minutes with this solution should always be strictly maintained.

- Add 3 mL of ice-cold **solution III** and mix vigorosly by inverting the contents of the tube 4-5 times. Store on ice for 5-10 mins. (Refer to Table **1**).

NOTE I: Immediately after the addition of ice-cold solution III, a white fluffy flocculent precipitate should appear. Precipitation is enhanced by using a chilled neutralization buffer and incubating the tube on ice.

NOTE II: It is important that the solution be thoroughly but gently mixed to ensure complete precipitation.

- Centrifuge the tube containing lysate at 10,000 rpm in a Sorvall SS34 rotor for 15 min.

NOTE: A tight white pellet should be observed sticking on the wall at the side of the tube.

- Use a pipette tip to transfer the supernatant gently into a fresh tube. Take care not to touch the pellet.

NOTE: Be careful during the transfer of the supernatant as the pellet can suddenly slip from the tube wall.

- Add 5.4 mL of isopropanol to the supernatant. Mix the contents by inversion. Incubate for 30 mins to an hour at room temperature.

NOTE: Slight turbidity is noticed after the addition of isopropanol.

- Spin the tube in a centrifuge at 10,000 rpm for 25 min at room temperature in a Sorvall SS34 rotor.

NOTE: The centrifugation step, if done at 4°C, would have caused the SDS to be precipitated along with the nucleic acids. At 25°C, i.e., room temperature, precipitation of SDS is prevented, which is desired.

- Discard the supernatant by draining it carefully by gentle aspiration as described above. Remove the last traces of liquid by storing the microfuge tubes on a paper towel in an inverted position for 1 minute.
- To the pellet, add 5 mL of 70% ethanol. Mix well by inverting the tube several times and wash thoroughly. Vortexing at this stage is also carried out if heavy salt contamination is suspected. If the pellet becomes loose after washing, centrifuge at 10,000 rpm for 5 minutes at 4°C in the same way as described above.
- Drain off the 70% ethanol by aspiration and keep the tube on a paper towel in an inverted position on the paper towel for 1 minute.
- Dry the DNA pellet by standing the tube at 37°C for 5-10 minutes with its mouth open.

NOTE: Do not over dry the pellet as it will sometimes be very difficult to dissolve it by TE buffer in the next step.

- Add 50 µL of 1X TE to the pellet when it turns transparent, and resuspend by tapping the tube.
- Prepare a 1% agarose gel (See ***CHAPTER 5***).
- Prepare the sample for loading in the gel. Mix 5µL of the DNA sample with an appropriate volume of gel-loading dye on a parafilm strip. Then, load and run the gel at 100V for 30-40 minutes.
- Visualize the crude preparation with the UV gel documentation system.

Expected Observations and Result

Typically, more than one band of plasmid DNA is usually visualized following gel electrophoresis, signifying the existence of more than one topological isomer of the plasmids. These forms include covalently closed supercoiled, nicked (break in the phosphodiester linkage in one of the strands of the plasmid DNA), and linear (break in the phosphodiester linkage at the same position in both strands of the plasmid DNA). Among these forms, the native form of the plasmid is represented by the supercoiled conformation, whereas the other forms are generated due to mechanical agitations at various steps during the isolation procedure. Supercoiled forms migrate at the fastest speed, and nicked forms of the plasmid migrate at the slowest speed. Linear forms of plasmid DNA are found to migrate with an intermediate mobility. Following electrophoresis, therefore, one to three bands of the plasmid DNA sample of variable intensities are typically visible, which depends on how much nicked and linear forms are present in the plasmid DNA preparation. In addition, total cellular RNA is also present in the sample, and it migrates much faster as a big blotch than the plasmid bands in the

gel. This RNA contamination is removed by treating the sample with Dnase-free RNase treatment to the sample as described below:

Purification of DNA

- Add 20 µL of DNase-free RNase (to achieve a final concentration of 100 µg/ml) from a stock solution of 10 mg/ml. Incubate at 37°C for 30 minutes to an hour, followed by 20 minutes at 55°C.
- At this stage, divide the dissolved DNA in two microfuge tubes so that each tube contains 500 µl of Rnase-treated sample. Add an equal volume of Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex the tube several times vigorously for complete mixing.

NOTE: This step removes and eliminates the contaminating proteins from plasmid DNA preparation. When the phenol-chloroform-isoamyl alcohol mixture is mixed with the aqueous DNA sample and allowed to stand at room temperature, typically two phases are formed – an upper aqueous (lower specific gravity) and the lower organic phase (higher specific gravity). During the mixing step, phenol denatures proteins and solubilizes denatured proteins and lipids, which are dissolved in chloroform. During the denaturation process, the proteins make complexes with phenol to form a phenol-protein complex, which partitions to the (lower) organic phase as well as in the interphase between aqueous and organic phases. DNA at pH 8.0 is selectively partitioned to the aqueous (upper) phase. Phenol gives a fuzzy interface, which is sharpened by the presence of chloroform. Isoamyl alcohol reduces foaming during the entire procedure.

- Spin the tube by centrifuging for 5 minutes at full speed at room temperature.
- The upper aqueous phase containing the DNA is withdrawn with a pipette tip into a fresh tube. Be careful not to contaminate the aqueous phase with the interface that appears as a white layer containing denatured proteins.

NOTE: It is usually a good idea to deliberately leave a little bit of the aqueous phase just above the white interphase during the transfer of the aqueous phase from the first-time extraction with phenol-chloroform-isoamyl alcohol. This will result in a protein-free preparation of the plasmid DNA, though it will incur some DNA loss.

- Repeat the above process.
- Add an equal volume (500 µl) of chloroform:isoamyl alcohol (24:1) to the aqueous phase and extract the DNA as above.

NOTE: Chloroform will dissolve any traces of phenol from the aqueous layer.

- Precipitate DNA by adding 1/10th volume of 3M Sodium acetate pH-5.3 (50 µL) and 2 volumes of absolute ethanol (1000 µL). Mix well and incubate at -80°C for half an hour or at -20°C overnight.
- Collect the precipitated nucleic acid by centrifugation at maximum speed in a microfuge at 4°C for 20 minutes.
- Remove the supernatant by decanting or aspiration. Remove all the liquids by inverting the tube on a paper towel for one minute.
- Add 1 ml of 70% EtOH (to remove salts). Vortex the solution and wash thoroughly.
- Recover the DNA pellet by centrifuging the tube in a microfuge at 4°C for 2 minutes. Remove the supernatant as before.
- The pellet is dried for 5-20 minutes at room temperature.
- Dissolve the pellet with 50 µL of TE.

Critical Parameters

- A fixed set of volumes of the solutions I, II, and III in plasmid DNA isolation are optimized for different volumes of culture in LB medium and routinely used. Inefficient lysis may occur if any of the parameters are deviated from the regular protocol. Then, one has to compromise with the quality of plasmid preparation.
- Larger culture volumes are used for large-scale isolation of low-copy plasmids. It may be beneficial to increase the lysis buffer volume in order to increase the efficiency of alkaline lysis, thereby increasing the DNA yield.

Precaution

- Gloves should be worn during the entire isolation procedure.
- The temperature and incubation period should be maintained strictly as stated.
- Ethidium bromide (EtBr) is carcinogenic. Hence, extreme care needs to be taken while handling this chemical. Gloves should be worn all the time.
- Carefully dispose of the electrophoresis buffer and the agarose gel after analysis.
- Temperature and incubation time needs to be adhered to according to the protocol.
- Alkaline lysis solution- II (SDS- NaOH) is prepared fresh and mixed gently.

For a thorough troubleshooting guide, please consult Table **2** below:

PROTOCOL 3.3: ISOLATION AND PURIFICATION OF PLASMID DNA BY ALKALINE LYSIS METHOD: MAXIPREPARATION

This protocol is used for the isolation and purification of plasmid DNA on a large scale from (200-500 mL) bacterial cultures. Highly purified plasmid results from using this procedure yielding milligram quantities of DNA. Note that the mini-

preparation, midi-preparation, and maxi-preparation of plasmid DNA follow essentially the same procedure by exploiting the size and structural differences between chromosomal and plasmid DNA for preparing crude lysates from bacterial cells containing plasmid DNA grown in liquid culture and purifying plasmid DNA from contaminating RNA and protein. These contaminants should be removed carefully. Failure to remove them from the plasmid preparation would affect the downstream procedures like transfection of higher eukaryotic cells, 5′-end labeling with T4 polynucleotide kinase, PCR, *etc.*, all of which require very clean and purified DNA. There are three procedures for purifying DNA from crude lysate:

- CsCl /ethidium bromide density gradient centrifugation
- Polyethylene glycol (PEG) precipitation
- Chromatographic methods
  - Anion Exchange Chromatography
  - Size-Exclusion Chromatography

In this book, the first two processes will be discussed.

In the protocol of isolating plasmid DNA on a large scale, lysozyme is employed to lyse the bacterial cells before exposing the cells to SDS and NaOH. Once lysozyme treatment is over, the cell lysate is treated with NaOH/SDS solution and potassium acetate as usual and centrifuged to separate plasmid DNA from cell debris containing proteins and chromosomal DNA. The supernatant is then treated with isopropanol to recover the plasmid DNA [1, 4].

Materials

Host *E. coli* bacteria bearing plasmid previously grown in LB plate supplemented with an appropriate antibiotic.

LB medium or enriched medium (*e.g.*, super broth or terrific broth) containing ampicillin or other appropriate antibiotics.

Buffers/Reagents/Solutions

Antibiotic for plasmid selection: Ampicillin (stock 50-100 mg/mL).

Alkaline lysis solution I: 50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0 (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Alkaline lysis solution II: 0.2 N NaOH, 1% (w/v) SDS (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Alkaline lysis solution III: 5 M potassium acetate (60 mL), glacial acetic acid (11.6 mL).

(see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

25 mg/mL hen egg white lysozyme

Isopropanol

70% and 95-100% ethanol,

TE (pH 8.0) buffer,

DNase-free RNase (stock 10 mg/ml)

Phenol Chloroform Isoamyl Alcohol Mix (25:24:1)

3M Sodium Acetate

Equipment

High-speed centrifuge such as Sorvall GSA, GS-3, or Beckman JA-Avanti J-E, Sorvall SS-34 or Beckman JA-10 or JA-17 rotor (or equivalent), incubator shaker, laminar flow hood, microcentrifuge, water bath (37°C), and micropipettes.

Glassware/Plastic Ware

Centrifuge tubes with ≥20-ml capacity (*e.g.*, 50 mL Oak Ridge centrifuge tubes), microcentrifuge tubes and tips.

Procedure

- On an LB agar plate containing ampicillin (or an appropriate antibiotic), streak the bacterial strain (harboring the plasmid). Incubate the plate in inverted condition at 37°C overnight to get a well-isolated single colony.

NOTE: Use aseptic techniques during streaking and inoculation.

- Transfer a well-isolated single colony into 5 mL of LB/TB/YT medium (in a 25 mL screw-capped tube) containing 50 μg/mL ampicillin or any other appropriate antibiotic. Grow at 37°C with vigorous shaking for 14-16 hrs to get a thick and saturated culture.
- Transfer 1mL of overnight culture to inoculate 500 mL LB/TB/YT medium or enriched medium containing appropriate antibiotic in a 2-liter flask. Grow at 37°C until the culture is saturated ($OD_{600} \cong 4.0$).

NOTE I: The yields of DNA can be increased by maximizing the aeration when a flask with a large surface area is used. For example, baffle flasks should be used if the culture volume exceeds >2 liters and vigorously shaken at >400 rpm.

NOTE II: Using enriched media like M9, terrific broth, and LB medium containing 0.1% (w/v) glucose can increase plasmid yields from 2- to 10-fold since different plasmids respond to the media by utilizing the nutrients differently.

NOTE III: From a 500-mL culture grown in LB medium, 1 to 5 mg of high copy number plasmid DNA is yielded routinely. When dealing with the low-copy number plasmids, the cultures of cells should be treated with chloramphenicol to amplify the plasmid.

- Transfer the culture into the 500 mL centrifuge bottles. The cells are harvested by centrifuging the culture at 6000X*g* (~6000 rpm in Sorvall GSA/GS-3 or Beckman JA-10 rotors) for 10 min at 4°C. Discard the supernatant. Keep the open centrifuge bottle in an inverted position on a paper towel for 1-2 minutes to drain the last traces of the medium.

NOTE I: At this stage, the pellets can be stored indefinitely at -20°C *or -70*°C.

NOTE II: It is important that the last traces of the medium be removed from the bacterial pellet. If the medium is not completely removed, the plasmid DNA yielded from such a procedure will inhibit the activity of the restriction endonuclease from digesting it. This is because the remains of the cell wall in the last traces of the medium will be carried over as contaminants throughout the procedure, finally affecting the purity of the yielded DNA. This, in turn, will eventually interfere with the activity of the restriction endonuclease.

- Add 4 mL of ice-cold **Solution I** and resuspend well by vortexing so that no clumps remain. Transfer this entire suspension to a 50 mL Oak Ridge centrifuge or other high-speed centrifuge tubes with a 50-mL capacity. (Refer to Table **1**).

NOTE: The presence of cell clumps in the suspension will ultimately affect the plasmid yield. This is because the number of cells that get exposed to lysis solution will get reduced if not completely resuspended. Hence, no visible clumps of bacteria should remain after proper resuspension of the pellet in solution I. Otherwise, a low yield of the plasmid will result if the pellet is not completely dispersed.

NOTE II: The suspension should appear hazy and viscous after the addition of solution I.

- Add 1 mL of 25 mg/mL hen egg white lysozyme in glucose/Tris/EDTA solution. Incubate for 10 min at room temperature.

NOTE I: Glucose maintains the osmolarity of the medium. Tris-HCl is used in buffering the medium in the range of pH-12 in step 7, especially when by the addition of NaOH, the pH of the solution is greatly increased, and also intensifies the efficiency of precipitation in step 7 when the pH is lowered by the addition of potassium acetate.

NOTE II: Lysozyme assists in the destruction of bacterial cell walls and subsequent release of plasmid DNA. Bacterial debris and soluble proteins are precipitated in step 7. Plasmid recovery reduces by 5% to 10% when lysozyme is not used in the preparation. Lysozyme helps to recover those plasmid DNA that might get trapped in partially degraded cell material, which ultimately gets lost by precipitation at step 7.

NOTE III: Treating the lysate with RNase A is the most efficient and economical method for degrading RNA. This can be accomplished at any step in the preparation of crude lysate, but it is most convenient to do it at step 5 by adding RNase A to the glucose/Tris/EDTA solution to a final concentration of 50 µg/ml.

- Add 10 mL of freshly prepared **Solution II**. Mix the solution by gently stirring with a pipet until it becomes homogeneous and clear. Store the tube on ice for 10 min. (Refer to Table **1**).

NOTE I: DO NOT VORTEX at this stage. After proper mixing, the solution should become very viscous.

NOTE II: The addition of Solution II to the cell resuspension turns it from opaque to transparent. Vigorous stirring or vortexing of the lysate should be strictly avoided at this step since bacterial chromosomes will shear into fragments and contaminate the plasmid DNA preparation, which in turn will be difficult to eliminate. Because plasmids are supercoiled, both DNA strands remain entangled even after denaturation. Hence, this step is considered the most crucial step in plasmid isolation, where gentle mixing of the solution is essential.

NOTE III: Release of the maximal amount of plasmid DNA from the cell takes only 10 minutes, whereas the release of chromosomal DNA is minimized. This, in turn, reduces the exposure of the plasmid to denaturing conditions. Hence the incubation time of 10 minutes with this solution should always be strictly maintained.

- Add 7.5 mL of **Solution III**and mix well with a pipet until viscosity is reduced and a large precipitate is formed. Incubate on ice for 10 min. (Refer to Table **1**).

NOTE I: Immediately after the addition of ice-cold solution III, a white fluffy flocculent precipitate should appear. Precipitation is enhanced by using a chilled neutralization buffer and incubating on ice.

NOTE II: It is important that the solution be thoroughly but gently mixed to ensure complete precipitation.

- Centrifuge for 10 min at 20,000 x *g* (13,000 rpm in Sorvall SS-34; 12,500 rpm in Beckman JA-17 rotor) at 4°C.

NOTE I: Tight white pellet should be observed on the wall of the tube.

NOTE II: The pellet comprising of chromosomal DNA, SDS-protein complexes, and other cellular debris appears large and fairly compact, while plasmid DNA remains in the translucent supernatant. Sometimes, floating material is observed in the supernatant, which can be reduced by the addition of ~0.5 ml chloroform before the centrifugation step.

- Transfer the supernatant into a sterile centrifuge tube. This can be done by carefully withdrawing the supernatant by a glass pipet. In case of any visible floating material, the supernatant is filtered through several layers of cheesecloth.

NOTE: In case of the presence of any material floating in the supernatant, or if it appears cloudy, repeat the centrifugation (step 9) before adding isopropanol.

- Add 0.6 volume (14 mL) of isopropanol and mix well by inverting the tube. Incubate at room temperature for 10 min.

NOTE: The suspension should turn turbid slightly.

- The nucleic acids are precipitated by centrifuging for10 min at 15,000 x *g* (11,500 rpm in SS-34 rotor; 10,500 rpm in JA-17 rotor) at room temperature.

NOTE: The centrifugation is done at room temperature instead of 4°C because lowering the temperature to 4°C will cause the SDS to precipitate along with the nucleic acids. Carrying out this step at 25°C would prevent the precipitation of SDS.

- Discard the supernatant and wash the pellet with 2 mL of 70% ethanol; centrifuge briefly at 15,000X*g* at room temperature. Carefully remove ethanol without disturbing the pellet. Dry the pellet under vacuum or at 37ºC.

NOTE: The pellet can be stored indefinitely at 4ºC.

- Add 50 µL of 1X TE to the pellet when it turns transparent, and resuspend by tapping the tube.
- Prepare a 1% Agarose gel (See ***CHAPTER 5***).
- Prepare the sample for loading in the gel. Mix 5µL of the DNA sample with an appropriate volume of gel-loading dye on a parafilm strip. Then, load and run the gel at 100V for 30-40 minutes.
- Visualize the crude preparation with the UV gel documentation system.

Expected Observations and Result

Typically, more than one band of plasmid DNA is usually visualized following the gel electrophoresis, signifying the existence of more than one topological isomer of the plasmids. These forms include covalently closed supercoiled, nicked (break in the phosphodiester linkage in only one strand of the plasmid DNA), and linear (break in the phosphodiester linkage at the same position in both strands of the plasmid DNA). The native form of the plasmid is represented by the supercoiled form, whereas other forms are generated due to mechanical agitations at various steps during the isolation procedure. Supercoiled forms migrate at the fastest speed, and nicked forms of the plasmid migrate at the slowest speed. Linear forms of plasmid DNA are found to migrate with an intermediate mobility. Following electrophoresis, therefore, one to three bands of the plasmid DNA sample of variable intensities are typically visible, which depends on how much nicked and linear forms are present in the plasmid DNA preparation. In addition, total cellular RNA is also present in the sample, which migrates much faster as a big blotch than the plasmid bands in the gel. This RNA contamination is removed by treating the sample with Dnase-free RNase treatment to the sample as described below:

Purification of DNA

- Add 50 µL of Dnase-free RNase (to achieve a final concentration of 100 µg/mL) from a stock solution of 10 mg/mL. Incubate at 37°C for 30 minutes to an hour, followed by 20 minutes at 55°C.
- Add an equal volume (5 mL) of Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex the tube several times vigorously for complete mixing.

NOTE: The plasmid DNA preparation is purified from the contaminating proteins by following this step. When the phenol-chloroform-isoamyl alcohol mixture is mixed with the aqueous DNA sample and allowed to stand at room temperature, typically two phases are formed – an upper aqueous (lower specific gravity) and the lower organic phase (higher specific gravity). During the mixing step, phenol denatures proteins and solubilizes denatured proteins and lipids, which are dissolved in chloroform. During the denaturation process, the proteins make complexes with phenol to form a phenol-protein complex, which partitions to the (lower) organic phase as well as in the interphase between aqueous and organic phases. DNA at pH 8.0 is selectively partitioned to the aqueous (upper) phase. Phenol gives a fuzzy interface, which is sharpened by the presence of chloroform. Isoamyl alcohol reduces foaming during the entire procedure.

- Centrifuge for 5 minutes at 10,000 rpm at room temperature for 15 minutes in a Beckman JA20.1 (or equivalent) rotor.
- The upper aqueous phase containing the DNA is withdrawn with a pipette tip into a fresh tube. Be careful not to contaminate the aqueous phase with the interface that appears as a white layer containing denatured proteins.

NOTE: It is usually a good idea to deliberately leave a little bit of the aqueous phase just above the white interphase during the transfer of the aqueous phase from the first-time extraction with phenol-chloroform-isoamyl alcohol. This will result in a protein-free preparation of the plasmid DNA, though it will incur some DNA loss.

- Repeat the above process.
- Add an equal volume (5 mL) of chloroform:Isoamyl alcohol (24:1) to the recovered aqueous phase and extract the DNA as above.

NOTE: Chloroform will dissolve any traces of phenol from the aqueous layer.

- Precipitate DNA by adding $1/10^{th}$ volume of 3M Sodium acetate pH-5.3 (500 µL) and 2 volumes of absolute ethanol (10 mL). Mix well and incubate at -80°C for half an hour or at -20°C overnight.
- Collect the precipitated nucleic acid by centrifugation at maximum speed in a microfuge at 4°C for 20 minutes.
- Remove the supernatant by decanting or aspiration. Remove all the liquids by inverting the tube on a paper towel for one minute.
- Add 5 mL of 70% ethanol and wash the pellet to remove salts. Vortex the solution.
- Recover the DNA pellet by centrifuging the tube in a microfuge at 4°C for 2 minutes. Remove the supernatant as before.
- Dry the pellet for 5-20 minutes at room temperature.

- Resuspend the pellet in 1 mL TE.

Critical Parameters

- A fixed set of volumes of the solutions I, II, and III in plasmid DNA isolation are optimized for different volumes of culture in LB medium and routinely used. Inefficient lysis may occur if any of the parameters are deviated from the regular protocol. Then, one has to compromise on the quality of plasmid preparation.
- Larger culture volumes are used for large-scale isolation of low-copy plasmids. It may be beneficial to increase the lysis buffer volume in order to increase the efficiency of alkaline lysis, thereby increasing the DNA yield.

Precaution

- Gloves should be worn during the entire isolation procedure.
- The temperature and incubation period should be strictly maintained.
- Ethidium bromide (EtBr) is carcinogenic. Hence, extreme care needs to be taken while handling this chemical. Gloves should be worn all the time.
- Dispose of the electrophoresis buffer and the agarose gel in a proper designated area, and do not contaminate elsewhere.
- Temperature and incubation time needs to be adhered to according to the protocol.
- Alkaline lysis solution- II (SDS- NaOH) is prepared fresh and mixed gently.

For a thorough troubleshooting guide, please consult Table **2** below:

3.2.2. Isolation and Purification of Plasmid DNA By Boiling Method

In this method, host bacterial cells are suspended in a medium containing Triton-X-100 and lysozyme, followed by exposure to high heat (100°C). High heat not only breaks open the bacterial cells but also denatures the strands of the plasmid DNA, chromosomal DNA, and proteins. Since plasmids are covalently closed and their strands are topologically intertwined, they tend to stay together. During the cooling of the medium in the later steps, the two separated strands of the plasmid DNA reanneal with each other to form a superhelical molecule. Strands of broken chromosomal DNA, in contrast, fail to renature owing to the fact that they are not intertwined. Thus, the plasmid DNA and RNA can be easily separated by centrifugation from the bacterial chromosome, denatured proteins, and lipids that remain attached to the cell membrane. The plasmid DNA is then recovered by precipitation using isopropanol [4].

The boiling method works well with all the plasmids smaller than 15 kb and can be used in mini (1-5 ml) to maxi (up to 250 ml) scale. Not all the strains of *E. coli*. can yield plasmids using this method. However, the preparation of plasmid DNA

by boiling method is not a method of choice for those strains that produce a large amount of carbohydrates. Although this is a quick method of preparing plasmid DNA, the quality of DNA is lower than that obtained from the alkaline lysis method. This procedure is mainly used to prepare plasmid DNA from a small number of cultures ranging from 1 to 24 cultures [4].

PROTOCOL 3.4.: PREPARATION OF PLASMID DNA BY BOILING METHOD: MINIPREPARATION

This protocol is used for the isolation and purification of plasmid DNA in minimum scale (1-2 mL) bacterial cultures by boiling method. The yield of the resulting DNA is enough to carry out screening by electrophoresis, subjected to restriction endonuclease digestion and other routine applications of molecular cloning. However, the quality of the yielded DNA is not suitable for transfection into mammalian cells or DNA sequencing applications, which can be done only after purification by PEG (see ***PROTOCOL 3.6***).

Materials

Host *E. coli* bacteria bearing plasmid previously grown in LB plate supplemented with an antibiotic.

LB medium containing ampicillin or other appropriate selective agent.

Buffers/Reagents/Solutions

Hen egg white lysozyme (10 mg/mL)

STET: 100 mM NaCl, 10 mM Tris-HCl pH-8.0, 1 mM EDTA, 5% (V/V) Triton-X-100 (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Sodium acetate (3M, pH-5.3)

Isopropanol, ice-cold

TE buffer

DNase-free RNase (10 mg/ml)

Equipment

1.5-ml disposable microcentrifuge tubes, boiling water bath (100^{o}C), and microcentrifuge.

Glassware/Plastic Ware

Microcentrifuge tubes and microtips.

Procedure

- Take a single bacterial colony and transfer it to a 5 ml sterile LB medium. Incubate at 37°C with shaking until the culture reaches the mid-log phase (time taken ~6-8 hours) (a freshly saturated overnight culture works even better).
- Take 1.5 ml of the saturated culture and transfer it to a 1.5-ml microcentrifuge tube. Spin the tube in a microcentrifuge for 20 sec at full speed, either at (room temperature) or at 4°C. Discard the supernatant with a pipette or by gentle aspiration (see Fig. **2**), leaving the pellet as dry as possible.

NOTE I: Longer centrifugation at higher speed and time would make it difficult to resuspend cells.

NOTE II: If the medium is not completely removed, the plasmid DNA yielded from such a procedure will inhibit the activity of the restriction endonuclease from digesting it. This is because the remains of the cell wall in the last traces of the medium will be carried over as contaminants throughout the procedure, finally affecting the purity of the yielded DNA. This, in turn, will eventually interfere with the activity of the restriction endonuclease.

- Resuspend the bacterial cell pellet in 300 µl of STET solution. There should be no clumps after the resuspension of the pellet, which can be achieved by vortexing. Add 20 µL of freshly prepared lysozyme (10 mg/mL stock, final concentration becomes 200 µg/300 µl).

NOTE: The yield of plasmid DNA will depend on the number of cells getting exposed to the lysozyme. Hence, the cell pellet needs to be completely resuspended without leaving any clumps, which otherwise reduces the cell number needed for lysis.

- Incubate on ice for 30 sec to 10 min.

NOTE: Time of incubation is an important criterion in this procedure, which ultimately affects the yield or quality of the plasmid DNA. Hence time maintenance should be done strictly.

- Place the tube in a boiling water bath (100°C) for 1 to 2 min.

NOTE: The cell wall becomes weakened by the action of heat and detergents. They then tend to break, causing the plasmid DNA and RNA to be released on one

hand. On the other hand, the larger bacterial chromosome remains attached or trapped inside the lysed cells and is not released.

NOTE II: Longer exposure of supercoiled DNA to heat leads to irreversible denaturation. This, in turn, results in a cyclic coiled conformation that is resistant to digestion with restriction enzymes. Moreover, the migration of this form of DNA through agarose gels is much faster than that of supercoiled DNA, and it is also stained poorly with ethidium bromide. Keeping the lysate at exactly 100°C for the exact recommended time will reduce the quantity of cyclic coiled DNA.

- Spin the tube in a microcentrifuge for 15 to 30 min at maximum speed.

NOTE: At this stage, the pellet containing chromosomal DNA and bacterial debris appears to be fairly gummy. The plasmid DNA and RNA are present in the supernatant.

- Take the supernatant carefully into a fresh microfuge tube, taking care not to disturb the pellet. Add 20 µl of 2.5 M sodium acetate (pH-5.2) and 220 µl (an equal volume) of cold isopropanol. Mix the solution by vortexing and allow the mixture to stand at −20°C for 15 to 30 min.

NOTE: The cold isopropanol helps precipitate the plasmid DNA and cellular RNA. 2 to 5 min is actually sufficient for precipitation.

- Centrifuge for 5-10 minutes in microcentrifuge at maximum speed.
- Discard the supernatant by aspirating as described in step 2 or by decanting the supernatant, followed by keeping the tube in an inverted position on a paper towel for 1 minute.

NOTE: A smudge observed on the specific part of the centrifuge tube is that of the pellet of the nucleic acid.

- Rinse and wash the nucleic acid pellet with 200 ml of chilled 70% ethanol well. Centrifuge for 5 minutes at the maximum speed if the pellet becomes loose. Remove the supernatant by gentle aspiration or by decanting the supernatant, followed by keeping the tube in an inverted position on a paper towel for 1 minute.
- Centrifuge the tube briefly for 30 seconds. Remove all the beads of 70% ethanol that collect at the bottom of the tube from the inner surface by aspiration. Air dry the pellet briefly for 5 minutes, avoiding over drying the pellet.
- The pellet is resuspended in 50 µl TE buffer containing DNase-free pancreatic RNase and vortex briefly. Store the sample at -20°C.

NOTE: The addition of RNase A is optional. Contaminating RNA may often interfere with the detection of DNA fragments on the agarose gel. The contaminating RNA can be destroyed by adding 1 µl of a 10 mg/ml RNase solution (DNase-free).

PROTOCOL 3.5: PREPARATION OF PLASMID DNA BY BOILING METHOD: LARGE-SCALE PREPARATION

This protocol is used for the isolation and purification of plasmid DNA in large-scale (500 mL) bacterial cultures by boiling method. The resulting DNA is not pure enough and may be further purified either by column chromatography or by centrifugation through CsCl-ethidium bromide gradients (see ***PROTOCOL 3.7***).

Materials

Host *E. coli* bacteria bearing plasmid previously grown in LB plate supplemented with an antibiotic.

LB medium containing ampicillin or other appropriate selective agent.

Buffers/Reagents/Solutions

Hen egg white lysozyme (10 mg/mL) in 25 mM Tris-HCl pH-8.0 (Should be prepared fresh).

STET: 10 mM Tris-HCl pH-8.0, 100 mM NaCl, 1 mM EDTA, 5% (V/V) Triton-X-100 (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

Sodium acetate (3M, pH-5.3)

Isopropanol, ice-cold

TE buffer

DNase-free RNase (10 mg/ml)

Equipment

High-speed centrifuge such as Sorvall GSA, GS-3, or Beckman JA-Avanti J-E, Sorvall SS-34 or Beckman JA-10 or JA-17 rotor (or equivalent), incubator shaker, Laminar flow hood, microcentrifuge, boiling and ice-water baths, and micropipettes.

Glassware/Plastic Ware

Centrifuge tubes with ≥20-ml capacity (*e.g.*, 50 mL Oak Ridge centrifuge tubes), microcentrifuge tubes, and microtips.

Procedure

- Inoculate 5 ml sterile LB medium with a single bacterial colony. Incubate the tube in a shaker incubator at 37°C until the culture reaches mid-log phase (time taken ~6-8 hours) (a freshly saturated overnight culture requires less time).
- 5 ml of the saturated culture is used to inoculate a 500 ml liquid LB/YT/Terrific broth with appropriate antibiotic in a 2-liter flask and grown overnight (18-20 hours) at 37°C with vigorous shaking using 250 rpm on a rotary shaker.
- The cultures are transferred into centrifuge bottles. The cells are harvested by centrifuging the culture at 6000x*g* (~6000 rpm in Sorvall GSA/GS-3 or Beckman JA-10 rotors) for 10 min at 4°C. Discard the supernatant. Place the open centrifuge bottle in an inverted position on a paper towel for 1-2 minutes to drain the last traces of the medium.

NOTE I: At this stage, the pellets can be indefinitely stored at -20 °C or -70°C.

NOTE II: If the medium is not completely removed, the plasmid DNA yielded from such a procedure will inhibit the activity of the restriction endonuclease from digesting it. This is because the remains of the cell wall in the last traces of the medium will be carried over as contaminants throughout the procedure, finally affecting the purity of the yielded DNA. This, in turn, will eventually interfere with the activity of the restriction endonuclease.

- The bacterial cell pellet is resuspended in 20 mL of STET solution. Resuspend the pellet completely (leaving no clumps) by vortexing or pipetting up and down several times to achieve complete suspension. Transfer the suspension to a sterile glass tube or flask.

NOTE: The yield of plasmid DNA will depend on the number of cells getting exposed to the lysozyme. Hence, the cell pellet needs to be completely resuspended without leaving any clumps, which otherwise reduces the cell number for lysis.

- Add 2 mL hen egg white lysozyme (10 mg/mL in 25 mM Tris-HCl pH-8.0) and mix by inverting the solution several times. Use a clamp to hold the flask or tube over an open flame of a Bunsen burner until the liquid just starts to boil. Shake the flask continuously during the heating procedure.
- Immediately immerse the bottom half of the flask in a large (preferably 2 liter)

beaker of boiling water bath for exactly 40 to 60 seconds.

NOTE I: To prevent boiling over, the level of the solution in the tube should not touch the top of the tube. Instead there should be a gap between the top of the solution and that of the tube. For rapid heating of the entire solution, the tube should be submerged deep enough into the boiling water.

NOTE II: Longer exposure of supercoiled DNA to heat leads to irreversible denaturation. This, in turn, results in a cyclic coiled conformation that is resistant to digestion with restriction enzymes. Moreover, the migration of this form of DNA through agarose gels is much faster than that of supercoiled DNA, and it is also stained poorly with ethidium bromide. Keeping the lysate at exactly 100°C for the exact recommended time will reduce the quantity of cyclic coiled DNA.

- Cool the tube by incubating it in an ice-water bath for 5 minutes.

NOTE: The cell wall becomes weakened by the action of heat and detergents, which finally cause them to break, causing the plasmid DNA and RNA to be released. The bacterial chromosome, being large, remains attached to or trapped inside, preventing its release from the lysed cells.

- Transfer the viscous suspension of the flask into a centrifuge tube and centrifuge for 20 minutes at ≥25,000×*g*, preferably in a swinging-bucket rotor (*e.g.*, 12,000 rpm in a Sorvall HB-4 rotor) at room temperature.

NOTE: Centrifugation can be carried out both in swinging buckets and fixed-angle rotors. However, the chromosomal DNA and denatured proteins get concentrated at the bottom of the tube in the form of a more compact pellet in a swinging bucket than in a fixed-angle rotor. However, fixed-angle rotors e.g., Sorvall SS-34 at 47,000 ×g (20,000 rpm), Beckman JA-17 at 40,000 × g (17,000 rpm), Beckman 70Ti at 200,000 ×g (44,000 rpm), or SW-41 at 100,000 × g (25,000 rpm) can also be used. Since the solution contains denatured chromosomal DNA, it becomes extremely viscous after boiling. Hence, extreme care should be taken while pouring the contents into centrifuge tubes.

- Pour as much supernatant as possible into a clean centrifuge tube, leaving out the remaining viscous liquid.

NOTE: At this stage, plasmid DNA can be purified from the supernatant by CsCl/ethidium bromide equilibrium gradient centrifugation without any further treatment. However, for purification, either by PEG precipitation or by column chromatography, the plasmid DNA needs to be precipitated with isopropanol before purification.

- Calculate the volume of the supernatant by measuring it. Then, add 0.6 volume (13.2 mL) of isopropanol in it. Mix thoroughly by inverting the tube several times, and incubate for 5 to 10 min at room temperature.

NOTE: Centrifugation at 4°C may cause the salt to precipitate.

- Centrifuge the tube for 15 min at 15,000 × *g* (11,500 rpm in a Sorvall SS-34 rotor; 10,500 rpm in a JA-17 rotor) at room temperature. Take a pellet containing the DNA.
- Decant the supernatant carefully and invert the tube on a paper towel to allow the last traces of the supernatant to drain away. Wash the pellet and the walls of the tube with 70% ethanol and spin the tube at room temperature. Discard the supernatant by draining off the ethanol or by using a Pasteur pipette, which is attached to a vacuum line to remove any remaining traces of liquid left in the tube. Invert the tube on a pad of paper towels on the lab bench for a few minutes until there are no traces of ethanol leaving the pellet damp.

NOTE: The pellet can be stored indefinitely at 4°C.

- Dissolve the pellet of nucleic acids in 3 mL TE buffer (pH-8.0) containing DNase-free pancreatic RNase and vortex briefly. Store the sample at -20°C.

NOTE: The addition of RNase A is optional. Contaminating RNA may often interfere with the detection of DNA fragments on the agarose gel. The contaminating RNA can be destroyed by adding 1 µl of a 10 mg/ml RNase solution (DNase-free).

- Purify the crude plasmid DNA either by precipitation with polyethylene glycol (***PROTOCOL 3.6***) or by equilibrium centrifugation in CsCl-ethidium bromide gradients (***PROTOCOLS 3.7***).
- Check plasmid DNA by gel electrophoresis and also analyze it by restriction enzyme digestion.

3.3. PURIFICATION OF PLASMID DNA

PROTOCOL 3.6: PLASMID DNA PURIFICATION BY PEG PRECIPITATION

Procedure

This purification procedure poses several advantages over CsCl Density gradient centrifugation, which involves ultracentrifugation and the use of ethidium bromide (a mutagen). This method exploits the inverse relationship of the macromolecular size and concentration of PEG that is required for precipitation.

This is a convenient method for plasmid DNA purification as polyethylene glycol (PEG) precipitation is a rapid and reliable process. One can halt at any step in this procedure without any change in the quality and quality of the plasmid DNA, making this method an attractive and convenient one. In this method, the crude lysate is treated with RNase, NaOH/SDS, and potassium acetate to remove RNA and chromosomal DNA contaminants. Plasmid DNA is recovered from the supernatant and precipitated with PEG8000. This method prepares plasmid DNA free of any contaminants [4].

Materials

Pellet from the crude lysate of plasmid-bearing bacterial cell culture (see ***PROTOCOL 3.3.*** and ***3.4***)

Glucose/Tris/EDTA solution: 50 mM Glucose, 25 mM Tris-HCl pH-8.0, 10 mM EDTA (see ***UNIT 3.5*****: Recipes of Reagents and Solutions**).

0.2 M NaOH/1% (w/v) SDS (prepare fresh from 10 M NaOH and 10% (w/v) SDS stocks)

10 mg/ml DNase-free RNase

3M potassium acetate solution, pH ~5.5

Buffered phenol

24:1 (V/V) chloroform/isoamyl alcohol

10 M ammonium acetate

PEG solution: 30% (W/V) PEG 8000, 1.6 M NaCl (Store at room temperature) (see ***UNIT 3.5*****: Recipes of Reagents and Solutions**).

3 M sodium acetate, pH 5.5

Equipment

High-speed centrifuge such as Sorvall GSA, GS-3, or Beckman JA-Avanti J-E, Sorvall SS-34 or Beckman JA-10 or JA-17 rotor (or equivalent), Sorvall HB-4 rotor.

Glassware and Plasticware

Microcentrifuge tubes and microtips

Procedure

Eliminate the contaminants from the nucleic acid pellet obtained from the crude lysate.

- Dissolve the nucleic acid pellet (obtained in the final step of crude lysate preparation) in 1 ml glucose/Tris/EDTA solution.
- 2.5 μL RNase A (final concentration of 20 μg/ml) is then added to the dissolved pellet. Incubate for 20 min at 37°C.
- Add 2 ml freshly prepared 0.2 M NaOH/1% SDS to the resulting solution. Mix gently by inverting the tube and incubate at room temperature for 5 to 10 min.
- Add 1.5 ml of 3 M potassium acetate solution, invert the tube for thorough mixing, and incubate at room temperature for 5 to 10 min.
- Spin the tube by centrifuging at 20,000X*g* (11,000 rpm in SS-34 rotor; 12,500 rpm in HB-4 or JA-17 rotor) for 10 minutes at room temperature. Take the supernatant.

The white pellet comprising the SDS-potassium complex may also contain remains of the chromosomal DNA from the crude lysate. The necessity to purify the plasmid DNA obtained from using this method will depend on the downstream applications to which it will be subjected. In some cases, it may not be necessary to remove the chromosomal DNA completely.

- Transfer the supernatant to a clean tube.
- Extract the supernatant containing plasmid DNA with an equal volume (≈5 ml) of buffered phenol, followed by another extraction with 5 ml of 24:1 chloroform/isoamyl alcohol.
- Centrifuge at 10,000 X g (8000 rpm in HB-4 or Sorvall SS-34 rotors, 8500 rpm in JA-17 rotor) for 10 minutes at room temperature. Transfer the aqueous phase to a fresh centrifuge tube.
- Add 1⁄4 volume of 10 M ammonium acetate (2 M final concentration) and 2 volumes of 100% ethanol to the aqueous phase. Mix thoroughly by inverting the tube several times. Incubate the tube in dry ice for 10 minutes.
- Precipitate DNA by centrifuging the tube for 10 min at 10,000 X *g* (8000 rpm in HB-4 or SS-34 rotor; 8500 rpm in JA-17 rotor) at 4°C.
- Rinse the pellet with 70% ethanol and dry briefly under vacuum.

SPECIAL INFORMATION PANEL: POLYETHYLENE GLYCOL (PEG)

• PEG consists of a simple repeating unit H(OCH2 CH2)$_n$ OH, which is a straight chain polymer. The number of repeating units present in the molecule dictates its molecular weight. PEG has diverse applications in molecular cloning [1, 4].
• PEG efficiently precipitates both linear and circular DNA according to their size in the presence of 10 mM $MgCl_2$ at room temperature. It bears an inverse relation with the size of the DNA fragments. Hence, the concentration of PEG required for precipitation will depend on the size of the DNA. However, PEG/$MgCl_2$ is unable to quantitatively precipitate linear DNAs of size less than 150 bp [1, 4].
• DNA/RNA hybridization, blunt-end ligation of DNA molecules, and 5′-end-labeling of DNA with bacteriophage T4 are enhanced in the presence of PEG as it efficiently causes renaturation of complementary DNA strands.
• Fusion of cultured cells with bacterial protoplasts is caused by PEG.
• Precipitation and purification of bacteriophage particle.

Precipitate the plasmid DNA with PEG

- Dissolve the pellet in a solution containing 2 ml TE buffer and add 0.8 ml PEG solution. Incubate 1 to 15 hr at 0°C.

NOTE: An increase in the time of incubation with PEG, either at 0° to 4°C, increases the recovery of the percentage of plasmid DNA. Usually, after an hour of incubation with PEG at 0°C, more than 50% (~0.5 to 3 mg) can be recovered by centrifuging the solution. Whereas, complete recovery of plasmid DNA is possible when the supernatant is incubated for more than 12 hr at 4°C

- Retrieve the plasmid DNA by centrifuging 20 min at 10,000 X *g* (8000 rpm in HB-4 or SS-34 rotor; 8500 rpm in JA-17 rotor; 10,000 rpm in most microcentrifuges) at 4°C.
- Wash the pellet with 70% ethanol and centrifuge the tube if necessary for a tighter pellet. Remove the supernatant by aspiration and drain the remaining traces of 70% ethanol by inverting the tube on a paper towel.
- Dissolve the DNA pellet in 1 ml TE buffer.
- Add 1/10th volume of 3 M sodium acetate, pH 5.5, and 2.5 volumes of chilled absolute ethanol to the tube for DNA precipitation.

NOTE: Precipitation with 3M sodium acetate and absolute ethanol in the last step, followed by dissolving the pellet in TE, is important to remove the traces of PEG molecules, which may be present in the plasmid preparation.

- Rinse the pellet with 70% ethanol and finally dissolve the pellet in 50-100 mL TE.

PROTOCOL 3.7: PURIFICATION OF PLASMID DNA BY CSCL/ ETHIDIUM BROMIDE EQUILIBRIUM CENTRIFUGATION

Procedure

This purification procedure relies on the use of cesium chloride (CsCl) in combination with ethidium bromide (a potent mutagen). A mixture of these two reagents is then subjected to a long ultracentrifugation step to establish the equilibrium density gradient. In this procedure, cesium chloride (CsCl) and ethidium bromide are mixed with the crude bacterial cell lysate, which is centrifuged to equilibrium. The principle of this procedure relies on the differential binding ability of the different forms of DNA, supercoiled plasmid DNA, and linear chromosomal DNA, to the intercalating agent ethidium bromide. Differential binding of ethidium bromide lowers the density of any given DNA. However, the plasmid DNA binds less ethidium bromide than chromosomal DNA as it is a covalently closed molecule. Moreover, due to the size difference, the banding pattern of the ethidium bromide-bound plasmid DNA molecules and the chromosomal DNA fragments will be in different regions in the gradient. For *e.g.*, plasmid DNA typically forms a band in a region of greater density (relatively lower position in the gradient) than chromosomal DNA in the gradient. Fragmented chromosomal DNA occupies a position in the gradient with a lower density (relatively upper position in the gradient). This method yields very pure and high-quality DNA that is free of most contaminants. After appropriate separation, the bands of plasmid DNA are recovered by puncturing the tube at a specific position in the tube. (Fig. **3**). The plasmid DNA is next passed through a cation exchange column followed by ethanol precipitation to remove ethidium bromide and CsCl, respectively [1, 3, 4].

Materials

Pellet from the crude lysate of plasmid-bearing bacterial cell culture (see ***PROTOCOL 3.3.*** and ***3.4***).

TE buffer, pH 7.5

Cesium chloride

Ethidium bromide (10 mg/ml stock)

CsCl/TE solution (see ***UNIT 3.5*****: Recipes of Reagents and Solutions**).

Dowex AG50W-X8 cation-exchange resin (see ***UNIT 3.5*: Recipes of Reagents and Solutions**).

TE buffer pH 7.5, 0.2 M NaCl

70% and absolute ethanol

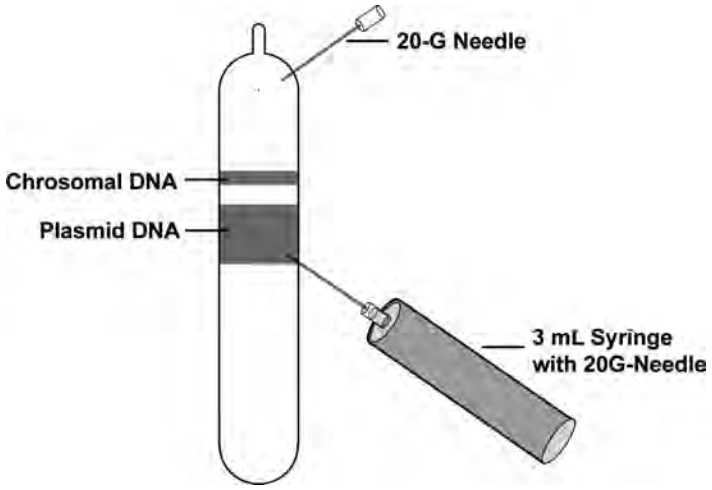

Fig. (3). Collection and retrieval of plasmid DNA from CsCl gradient.

Equipment

Beckman VTi65 or VTi80 rotor (or equivalent), 5-ml quick-seal ultracentrifuge tubes.

3-ml syringes with 20-G needles.

Additional reagents and equipment for ethanol precipitation, regular cold centrifuge/rotors and its tubes, microcentrifuge tubes.

Procedure

- Add 4 ml TE buffer to the tube containing the pellet of the nucleic acids from a crude cell extract. Mix to dissolve the pellet. To this suspension, add 4.4 g CsCl and mix thoroughly to allow complete dissolution of CsCl. Then, further add 0.4 ml of 10 mg/ml ethidium bromide to this mixture and again solubilize it completely by thoroughly mixing.

NOTE: A deep red flocculent precipitate of ethidium bromide complexed with remaining protein is observed in the solution. This precipitate can be removed by centrifuging the lysate–CsCl/ethidium bromide solution at ~2000 × g for 5

minutes at room temperature. The protein–ethidium bromide complex forms a disc at the top of the solution. The rest of the solution can be discarded either by pipetting out from beneath the disc or carefully drained in such a way that the floating disc adheres to the side of the tube.

- Transfer the resulting solution to a 5-ml ultracentrifuge tube. Fill the tube with CsCl/TE solution up to the top of the tube and finally seal the tube. Centrifuge the tube at 500,000 X *g* (77,000 rpm in VTi80 rotor) for 3.5 hours at 20°C. Alternatively, the centrifugation step can be carried out at 350,000 × *g* (65,000 rpm in VTi80 rotor; 58,000 rpm in VTi65 rotor) for 14 hours at 20°C.

NOTE: This centrifugation step must be carried out at a temperature not lower than 15°C. This gradient is formed by using a high concentration of CsCl and a high centrifugal force. The temperature factor is important since, at lower temperatures, during the run, a finite amount of CsCl migrates at the bottom of the tube and precipitates from the solution (where the density is highest). This happens due to the lower solubility of CsCl. This, in turn, moves the center of mass toward the bottom of the tube. This movement can have drastic effects such as rotor imbalance, breakage of the rotor, major damage in the centrifuge, and serious personal injury to the user. In contrast, at higher temperatures, owing to the higher solubility of CsCl, this problem does not arise, and equilibrium is achieved very quickly.

NOTE II: Apart from vertical rotors, other rotors like fixed-angle and swinging-bucket rotors can be used. However, a longer time is required for centrifugation when using these rotors.

- Remove the tube from the centrifuge carefully without disturbing the separated gradient. First, insert a 20-G needle gently into the top of the tube. Recover the plasmid band (the lower of the two bands) by inserting a 3-ml syringe with a 20-G needle attached into the side of the tube approximately1 cm below the plasmid band, as shown in Fig. (**3**). Insert the needle with the beveled side up.

NOTE I: The gradient must be handled very carefully, avoiding any disturbance to the tube and the gradient. The top of the first needle should not be covered as it is required for air displacement when the volume of solution is withdrawn.

NOTE II: Only the plasmid band will be visible if appropriate care has been taken in the previous steps to remove the chromosomal DNA. In ordinary light, plasmid DNA in huge amounts will be visible, whereas with low-intensity shortwave UV light and by-side illumination, smaller amounts will be visualized.

NOTE III: Longer exposure of the DNA–ethidium bromide complex to UV light

will cause the DNA to get damaged. A diffuse region of fluorescence at the bottom of the tube indicates the presence of RNA.

NOTE IV: It is important to check the condition of the needle to determine whether it is clear and does not pose any resistance required when drawing off the plasmid DNA. During the puncture procedure, if a piece of the plastic tube gets inserted within the bore of the needle, it tends to get clogged. Drawing harder on the syringe can create turbulence causing the obstruction to be sucked in and mixing the gradient. In this case, using another needle will be a wise choice to draw off the band.

CAUTION: In case of a needle being clogged, removing it will cause the tube to empty through the hole that remains. The air inlet needle can also become clogged; if it does, then remove it to allow air to enter through the remaining hole. The plasmid DNA can get sheared when it is drawn through a very small opening in a clogged needle.

CAUTION: Protect eye injury by wearing UV-blocking glasses or face shields. Gloves and lab coats should be worn when handling ethidium bromide.

- A second ultracentrifugation step is required for higher-purity plasmid DNA yield from contaminating RNA or chromosomal DNA (when present in the preparation). In such a case, the plasmid DNA collected from the first centrifugation step is added to another ultracentrifuge tube, which is filled with CsCl/TE solution containing 1.0 mg/ml ethidium bromide as done earlier, and then steps 2 and 3 are again repeated.
- To remove ethidium bromide from the collected and recovered plasmid DNA, chromatographic separation of the DNA-ethidium bromide mixture through the Dowex AG50W-X8 column is carried out. Measure the volume of the recovered plasmid DNA-ethidium bromide mixture carefully. Load 1.5 to 2 volumes (of the plasmid DNA) of pre-treated Dowex AG50W-X8 resin (see ***UNIT 3.5*: Recipes of Reagents and Solutions**) in a glass/plastic column. Equilibrate the column by washing it with several volumes of TE buffer/0.2 M NaCl.

NOTE: Commercially purchased plastic column or Pasteur pipet plugged with a little glass wool can be used to set up the column.

- The syringe containing the plasmid DNA/ethidium bromide solution is loaded directly on top of the resin bed. Care should be taken not to disturb the resin.
- Collect the flow through immediately during and after loading the column. Wash the column with two volumes (of the volume of plasmid solution loaded) of TE buffer/0.2 M NaCl.

NOTE I: The final volume of the solution should be three times that of the plasmid DNA volume, which is collected from the gradient column. DNA will flow through in the volume recommended, while ethidium bromide will be retained in the resin and form a red band at the top. In this process, the CsCl will get diluted sufficiently, allowing the DNA to be precipitated.

NOTE II: Another procedure to remove ethidium bromide is by plasmid DNA–ethidium bromide solution extraction with an equal volume of TE-saturated n-butanol. Mix vigorously by vortexing these two solutions to maximize the efficiency of extraction. The upper organic phase is discarded, and the lower aqueous phase is repeatedly extracted until no red color remains. The solution is diluted 3-fold with TE buffer in order to dilute the CsCl.

- Add two volumes of absolute (100%) ethanol at room temperature or -20°C. Centrifuge for 10 minutes at 10,000 X *g* at 4°C. The plasmid DNA will be obtained as a precipitate.

NOTE: At temperatures lower than-20°C, the CsCl in the solution will precipitate. Dialysis of the plasmid DNA against 500 to 1000 volumes of TE buffer provides an alternative to ethanol precipitation. The dialysis buffer should be changed every 2 hours between changes at room temperature or 4 hr at 4°C.

- Wash the pellet with 70% ethanol and dry under vacuum. Finally, resuspend the pellet in TE buffer and store at 4°C.

Precautions

- Ethidium bromide, which is a mutagen, should be handled carefully with gloves and be disposed of properly to prevent environmental hazards. Different institutions have different methods that should be consulted before disposal.
- Serious eye injury can be prevented by wearing UV-blocking glasses or face shields while working with UV light.
- Check for any clogging in the needle before using it to withdraw the plasmid band from the tube. A resistance-free needle is required in the syringe when drawing off the plasmid DNA. Shearing of the plasmid DNA occurs when it is drawn through a very small opening in a clogged needle.
- While using the column for removing the ethidium bromide, contaminated organic solvent waste is generated. An appropriate procedure for its disposal should be followed.

SPECIAL INFORMATION PANEL: CESIUM CHLORIDE AND CESIUM CHLORIDE EQUILIBRIUM DENSITY GRADIENTS

Cesium is a heavy atom and was discovered in 1855 by Wilhelm Bunsen, a German chemist. This property of cesium has been utilized to isolate DNA from density gradients formed from concentrated solutions of CsCl after a few hours of centrifugation [1, 4]. After a few hours of centrifugation, when equilibrium is achieved, all DNA molecules of a given molecular weight accumulate at a region of the gradient in the centrifuge tube where its density and that of CsCl match. At the equilibrium, DNA molecules do not experience any net centrifugal forces and, therefore, do not move any further. In other words, the density of the initial concentration of CsCl depends on the density of the macromolecules or particles under investigation in such a way that the density of the CsCl solution in the gradient formed in the centrifugation tube should match that of the macromolecules [1, 4]. The buoyant density of a macromolecule is defined as the concentration of CsCl (in g/cm^3) at that exact point in the density gradient at which the macromolecule floats. For example, the initial density of the CsCl solution is chosen so as to match the buoyant density of most of the double-stranded linear DNAs. Since the buoyant density of double-stranded linear DNAs is ~ 1.70 g/ml, gradients are usually formed from a CsCl solution whose initial density is also 1.70 g/ml.

The density of double-stranded linear DNA in CsCl is a function of its base composition.

Where,

$\rho = (0.098)\ [G+C] + 1.660$ g/cm3

p = buoyant density of DNA

[G+C] = mole fraction of G+C in double-stranded DNA

3.4. STORAGE OF PLASMID DNA

It is important that both the procured and constructed plasmid DNA be maintained in the laboratory for both short and long terms. Various means are typically used to maintain the plasmid DNA in the laboratory. These are discussed below:

Short-Term Storage: Bacterial strains containing plasmids can simply be maintained by growing on selective antibiotic plates and storing at 4°C. This way of storage works well for about one month [4].

Permanent Storage: A saturated culture (containing appropriate selective agent/antibiotic) of bacteria harboring the plasmid DNA is used for this method. Add 1 to 2 ml saturated culture of bacteria (along with plasmid DNA) to a sterile

100% glycerol or DMSO-based solution. Mix well by vortexing and freeze at -70°C freezer. When the plasmid DNA is required, it should be recovered by growing the same on a selective LB/YT plate by streaking a piece of frozen culture with a sterile long toothpick. The resident plasmid DNA is then isolated from these cells and would be subjected to further analysis and applications [4].

Another approach to storing plasmid DNA is in the buffer TE for several weeks by storing at 4°C or for several years by storing at -20°C or -70°C.

NOTE: Since *DMSO is less viscous than glycerol, it is easier to pipet out while preparing for frozen stocks. This happens to be the only advantage of using DMSO over glycerol. Use spectrophotometric-grade DMSO from the tightly sealed bottle.*

3.5. Recipes Of Reagents And Solutions

Alkaline Lysis Solution I (Glucose/Tris/EDTA (GTE))

50 mM glucose

25 mM Tris-HCl, pH 8.0

10 mM EDTA

Prepare the solution from respective stock solutions of 1 M Glucose, 1 M Tris-HCl ph-8.0, and 0.5 M EDTA pH-8.0. Sterilize the solution by filtration and store at 4°C temperature. Alternatively, sterilization by autoclaving is also permitted at 15 psi for 15 minutes. Store at 4°C.

Alkaline Lysis Solution II (NaOH/SDS)

0.2 N NaOH

1% (W/V) sodium dodecyl sulfate (SDS)

Prepare the solution immediately before use from 10 N NaOH and 10% SDS stock solution.

Alkaline Lysis Solution III (5 M Potassium Acetate Solution, pH 4.8)

5 M potassium acetate 60 mL

Glacial acetic acid 11.6 mL

Adjust pH to 5.5 with glacial acetic acid and make up the volume to 100 ml.

Sterilize by autoclaving and store at 4°C temperature until use.

STET Solution

8% (W/V) sucrose

5% (W/V) Triton X-100

50 mM EDTA pH-8.0

50 mM Tris-HCl, pH 8.0

Filter, sterilize, and store at 4°C

TE (Tris/EDTA) Buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA, pH 8.0

CsCl/TE Solution

100 ml TE buffer, pH 7.5

100 g CsCl

Store indefinitely at room temperature

Dowex AG50W-X8 Cation Exchange Resin

The cation exchange resin is prepared in batches. (200 to 400 ml packed resin) of Dowex AG50W-X8 resin (100-to 200-mesh; Bio-Rad) by following a series of washing steps.

Between each change of wash solution, the resin is collected in a filter paper kept in a large Buchner funnel.

- Ist. Wash: ≥10 volumes of 0.5 N NaOH is used to wash the resin until no color is observed in the wash solution (resin will retain its buff color).
- 2nd Wash: 5 to 10 volumes of 0.5 N HCl.
- 3rd Wash: 5 to 10 volumes of 0.5 M NaCl.
- 4th Wash: 5 to 10 volumes of distilled H_2O.
- 5th Wash: 5 to 10 volumes of 0.5 N NaOH.
- 6th and Final Wash: rinse with distilled H_2O till the pH = 9.0.
- Prepared resin is indefinitely stored in 0.5 M NaCl, 0.1 M Tris (pH 7.5) at 4°C.

Polyethylene glycol (PEG) solution

30% (W/V) PEG 8000

1.6 M NaCl

Filter sterilize through a 0.22μm filter. Store at 4°C indefinitely

DMSO solution

7% dimethyl sulfoxide (V/V)

Glycerol solution

65% glycerol (V/V)

0.1 M MgSO4

0.025 M Tris-HCl, pH 8

DMSO solution

7% dimethyl sulfoxide (V/V)

CONCLUSION

Plasmid isolation from bacteria is one of the first stepping stones toward a molecular biological experiment, and this chapter provides all the necessary information on plasmids. We have included three basic protocols for the isolation of plasmids from different volumes depending on the requirement. Also, the rationale involved in each step and critical points, such as how to handle the sample tube (gentle or vigorous movement) in a protocol, are mentioned vividly so that a newcomer knows the dos and don'ts at various steps in the process. The use of caesium chloride density gradient centrifugation and precipitation by polyethylene glycol procedures for the preparation of ultra-pure plasmid DNA samples was also included in the chapter. Special notes on certain chemicals or processes in the chapter can enlighten a beginner.

FURTHER READING

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CHAPTER 4

Isolation and Purification of Genomic DNA

Abstract: Isolation and purification of genomic DNA is an important procedure in modern molecular biology. This chapter begins with an introduction to eukaryotic genomic DNA followed by various optimized protocols of their isolation from various sources. The three most commonly used procedures of genomic DNA from bacterial cells, plant tissues, and whole blood in the laboratory are presented in vivid detail. Furthermore, we have included recipes for various reagents and solutions required for the isolation of genomic DNA samples. As usual, the critical parameters and notes to be taken into consideration are also mentioned along with the protocols after the appropriate steps.

Keywords: CTAB, DNA, Genomic, WBC.

4.1. INTRODUCTION TO GENOMIC DNA

DNA forms the genetic materials in nearly all living organisms, from viruses to humans, except for some viruses, where RNA constitutes their genetic materials. It was Friedrich Miescher who first successfully isolated DNA as early as 1869 [1]. Currently, genomic DNA isolation and purification is a routine procedure in many molecular biology applications and forensic analyses. Methods employed to isolate genomic DNA are dependent on the source, age, and size of the sample. The ability to extract genomic DNA in a pure form from various sources is of primary importance, enabling scientists and researchers to study the molecular basis of genetic diseases, develop diagnostic tools, and design novel drugs. The ability to isolate DNA from a minuscule quantity of samples from hair, dried blood, or a minute quantity of microbial samples makes it possible to carry out forensic and other applications successfully involving small or large-scale DNA sequence analysis that unequivocally identify criminal suspects, establish paternity, and detect novel bacterial and viral species.

In almost all animal and plant cell types, DNA is present except for the red blood cells. Since DNA is an essential molecule in all living cells, it is not surprising that elaborate protective layers exist in all cell types to safeguard it. Consequently, a comprehensive knowledge of the nature of these protective mechanisms is vital

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for developing successful ways of extracting high-quality genomic DNA in substantial quantities from these sources. Prokaryotes (exemplified by all bacteria and blue-green algae) are unicellular (acellular) organisms that lack intracellular compartmentalization. Consequently, the prokaryotic DNA does not have the protection of a nucleus and presumably floats around in a single-compartment cell, which is surrounded by a cell membrane and a wall that is made up of peptidoglycans. Their genomic DNA is protected from invading viral DNA by restriction enzymes that can selectively cleave foreign viral DNAs. Interestingly, the bacteria prevent their own DNA from being digested by their own enzymes in a unique manner. The methyl groups specifically present in the bacterial genomic DNA assign the bacterial DNA as the 'self' molecule, which restriction endonucleases are able to recognize, thereby preventing its digestion. The viral DNA, in contrast, lacks the 'methyl' marks that appear as 'foreign' molecules to the bacterial restriction enzymes, which promptly act to destroy these molecules.

Eukaryotic cells, including both plant and animal cells, in contrast, evolve compartmentalization and possess multiple cellular partitions. These cells harbor their genomic DNA within the well-shielded compartments of the nucleus, protecting it from the activities in the cytoplasm. While all animal and plant cells are surrounded by only cell membranes, plant cells have the additional protection of a cell wall. More interestingly, the fungi, despite being eukaryotic cells, possess a special kind of cell wall composed of chitin [1, 4]. Isolation of the genomic DNA from a specific source (such as bacteria, plant, fungal, or animal cells), therefore, must employ specific chemical/biochemical steps that selectively destroy these coverings (see below).

All prokaryotic and eukaryotic cells harbor enzymes dubbed deoxyribonuclease (DNase) that can potentially cleave and destroy the genomic DNA during its isolation from respective cells. Remarkably, the action of these nucleases is kept under control by the cell's regulatory mechanisms, and they typically do not attack the resident genomic DNA as long as these cells remain alive. However, during the isolation procedure, as soon as these cells are lysed, these control mechanisms cease to work, and the nucleases start to act in a reckless manner, which very often destroys the resident DNA. Consequently, all isolation procedures harness a strategy that promptly inactivates these nucleases. Notably, all nucleases utilize Mg^{2+} (rarely Mn^{2+}) as their co-factor. All cells harvesting/extraction buffers in the DNA isolation protocols routinely contains EDTA (Ethylene Diamine Tetra Acetic acid), which can promptly chelate the divalent Mg^{2+}/Mn^{2+} cations and readily inactivate the nucleases to safeguard the resident DNA [1, 4].

It is noteworthy that inside the cell, DNA remains associated with various DNA-binding proteins. Most isolation protocols, therefore, employ steps to remove proteins at some point during the isolation procedure [1]. Notably, DNA remains quite stable and unreactive against the action of heat, moderate change of pH of the medium, and several organic compounds (phenol/chloroform, *etc.*) and strong detergents. Most isolation protocols, therefore, involve the treatment of the solution containing cellular DNA with either heat or some of these compounds to selectively destroy and denature cellular proteins and enzymes.

Cellular DNA can be categorized into genomic DNA, mitochondrial or chloroplast DNA, and plasmid DNA (if a cell harbors a plasmid). While plasmid DNA molecules are relatively much smaller molecules, most genomic DNA molecules are much longer and have significantly higher molecular weight [1, 4, 5]. Despite being a relatively sturdy molecule, genomic (or even mitochondrial/chloroplast) DNA is always at risk of undergoing breakage or fragmentation during its removal processes from the nucleus. If the DNA undergoes shearing at too many places, its biological (a resident gene may become inactivated) and physicochemical (it will not spool effectively in the presence of ethanol and salt and becomes harder to capture effectively) properties become compromised. For the isolation of genomic DNA from all sources, it is therefore important to exercise relatively gentle methods during the last steps of DNA extraction and to avoid violent mechanical shaking or agitation that would otherwise shear the DNA (see the special information panel on **MINIMIZING DAMAGE TO LARGE DNA MOLECULES**).

Towards the end of all isolation procedures, the DNA fragments remain suspended in diluted form in the extraction medium. At this point, every protocol calls for a concentration strategy by which the dilute DNA can be concentrated. Typically, a dilute solution of DNA is concentrated by spooling or precipitation with ice-cold alcohol in the presence of salt [1, 4]. Sometimes, a small layer of alcohol is added to the top of the solution containing the cellular fragments. In this case, the DNA collects at the interface between the alcohol and the cell suspension. Subsequently, the DNA is captured or spooled onto a sterile wooden stick or glass rod. The alcohol allows the DNA fragments to stick together, enabling its withdrawal from the aqueous phase and promoting concentration.

All the plasmid and genomic DNA isolation protocols involve three fundamental approaches:

- Complete or partial lysis of the cells (Depends on the source organisms/cells).
- Removal of proteins and other contaminants.
- Recovery of the pure DNA.

The following sections describe the isolation of genomic DNA samples from various sources and plasmid DNA samples from bacteria.

4.2. ISOLATION OF GENOMIC DNA FROM BACTERIAL CELLS

Isolation of genomic DNA from bacterial cells is one of the few initial experiments done in a molecular biology laboratory course. The principal rationale of this procedure comprises breaking open the bacterial cells (referred to as lysis) either using mechanical (by grinding or sonication) or biochemical (using enzymes that destroy the peptidoglycan cell wall of the bacteria) means. Once the genomic DNA is released in the extraction medium, it is then separated from DNA-binding proteins, cellular RNA, lipids, and carbohydrates. Good quality genomic DNA, which is free from all other cellular contamination, is required by downstream reactions, including setting up restriction digestion reactions, polymerase chain reactions, *etc.* Therefore, integrity and purity of DNA are two essential features required for studying the structure and chemistry of DNA, examining DNA-protein interactions, and carrying out DNA hybridizations, gene cloning, and many more.

Five major steps involving the isolation/extraction of genomic DNA from bacteria involves:

Disruption or Lysis of the Bacterial Cells

In order to extract DNA, lysis of the bacterial cells is the first step towards releasing the nucleoid containing the genomic DNA into the extraction medium. Grinding/homogenization (mechanical means) or treatment with lysozyme (biochemical means) is generally used for the lysis of bacterial cells (Fig. **1**). Denaturation of the proteins and dissolution of the cell membrane are achieved by adding lysozyme, an ionic detergent. High salt (0.15 M NaCl) used in extraction buffer helps to maintain the integrity of the DNA [1, 4].

Inhibition of DNases

As mentioned in the introduction, the released genomic DNA always encounters the action by nucleases and is thus at a high risk of destruction by these cellular enzymes following lysis. Hence, the released genomic DNA needs to be protected from these enzymes. The majority of DNases require cofactors like divalent metal ions such as Mg^{2+} for their functioning. EDTA is a metal chelating agent that forms complexes (chelates) with several metal ions, including $Mg^{2+,}$ and thereby inactivates these nucleases [1, 4]. Consequently, the use of these metal chelators protects the genomic DNA from degradation by these DNases by chelating the Mg^{2+}.

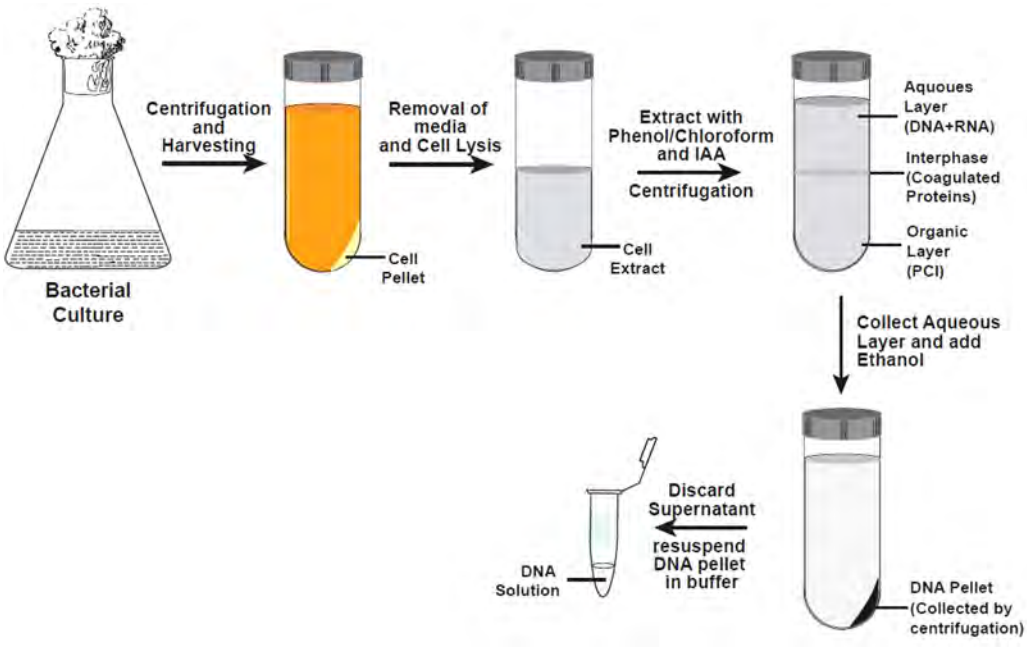

Fig. (1). Schematic diagram showing the major experimental steps involved in the isolation of genomic DNA from bacterial cells (see text for details).

Dissociation of Nucleoprotein Complexes

Broad-spectrum proteolytic enzymes like proteinase K and detergents like SDS and phenol interfere in DNA-protein interactions during the extraction. This interference becomes more efficient in an alkaline pH environment and high salt concentration [1, 4].

Removal of Intrusive Compounds

The cell extract contains proteins, RNA, and other cellular debris, which need to be removed to maintain the purity of the DNA. Typically, proteins are removed by treatment of the intermediate extract either with phenol-chloroform-isoamyl alcohol or with phenol-chloroform (Fig. **1**). RNA is routinely eliminated by the addition of pancreatic RNase A. Note that proteins are usually denatured in the presence of either SDS or high temperature but are unable to do so in case of stable enzymes like RNases and proteases. The addition of sodium acetate precipitates proteins by salting out [1, 4].

Precipitation of DNA

As mentioned in the introduction, the addition of salts in the presence of high concentrations of isopropanol or ethanol causes the nucleic acids, *i.e.*, DNA and

RNA, to precipitate from solutions (Fig. **1**). The DNA is recovered from the aqueous phase by precipitating with the addition of chilled (0°C/-20°C) ethanol after the proteins and RNA are removed [1, 4, 5]. The precipitate is then dissolved in buffer and again extracted with phenol or organic solvent to remove any remaining traces of contaminating protein in the preparation, followed by precipitation again with chilled ethanol.

PROTOCOL 4.1: ISOLATION AND PURIFICATION OF GENOMIC DNA FROM BACTERIAL CELLS

Materials

Overnight grown fresh culture of *E. coli* DH5α (or other) strain.

Chemicals/Reagents

Saline-EDTA (0.15 M NaCl, 0.1 M EDTA adjusted to pH 8.0)

Lysozyme solution (10 mg/mL),

10% sodium dodecyl sulfate (SDS)

10 mM Tris-HCl buffer (pH 8.0)

TE-saturated phenol: chloroform: isoamyl alcohol (25:24:1 Mixture. pH-8.0)

TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0)

Isopropanol

Dehydrated ethanol

Dnase-free RNase solution (10 mg/mL).

NOTE: Media and all buffers/reagents must be sterilized by autoclaving at 15 lbs for 15 min in a liquid cycle.

Equipment

Laminar flow hood, incubator-shaker, table-top or microcentrifuge, pH meter, and weighing balance.

Glassware/Plastic Ware

Conical flasks, petri plates, centrifuge tubes, microfuge tubes, micropipettes, and tips.

NOTE: All glassware and plasticware must be sterilized by autoclaving at 20 lbs for 20 min in dry cycle.

Procedure

The following protocol was used for mini preparation of *E. coli* chromosomal DNA.

- Pick a single colony of *E. coli* (DH5α strain) from a freshly grown plate and inoculate into 5 mL of LB broth in a 25 mL culture tube. Incubate the culture for 16-20 hours at 37°C with vigorous shaking (200-250 cycles/minute in a rotary shaker).
- Take 1.5-2.0 mL of this culture and transfer it to a sterile microfuge tube. Harvest the cells by centrifugation at 5,000-6,000 rpm for 5 minutes at 4°C. Save the cell pellet and decant the supernatant either by aspiration (see **Fig. 2 in *CHAPTER 3***) or by draining the tube and holding it in an inverted position to remove the last traces of media. Add 1 mL of 10 mM Tris-HCl buffer (pH-8.0) to the cell pellet and resuspend it by pipetting up and down several times; wash the pellet again by centrifugation.
- Add 0.8 mL saline EDTA buffer to the cell pellet and resuspend thoroughly by gentle pipetting up and down.
- Add 50 µL of freshly prepared 10 mg/mL lysozyme solution and mix properly by pipetting up and down several times. Avoid vortexing. Incubate at 37°C for 20 minutes.
- Add 0.2 mL 10% SDS, mix well by gentle inversion, and incubate in a water bath at 60°C for 15 minutes.
- Add 0.5 mL saturated equilibrated phenol into the tube and mix the content by inverting the tube 20 times. Centrifuge it at 10,000 rpm for 15 min at room temperature.

NOTE I: Avoid vortexing after the addition of equilibrated phenol since vortexing may lead to shearing and breakage of long genomic DNA. Mix and extract by gentle inversion of the tube as many as 20 times.

NOTE II: Two distinct phases appear right after centrifugation: a lower organic phase containing denatured proteins and an upper well-defined clear phase. The extraction is performed again in case no clear phase is visible.

- The upper aqueous phase is very carefully withdrawn using a tip and transferred into a fresh microfuge tube. Take an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), mix, and add it to the tube. Again, by inverting the tube gently, mix the contents of the tube. This mixing by inver-

sion is done for 20 times. Centrifuge it at 10,000 rpm for 15 minutes at room temperature.

- Using a tip, withdraw the upper aqueous phase and carefully transfer it to a sterile microfuge tube. Precipitate the DNA by adding either an equal volume of isopropanol or two volumes of absolute ethanol from the sidewall of the tube. Mix the contents by gentle inversion five times.
- Store the tubes at room temperature for 30 minutes. Spin in the centrifuge at 12,000 rpm for 15 minutes. Pour off the supernatant and remove the last traces of ethanol/isopropanol by inverting the tube on a sterile filter paper.
- Wash the pellet with 100 µL of 70% ethanol. To ensure this, mix by rotating the tube in a horizontal motion several times. Pellet the DNA by spinning the tube at 12,000 rpm for 5 min in a microfuge. Decant the supernatant, taking care not to disturb the pellet. If the pellet gets dislodged, then centrifuge again to get a tighter pellet. Carefully suck up the remaining liquid from the tube with a tip and air dry the DNA pellet for 15 min.
- Add 50 µL of 1X TE buffer to the DNA pellet. By gentle tapping motion, the pellet in the tube is dissolved. Keep the tube at 4 °C for future use.
- Pour a 0.7% agarose gel into the TAE buffer.
- Check the isolated DNA in an agarose gel. Mix 1-5 µL with gel loading dye (1X) and load in the well of the gel. At 100-120V, run the gel for 30-40 min. To confirm the size of the DNA, load the DNA marker also in another well.
- On a UV transilluminator, look for the DNA band and record the observation.

Observation

DNA can be seen as a red-orange fluorescent band in the gel under UV light very near the loading well.

Precautions

- Exercise precaution while operating the centrifuge. Always load a balance tube of equal weight.
- All the glassware and plasticware should be Dnase-free by sterilization. Use autoclaved glassware, tubes, and tips.
- Precaution should be taken while handling, as ethanol is highly flammable.
- The phenol-chloroform extraction should be carried out under a fume hood.
- Ethidium bromide is carcinogenic. Hence, precautions like wearing gloves and lab coats should be taken while working with it. It is also to be kept in mind that ethidium bromide waste should be disposed of in a designated sink or receptacle.
- All the reagents should be checked for any precipitate that might form at the bottom of a bottle containing a solution. The reagent bottle can be placed in a water bath maintained at 55-65 °C and then cooled to room temperature (15-25 °C). Recheck for any precipitate before use.

4.3. ISOLATION OF GENOMIC DNA FROM PLANT TISSUE

Isolation of deoxyribonucleic acid (DNA) from plant tissues is a challenging task since in divergent plant tissues, different amounts of metabolites and structural biomolecules are present. Among the two such classes of plant biomolecules are the complex polysaccharides and polyphenols that vary significantly between species, both of which affect the quality and quantity of the isolated DNA [2 - 4]. The presence of contaminating polysaccharides and polyphenols can interfere with the downstream applications with the yielded DNA. A protocol for isolating clean plant DNA is provided that uses cetyltrimethylammonium bromide (CTAB) buffer [4].

All extraction protocols of genomic DNA isolation from plant tissue involve cell lysis that causes the cell wall, both the nuclear and cell membranes, to get disrupted. DNA is then released into the extraction medium. This is usually followed by the removal of proteins, polysaccharides, polyphenols, lipids, and many secondary metabolites and, finally, recovery of the genomic DNA [1]. All methods employ CTAB-based extraction buffers that contain CTAB (cetyltrimethylammonium bromide or hexadecyltrimethylammonium bromide) and polyvinylpyrrolidone (PVP). CTAB and PVP have been successfully used to remove polysaccharides and polyphenols while purifying DNA from plant tissues. Polysaccharides are separated using the cationic detergent – CTAB, while PVP removes polyphenols [2, 4]. The rationale for using CTAB involves differential solubility of polysaccharides and DNA in a CTAB-containing medium that depends on the concentration of sodium chloride in the medium. Polysaccharides are insoluble, and DNA is soluble at 1.4 M of salt concentration, while, at a lower salt concentration of 600 mM, DNA is insoluble, but polysaccharides are soluble. Consequently, the phenomenon of differential precipitation of polysaccharides and DNA takes place by adjusting salt concentration in lysates with CTAB [2].

Role of Different Components

Following are the roles of various components used in DNA extraction protocol:

Cell Lysis Extraction Buffer

The extraction buffer contains cetyl trimethyl ammonium bromide (CTAB), 1.4 M NaCl, EDTA, 2-mercaptoethanol, and Tris-HCL. Being a cationic detergent, CTAB readily destabilizes the cell membrane, and 2-mercaptoethanol breaks the disulfide bonds between the cysteine residues, causing the proteins to denature. At high NaCl concentrations, both genomic DNA and tannins/polyphenols present in the crude extract remain in the supernatant while the polysaccharides become largely insoluble. The DNase activity is controlled by the use of the chelating

agent EDTA, which chelates the magnesium ions, which are required for its activity; sodium chloride aids in the precipitation of carbohydrates. The cell wall gets destabilized under these conditions, causing the cells to lyse while maintaining the genomic DNA in solution [2, 4].

Phenol Chloroform Extraction for Precipitation and Removal of Protein

The main undesirable contaminants are proteins and polysaccharides, which are removed by a subsequent phenol-chloroform extraction step in which the nucleic acid solution is extracted successively by phenol:chloroform:isoamyl alcohol (25: 24: 1) and chloroform:isoamyl alcohol (24:1), respectively (Fig. **2**). Each time the tube is centrifuged after extraction followed by transferring of the upper aqueous layer into a new tube, it must be made sure that the interface is not disturbed. Proteins and lipids get denatured and accumulate in the organic phase or in the marginal layer between the two phases. The nucleic acids remain in the top aqueous layer [2, 4]. Another way of removing proteins is by using the enzyme proteinase K, which, is again denatured by phenol *via* phenol chloroform extraction.

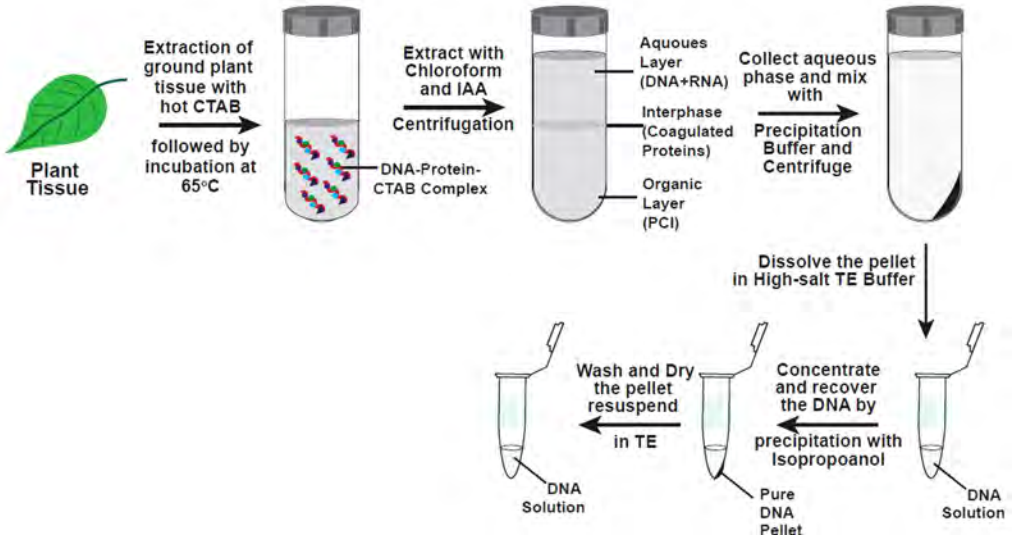

Fig. (2). Schematic diagram showing the major experimental steps involved in the isolation of genomic DNA from plant tissue (see text for details).

Precipitation of Nucleic Acids

The standard protocol for precipitating DNA is alcohol precipitation. This method makes use of salts and absolute ethanol. The commonly used salts include sodium acetate pH 5.2 (final volume 0.3M), sodium chloride (final concentration 0.2M),

ammonium acetate (2-2.5M), lithium chloride (0.8M), and potassium chloride. One-tenth volume of salt is added to the nucleic acid solution, followed by 2.5 times the volume of the solution (Fig. **2**) [1, 4]. At lower concentrations of Na salts, the nucleic acid is precipitated spontaneously while the polyphenols and polysaccharides remain in the solution. At this condition, the DNA can be precipitated by centrifugation. Washing the pellet with 70% alcohol removes the salts and alcohol remnants. Two-third volume of isopropanol can also be used instead of ethanol.

Resuspending DNA

TE (10 mMTris:1mM EDTA) or sterile distilled water is used to resuspend the nucleic acid pellet (Fig. **2**).

Purification of DNA

The DNA still has RNA as its contaminant, and hence, it is removed by incubating the nucleic acid solution with RNase A (10 mg/mL) at 37°C followed by phenol:chloroform extraction to remove the Rnase, reprecipitation, and resuspension [1, 4].

PROTOCOL 4.2: CTAB PROTOCOL FOR THE ISOLATION AND PURIFICATION OF GENOMIC DNA FROM PLANT TISSUES

DNA of high molecular weight from plant tissue is best isolated by this method. However, when working with liquid nitrogen, chloroform, and phenol, which are used here, precaution should be taken as severe burns are caused by phenol, and chloroform is carcinogenic.

Materials

Plant Leaves.

Chemicals and Reagents

CTAB Extraction Buffer (2% (W/V) CTAB, 100 mM Tris-HCl, pH-8.0; 20 mM EDTA, 1.4 M NaCl, 2% (V/V) 2-mercaptoethanol (2-ME), the requisite amount of 2-mE should be added right before the use)

CTAB/NaCl Solution (10% CTAB in 0.7M NaCl).

CTAB Precipitation Buffer (1% (W/V) CTAB, 50 mM Tris-HCl, pH-8.0; 10 mM EDTA

Extraction Buffer (100 mM Tris-HCl, pH-8.0; 100 mM EDTA, 250 mM NaCl, 100 µg/mL proteinase K)

High-Salt TE Buffer: 10 mM Tris-HCl, pH-8.0; 100 mM EDTA, 1 M NaCl

Chloroform/octanol or Chloroform/Isoamyl Alcohol (24:1 ratio) Mixture

Isopropanol

80% Ethanol

TE Buffer (10 mM Tris, pH 8.0, 1 mM EDTA)

Equipment

Water bath, micropipettes, mortar-pestle or pulverizer, low-speed centrifuge (at least 14,000 x g), centrifugal vacuum concentrator (*e.g.*, SpeedVac).

Glassware/Plastic Ware

Sterile tips, sterile microcentrifuge tubes, stands for tubes and flask, mortar and pestle, and polypropylene tubes.

NOTE: Tubes made of polycarbonate should not be used for phenol and chloroform extraction.

Procedure

Plant leaf samples are first washed thoroughly, cut into smaller pieces, and chilled in liquid nitrogen, followed by grinding cryogenically either in a mortar pestle or pulverizer. Freeze-dried plant tissue samples are ground at room temperature to a fine powder for extracting DNA and, so far, have been found to be the best method.

- Before starting the isolation procedure, add requisite amounts of 2-ME and proteinase K to the appropriate volumes of CTAB extraction buffer and extraction buffer to make the final concentrations of 2-mE and proteinase K to 2% and 100 µg/mL, respectively. Pre-heat this CTAB extraction buffer to 65°C.
- Chill a mortar pestle or pulverizer with liquid nitrogen. Use liquid N_2 to grind 1.0 g tissue to a fine powder.
- Transfer the ground plant tissue to a 50 mL polypropylene (resistant to organic solvent) centrifuge tube.
- Add 500 µl of preheated (at 65°C) CTAB extraction buffer to a tube containing homogenized tissue (100 mg). Mix thoroughly by vortexing.

- Incubate the tube for 30-60 minutes in a 60°C water bath with occasional mixing.
- Add an equal volume of chloroform/octanol or chloroform/isoamyl alcohol (24:1).
- Mix well by gentle inversion followed by centrifugation of the sample for 5 minutes at 7500Xg (10,000 rpm in a microcentrifuge) at 4°C to separate the aqueous and organic phases.
- Suck out the upper aqueous phase with a pipet tip very carefully to a new tube. Continue with the alternate extraction and centrifugation till a clear upper phase is obtained.
- Take the upper aqueous phase in a new tube. Add 1/10th volume of CTAB/NaCl solution and mix well by inversion.
- Extract with an equal volume of chloroform/octanol mix and recover the aqueous phase by centrifugation at 7500Xg for 5 minutes at 4°C, as mentioned above. Transfer the aqueous phase to a fresh tube.
- Add 1.0 volume of CTAB precipitation solution and mix well by gentle inversion. If a visible precipitate appears, proceed to step 12. If no visible precipitate is noted, the extract is incubated at 65°C for 30 minutes to precipitate the DNA.
- In a JA-20 rotor, spin the tube containing the sample at 500 X g (2000 rpm) for 5 minutes at 4°C.
- Take a pellet. Discard the supernatant (Save the supernatant without directly discarding it). Add high-salt TE buffer to dissolve the pellet. For 1 gm of starting plant material, add 0.5 to 1.0 mL of TE buffer. At this stage, the DNA molecules come into the solution, while the carbohydrates remain in the pellet. If the pellet does not dissolve readily, incubate the resuspended pellet at 65°C for 30 minutes.
- Precipitate the nucleic acid by adding 0.6 volumes of isopropanol and mix thoroughly. Centrifuge at 7500Xg at 4°C for 15 minutes.
- Decant the supernatant. Wash the pellet with 80% ethanol and centrifuge at 7500Xg at 4°C for 5 minutes.
- Air dry the pellet by keeping the lid of the tube open till the smell of the alcohol is removed. This will prevent the DNA from drying completely.
- Resuspend the DNA pellet in TE buffer (0.1 to 0.5 mL per gram of original plant material). The pellet may need to be warmed in order to dissolve.

Expected Observation

The gel was observed in a UV trans-illuminator (or Gel documentation system) for observing DNA bands.

Result

If bright and intense bands are visible near the wells in the gel, one can conclude that the yield of the isolated genomic DNA is good and that of high concentration. Owing to their high molecular weight, the major band of the genomic DNA remains visible at the top of the gel, usually because of their very low mobility. The intactness of the genomic DNA can be inferred if the DNA appears as a straight band in the gel.

CONCLUSION

The isolated genomic DNA of high concentration and intactness can be used for downstream reactions.

Precautions

- Use all the necessary precautionary measures when operating the centrifuge.
- The working bench and other areas of the lab need to be cleaned to prevent unwanted contamination when the work is done.
- Clean, dry, and autoclaved Dnase-free plastic wares that are resistant to organic solvents should be used. All buffers and reagents should be autoclaved to ensure they are free of DNase.
- Extreme care needs to be taken while handling flammable liquids like ethanol.
- Depending on the source of DNA, it is wise to repeat the extraction with chloroform/octanol to obtain pure DNA.
- When working with carcinogenic agents like ethidium bromide, it is advisable to wear protective clothing like lab coats and gloves.
- All the reagents should be checked for any precipitate that might form at the bottom of a bottle containing a solution. The reagent bottle can be placed in a water bath maintained at 55-65 °C and then cooled to room temperature (15-25 °C). Recheck for any precipitate before use.

SPECIAL INFORMATION PANEL: CTAB

• Cetyltrimethylammonium bromide (CTAB) is a cationic detergent used as a topical antiseptic and is sold under the trade names Savlon and Cetavlon.

• CTAB uses the property of differential precipitation of biomolecules from solutions of different ionic strengths. It precipitates nucleic acids and acidic polysaccharides from solutions of low ionic strength. Under these conditions, proteins and neutral polysaccharides remain in the solution. Whereas in solutions of high ionic strength, CTAB forms complexes with proteins and acidic polysaccharides but will not precipitate nucleic acids [2 - 4].

• Murray and Thompson, in 1980, made use of this unique property of this detergent in isolating DNA from plants that contain large quantities of polysaccharides.

• CTAB is used in two types of basic precipitation procedures:

• For preparation of genomic DNAs. The detergent in high ionic strength buffer (>0.7M NaCl) is added to bacterial or cell lysates. CTAB/polysaccharide/protein complexes are formed in these conditions. Sequential extraction with chloroform and phenol removes the complex, and DNA is recovered by precipitation with isopropanol or ethanol [2 - 4].

• For the preparation of phagemid, plasmid, and bacteriophage DNAs, CTAB is added to lysates of low ionic strength. The precipitated DNAs are collected by centrifugation, dissolved in solutions of high ionic strength, and purified by ethanol precipitation.

• Because CTAB precipitates in the cold, solutions containing the detergent should be stored at temperatures > 15°C.

4.4. ISOLATION OF GENOMIC DNA FROM WHOLE BLOOD

Isolation of genomic DNA from the blood constitutes one of the fundamental methods used in clinical diagnostics (such as DNA fingerprinting, paternity tests, and other genotypic studies), as blood is the only tissue that can be isolated from the human body in the least invasive means. Typically, blood is composed of red blood cells (RBCs), white blood cells (WBCs), and platelets. Amongst them, the genomic DNA is routinely isolated from the WBCs since they are the only cells that contain the nucleus [1, 4]. In contrast, the red blood cells (RBCs) typically lose their nuclei during the maturation event. Therefore, the initial step toward isolating the genomic DNA from WBCs would be to separate these cells from the whole blood. Treatment with detergents causes rupturing of the cell membrane, whereby intracellular contents are released. In the extract, DNA remains

contaminated with proteins, RNA, sugars, and fats. DNA is then eventually purified from these contaminants.

Therefore, following are the steps for the DNA purification procedure:

- Lysis of WBCs
- Precipitation of Proteins followed by its removal.
- Precipitation of genomic DNA
- Removal of residual contaminants
- Elution of pure genomic DNA

PROTOCOL 4.3: ISOLATION AND PURIFICATION OF GENOMIC DNA FROM WHOLE BLOOD

Principle

The principle of isolation of genomic DNA from whole blood involves the removal of the red blood cells followed by the lysis of white blood cells and their nuclei with lysis buffer. Contaminating proteins are removed by subsequent precipitation and short washing steps. The high molecular weight genomic DNA remains in the solution, which is then recovered by ethanol precipitation [4].

Materials

Blood Sample (fresh/frozen)

Chemicals/Reagents

Acid citrate dextrose solution B (ACD) (for freshly drawn or frozen blood samples)

0.48% W/V citric acid

1.32% W/V sodium citrate

1.47% W/V glucose

Lysis Buffer

10 mM Tris-Cl (pH 8.0),

0.1M EDTA (pH 8.0)

0.5% SDS

20 µg/mL DNase-free Pancreatic RNase

10M Ammonium acetate (used as an alternative to dialysis, step 9)

Dialysis Buffer

50 mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0) (used as an alternative to ethanol precipitation Step 9)

Phosphate-buffered saline (PBS for frozen blood samples)

Ethanol (used as an alternative to dialysis)

Phenol equilibrated with 0.5 M Tris-HCl (pH 8.0)

TE

Proteinase K (20mg/mL)

Equipment

Magnetic stirrer, pH meter, orbital shaker (incubator), micropipettes, vortex mixer (optional), hot plate/microwave oven, autoclave. Aspiration device attached to a vacuum line equipped with traps, water bath at room temperature and at 37°C, dialysis tubing, dialysis tubing clips, Rocking platform, tube mixer or roller apparatus, wide-bore pipettes (0.3 cm diameter orifice), Sorvall H1000B rotor (or equivalent) and centrifuge tubes cooled to 4°C for freshly drawn blood samples, Sorvall 55-34 rotor (or equivalent) and centrifuge tubes cooled to 4°C for frozen blood samples.

Glassware/Plastic Ware

Conical flask, measuring cylinder, beaker, culture flasks/tubes, 15 mL and 50 mL centrifuge tubes, microtips, disposable gloves.

Procedure

Collection: Human blood should be withdrawn under sterile conditions by a trained phlebotomist. Samples of blood can be either fresh or frozen.

Collection of Cells from Freshly Drawn Blood

A1. Collect 20 mL fresh blood in a tube containing either 3.5 mL acid citrate dextrose solution B (ACD) or EDTA.

NOTE: DNA can be prepared from fresh blood or from stored blood samples either for several days at 0°C or indefinitely at -70°C. It should be noted that downstream reactions like polymerase chain reaction get inhibited in case when blood was collected into a tube containing heparin. Hence, Heparin should never be used to collect blood.

A2. Take the blood in a centrifuge tube and spin in a Sorvall Hl000B rotor in a 50-mL swinging bucket at 1300Xg (2500 rpm) for 15 minutes at 4°C.

A3. Discard supernatant with a Pasteur pipette. The buffy coat is very carefully withdrawn and transferred to another centrifuge tube and spun again for the same time at the same speed. The pellet containing the red blood cells is discarded.

NOTE: The buffy coat consists of white blood cells of heterogeneous density.

A4. Use an aspirator to suck the residual supernatant from the buffy coat. Resuspend the buffy coat by adding 15 mL of lysis buffer. The solution is incubated for 1 hour at 37°C before proceeding to Step 2.

Collection of Cells from Frozen Blood Samples

B1. 20 ml of freshly withdrawn blood is collected in a tube containing either 3.5 mL acid citrate dextrose solution B (ACD) or EDTA, followed by storage at 0°C for several days or at -70°C for an indefinite period.

B2. In a water bath at room temperature, thaw the frozen blood sample and then transfer it to a centrifuge tube. Add an equal volume of phosphate-buffered saline at room temperature.

B3. Centrifuge tube in a Sorvall SS-34 rotor at 3500Xg (5400 rpm) for 15 minutes at room temperature.

B4. Aspirate the lysed red cells present in the supernatant. Add l5 mL of lysis buffer to resuspend the pellet. Keep the solution at 37°C for 1 hour and then proceed to the step 2.

Treatment of Lysate with Proteinase K and Phenol

- Transfer the lysate either from step A4 or from B4 to one or more centrifuge tubes in such a way that the volume of the lysate should not exceed one-third of the volume of the centrifuge tube.
- Add proteinase K (20 mg/mL) to a final concentration of 100 µg/mL. Mix by gently stirring the solution containing the enzyme and the viscous lysate using a glass rod.

- Keep the tube in a 50°C water bath and incubate the lysate for 3 hours. Intermittently swirl the tube of viscous solution.
- Place the tube on the working bench and let it stand at room temperature to cool down. Add an equal volume of phenol equilibrated with 0.1 M Tris-HCl (pH 8.0). Mix very gently, either placing the tube on a roller apparatus or on a tube mixer that mixes the solution by turning up and down for l 0 minutes or, in some cases, for 1 hour if the emulsion of the phases has not been formed.

NOTE: The concentration of EDTA in the lysis buffer plays a vital role in the separation of the phases during phenol extraction. The presence of 0.1 M EDTA permits easier separation of the phenolic and aqueous phases in contrast to 0.5 M EDTA (pH 8.0). In the latter case, separation of the two phases becomes difficult as the density of phenol and that of the buffer matches. Moreover, it has also been determined that DNA degradation by nucleases and heavy metals is minimized when using 0.1 M EDTA in the lysis buffer, hence providing a high degree of protection.

- Use a Sorvall SS-34 rotor to spin the tubes at 5000Xg (6500 rpm) for 15 minutes at room temperature
- Transfer the viscous aqueous phase to a fresh centrifuge tube. This phase is very slowly withdrawn with a wide-bore pipette of 0.3 cm diameter orifice. Take care not to disturb the interface, and ensure that hydrodynamic shearing forces are minimal.

NOTE: When the DNA solution is too viscous to be withdrawn, then it is wise to remove the organic phase with a long pipette, which is attached to an aspirator.

- Centrifuge the DNA solution at 5000Xg (6500 rpm in a Sorvall SS-34 rotor) for 20 minutes at room temperature. Protein and clots of DNA will sediment to the bottom of the tube. Transfer the supernatant containing the genomic DNA solution into a 50-mL centrifuge tube, leaving behind the protein and clots of DNA.
- Repeat the extraction with phenol twice and pool the aqueous phases from both steps.
- Two methods are employed to recover DNA. They are as follows.

Recovery of 150-200 kb Average-Sized Genomic DNA

- In a dialysis bag, pool all the aqueous phases. Use a dialysis tubing clip to close the bag, making sure that there is enough room in it. This is particularly done to accommodate an increase of the sample volume up to 1.5-2-fold during dialysis.

- At 4°C, carry out dialysis of the solution against 4 liters of dialysis buffer. Give 3 changes of the buffer at intervals of ≥ 6 hours.

NOTE: Time taken for complete dialysis is usually ≥24 hours. This is due to the high viscosity of the DNA solution.

Recovery of 100-150 kb Average-Sized Genomic DNA

- Pool all the aqueous phases into a centrifuge tube after the third phenol extraction. Add 0.2 volume of 10 M ammonium acetate and 2 volumes of ethanol at room temperature. Thoroughly mix the contents of the tube by swirling motion. A white precipitate of DNA will be observed immediately.
- Using a glass rod or Shepherd's crook (a Pasteur pipette whose end has been sealed and shaped into a U), spool the DNA from the ethanolic solution. This is done in such a way that the DNA is removed in one piece as a thread, leaving behind the oligonucleotides that are present as contaminants.
- Using a Sorvall SS-34 centrifuge, spin the tube containing the sample at 5000 X g (6500 rpm) for 5 minutes at room temperature. Discard the supernatant. Take precipitate.
- Add 70% ethanol to the precipitate and mix well. Centrifuge for 5 minutes at room temperature at 5000Xg (6500 rpm in a Sorvall SS-34). DNA will precipitate. Discard supernatant. Add 70% ethanol to wash the precipitate. This is done twice.
- Decant the contents of the tube slowly and remove all the remaining traces of 70% ethanol by aspiration. Air dry the pellet at room temperature by leaving the tube open on the lab bench until all the ethanol has evaporated.

NOTE: Resuspend the pellet when it turns from white to transparent. Otherwise, it is extremely difficult to dissolve desiccated DNA. Avoid drying the pellet completely.

- l mL of TE (pH 8.0) is used to dissolve the pellet when 0.1 mL cells are the starting material. Gentle rocking the contents of the tube helps to dissolve the DNA completely. In that case, the tube is placed on the rocking platform and rocked gently for 12-24 hours at 4°C. Make sure that the DNA has completely dissolved and store it at 4°C.
- Determine the DNA concentration of the sample.

Observation and Results

DNA isolated is then electrophoresed in agarose gels and observed under a UV Trans-illuminator to check the presence of DNA bands. The presence of the bands

is captured by the gel documentation system.

Precautions

- Preheat the water bath to 37°C before starting the procedure.
- Thoroughly mix each of the reagents. All the reagents should be checked for any precipitate that might form at the bottom of a bottle containing a solution. The reagent bottle can be placed in a water bath maintained at 55-65 °C and then cooled to room temperature (15-25 °C). Recheck for any precipitate before use.
- Use autoclaved tubes and tips for the procedure.

- Sterile conditions should be maintained while collecting the blood in an anticoagulant-coated tube (*e.g.*, EDTA). Heparin should not be used as an anticoagulant as this will inhibit the polymerase chain reaction using the genomic DNA as a template in the downstream step.

SPECIAL INFORMATION PANEL: MINIMIZING DAMAGE TO LARGE DNA MOLECULES

• High-molecular-weight (> 100 kb) DNA molecules are easily broken by shearing and other mechanical forces.
• Breakage and damage to the long DNA molecules can be caused during steps like extraction with organic solvents and precipitation with ethanol or butanol, excessively rapid and prolonged pipetting, and vigorous shaking [2, 3].
• The high molecular weight DNA molecules are rigid. Hence, they are vulnerable to shearing forces, which can cause double-stranded breaks. DNA shearing can occur during the vortexing or shaking of a solution, when solutions are being drawn into or expelled from pipettes with small diameters, or when dissolving long DNA molecules. All these activities can spur up the velocity gradient in the solution, causing the DNA to be sheared.
• General ways to minimize shearing are the following [2, 3].
  - Mix by shaking rather than stirring DNA solutions.
  - DNA concentration should be maintained at a higher level.
  - Electrostatic forces are reduced by using a high ionic strength buffer
  - Large DNA molecules become stiff.
  - Add spermine or polylysine, which are condensing agents.
  - Use agarose blocks to isolate and manipulate DNA.

4.5. Recipes Of Reagents And Solutions

Saline-EDTA

0.15M NaCl,

0.1M EDTA adjusted to pH 8.0)

CTAB Extraction Solution

2% (W/V) CTAB

100 mM Tris-HCl, pH 8.0

20 mM EDTA, pH 8.0

1.4 M NaCl

Store at room temperature (stable for several years)

CTAB Precipitation Solution

1% (W/V) CTAB

50 mM Tris-HCl, pH 8.0

10 mM EDTA, pH 8.0

Store at room temperature (stable for several years)

Extraction Buffer

100 mM Tris-HCl, pH 8.0

100 mM EDTA, pH 8.0

250 mM NaCl

100 μg/mL proteinase K (add fresh before use)

Store indefinitely at room temperature without proteinase K

High-salt TE Buffer

10 mM Tris-HCl, pH 8.0

0.1 mM EDTA, pH 8.0

1 M NaCl

Store at room temperature (stable for several years)

CTAB/NaCl Solution (10% CTAB in 0.7 M NaCl)

Dissolve 4.1 g NaCl in 80 mL water and slowly add 10 g CTAB (hexadecyltrimethyl-ammonium bromide) while heating and stirring. If necessary, heat to 65°C to dissolve. Adjust the final volume to 100 mL.

TE (Tris.Cl/EDTA) Buffer

10 mM Tris.Cl, pH 8.0

1 mM EDTA, pH 8.0

Acid Citrate Dextrose Solution B (ACD) (for Freshly Drawn or Frozen Blood Samples)

0.48% W/V citric acid

1.32% W/V sodium citrate

1.47% W/V glucose

Lysis Buffer

10 mM Tris-HCl (pH 8.0)

0.1 M EDTA (pH 8.0)

0.5% SDS

20 µg/mL DNase-free Pancreatic RNase. (Add the three ingredients in advance and store at room temperature. RNase is added to an appropriate amount of the lysis mixture just before use.

Dialysis buffer

50 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Prepare four lots of 4 liters of dialysis solution and store them at 4°C

CONCLUSION

Isolation and purification of eukaryotic genomic DNA from bacteria, plant tissues, and blood samples have been covered in this chapter. Genomic DNA, being large in size, is liable to break during the isolation procedures, which are minimized by various precautionary techniques mentioned here. While working on the protocols, one can find a thorough description and rationale of each step. To make this chapter user-friendly, the preparation and composition of the complex reagents are mentioned in the last section.

FURTHER READING

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CHAPTER 5

Analysis of DNA

Abstract: Analysis of isolated genomic and plasmid DNA samples is critical and vital to assess their quality and quantity. Here, we have included methods to analyze the plasmid and genomic DNA samples by gel electrophoresis and spectrophotometric methods. The principles and factors affecting both agarose and polyacrylamide gel electrophoresis are discussed. Along with this, protocols are cited for analyzing and recovering DNA from agarose and polyacrylamide gels. The recipe for the buffers and solutions required for each are mentioned for the convenience of beginner experimenters. Spectrophotometric estimation of isolated DNA, included at the end of the chapter, will provide insight into the purity of the DNA sample.

Keywords: Absorbance, Agarose gel electrophoresis, Extinction coefficient, Polyacrylamide gel electrophoresis, Spectrophotometer, TAE, TBE.

5.1. INTRODUCTION

It is important that following the isolation of plasmid DNA (described in ***CHAPTER 3***) or genomic DNA (described in ***CHAPTER 4***), the DNA samples must be examined and analyzed for their quality and quantity before subjecting them to further downstream applications. This examination and analysis are crucial for many downstream applications, such as analysis by restriction endonuclease digestion and the amplification of the target DNA by polymerase chain reaction. The two most commonly used methods widely in molecular biology laboratories to analyze nucleic acids are i) gel electrophoresis and ii) spectrophotometric analysis.

Gel electrophoresis involves the movement of ions and electrically charged macromolecules through a porous matrix when an electric field across the matrix is applied. The most commonly used matrices in the gel electrophoresis of nucleic acids are agarose and polyacrylamide. The choice of these gel matrices and a given concentration of gel depends on the size of nucleic acid molecules, as the concentration of the agarose or acrylamide determines their pores sizes and subsequently further dictates the mobility of a given DNA molecule through these matrices. Agarose gel electrophoresis takes advantage of the uniform negative

charge of all kinds of DNA due to the presence of phosphate groups. Therefore, the mobility of a given kind of DNA observed in agarose gels accurately reflects its molecular mass when migrating in an electric field [1, 3]. Therefore, agarose gel electrophoresis is the method of choice used for the separation of nucleic acids based on their sizes, for the quantification and purification of a specific nucleic acid fragment from their mixture, and for the analysis of DNA restriction fragments. Polyacrylamide gel electrophoresis (PAGE) consists of another method of separating DNA fragments/proteins in an electric field that relies on different kinds of matrix made from the polymerization of acrylamide, which also depends on size, structure, and molecular weight (MW). Notably, PAGE provides a very high resolution of DNA molecules from as low as 10 bp to 3,000 bp long. DNA mobility observed in polyacrylamide gels is essentially independent of the electric field strength used for electrophoresis [3].

The nitrogenous bases of DNA absorb ultraviolet rays maximally at the wavelength of 260 nm. This is the principle on which spectrophotometric analysis is based. According to Lambert-Beer law, the amount of absorption of UV radiation by the nitrogenous bases is directly proportional to the extinction coefficient, the concentration of the chromophore, and the path-length through which the UV light passes (see ***UNIT 5.8***) [3]. When the extinction co-efficient (since DNA is the chromophore in every case) and path-length (since a single type of cuvette is always used) are held constant, the amount of UV radiation remains directly proportional to the concentration within a linear range of absorption values (see ***UNIT 5.8***). Using this rationale, the concentration of an unknown sample of plasmid or genomic DNA can be determined precisely from which quantity and purity of the DNA may be deduced. Most of the downstream processes, like restriction digestion, PCR, *etc.*, require pure and a specific quantity of DNA, which can be determined spectrophotometrically.

5.2. SEPARATION AND PURIFICATION OF DNA FRAGMENTS IN AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis in an agarose gel is a basic but highly effective and widely used method for the separation, identification, and purification of 0.5 kb to 25 kb DNA fragments. Essentially, this method involves the preparation of an agarose gel whose concentration depends on the size of the DNA fragments to be separated. This is followed by the introduction of the DNA samples to be analyzed into the sample wells (dubbed loading) and electrophoresis for a specific time that separates/resolves the DNA fragments according to their size [3, 4]. Finally, the gel is stained with ethidium bromide to visualize the separated and resolved DNA bands (Fig. **1**).

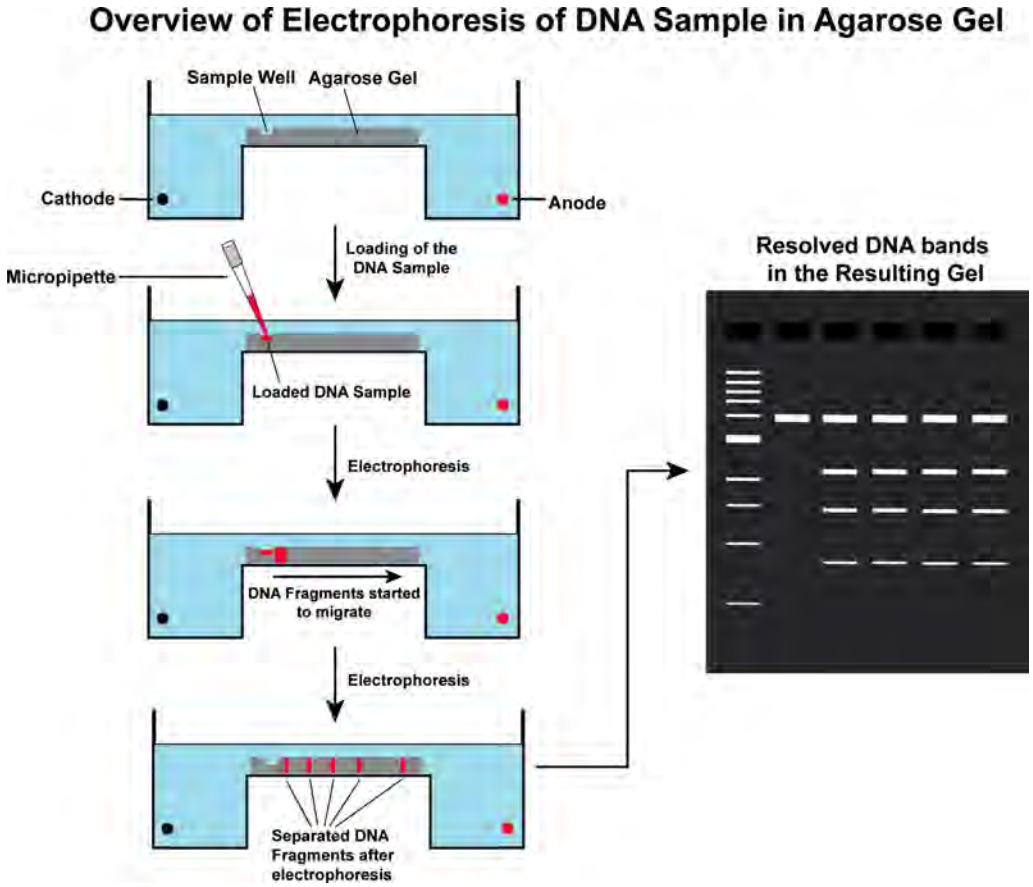

Fig. (1). Schematic diagram showing different steps involved in agarose gel electrophoresis.

Agarose as a Gel Matrix

Agarose is a linear polysaccharide that consists of alternative residues of D-galactose and 3, 6-anhydro-L-galactopyranose joined by α(1→3) and β(1→4) glycosidic linkages (Refer to Fig. **2**) that are isolated and purified from agar or agar-bearing marine algae (sea kelp). At high temperatures, agarose forms helical fibers. When cooled, these helical fibers get supercoiled with a radius of 20-30 nm, ultimately leading to the formation of a web-like structure consisting of cylindrical paths or channels. The diameters of these channels vary from 50 nm to> 200 nm [2 - 4].

When agarose is dissolved in boiling water, it leads to the complete dissolution of solid agarose powder in the aqueous medium. Subsequent cooling of this liquid medium below 45°C leads to the appearance of a semi-solid gel matrix owing to the formation of extensive hydrogen bonds between the chains of agarose

molecules. The concentration of agarose dictates the pore size that will be formed during the gelling process. Large pores result from a low concentration of agarose that allows high molecular weight DNA molecules to move efficiently. Conversely, low molecular weight DNA can move through smaller pores, which are formed using a high concentration of agarose. Agarose with even larger diffusion pores are used in gel electrophoresis to allow the passage of very large molecules of DNA. Low electro-endosmotic agarose is preferred for analytical purposes to separate DNA [2 - 4].

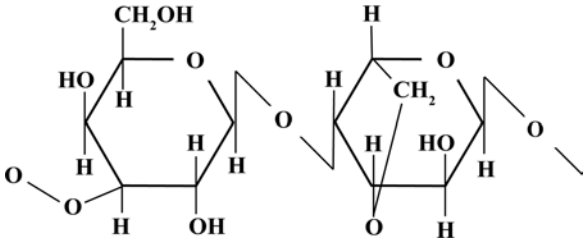

Fig. (2). Chemical structure of agarose.

5.3. Important Aspects Relevant to the Analysis of DNA Molecules in Agarose Gel

In this procedure, a slab of agarose gel matrix is first prepared (by a process called gel casting) by boiling the agarose powder in 1X (typically, working concentration is indicated by 1X) of electrophoresis buffers (either in TAE or TBE buffer - see ***RECIPES*** at the end of the chapter for their compositions). During the casting of the gel slab, a series of square slots/wells are formed at one side of the slab using a device called a comb. The DNA that needs to be analyzed is subsequently loaded in these slots/wells and electrophoresed [2 - 4].

Since the ubiquitous presence of DNase/RNase in normal water can degrade the DNA/RNA molecules, specific electrophoresis buffers such as TAE (Tris-acetate-EDTA) or TBE (Tris-borate-EDTA) prepared in deionized (such as Milli-Q®) water are generally used to prepare the gel. Furthermore, to prevent the DNA samples from being floated out from the well/slots during the loading procedure prior to electrophoresis, DNA is first mixed with a loading dye solution. This dye solution contains glycerol, ficoll, or sucrose, which makes the sample heavier than the electrophoresis buffer and allows the sample (along with loading dye) to sink into the bottom of the well. Furthermore, the loading buffer also contains a tracking dye (bromophenol blue and xylene cyanol), which allows visualization of the track of the travel path of the DNA during electrophoresis since, otherwise, the DNA sample is typically colorless. The track made by the path of the tracking dye while the gel is running (also called the dye front) enables the user to follow the approximate distance migrated by a given DNA sample [3, 4].

Finally, to visualize DNA, agarose gels are usually stained with ethidium bromide, a DNA intercalating agent, and illuminated with UV-β rays (280-320 nm). Part of the energy of this UV is absorbed by the DNA ethidium bromide complex, and the rest is transferred to the ethidium bromide molecule (due to their physical proximity owing to intercalation). Ethidium bromide subsequently absorbs this light energy and eventually fluoresces back in the range of 590-620 nm light (red-orange range of the visible spectrum). As a result, the DNA-ethidium bromide complex appears as an orange-colored band inside the gel. The size of a DNA sample can be deduced from its extent of migration through the gel, and typically, this is accomplished *via* the migration of a standard DNA marker (called DNA Ladder). DNA and RNA size markers contain a mixture of DNA (or RNA) fragments of known length, making them suitable for estimating the fragment length of concurrently run unknown DNA of interest and its relative quantity. This is done by comparing the relative band intensities of the known and unknown DNA bands. Notably, this technique can also be employed to isolate any particular DNA fragments by using various techniques, including phenol extraction, freeze-squeeze of gel slices, digestion of the gel using agarase, electroelution onto dialysis membranes or ion exchange paper, and electrophoresing through melting of low-gelling-point agarose followed by ion-exchange chromatography [3, 4].

SPECIAL INFORMATION PANEL: ETHIDIUM BROMIDE (EtBr)

• Ethidium bromide (3, 8-diamino-6-ethyl-5-phenylphenathridium bromide) has been playing a very significant role in molecular biology for a long time.
• Its property to intercalate between the stacked bases of double-stranded nucleic acids allows it to be used in the direct visualization of DNA in gels placed in UV light.
• One ethidium molecule gets intercalated per 2.5 bp in the helix, independent of the base composition of the DNA. The planar stacked tricyclic phenanthridine ring system of the drug lies perpendicular to the helical axis and makes van der Waals contact with the base pairs above and below. The base pairs get displaced by 3.4°A along the helix axis, which causes a 27% increase in the length of double-stranded DNA.
• The geometry of the bound dye is such that it displays an increased fluorescent yield compared to the dye in free solution. Ultraviolet (UV) radiation at 254 nm is absorbed by the DNA and transmitted to the dye, while radiation at 302 nm and 366 nm is absorbed by the dye itself. In both cases, a fraction of energy is re-emitted at 590 nm in the red-orange region of the visible spectrum.
• As more drug is added, more uncoiling of the helix takes place at the point of insertion of the drug, forcing the circles to form left-handed from right-handed supercoils and a change in the average pitch of the helix and increase in the number of base pairs per turn. The binding affinity of the dye for closed circular DNA decreases progressively as the number of reversed supercoils increases, and hence, closed circular DNAs bind less drug at saturation than nicked circles.
• From 1966 to the mid-l980s, the only reliable method available to purify closed circular DNAs was equilibrium CsCl-ethidium bromide centrifugation (See special information panel on CsCl in **Chapter 4**). The buoyancy of DNA in CsCl density gradients reduces upon binding of the dye. The magnitude of this decrease is a function of the average number of drug molecules bound per base pair. Because of the restricted binding of ethidium bromide to positively supercoiled DNAs, closed circular DNAs come to equilibrium at a denser position (-1.59 g/cc) in CsCl-ethidium bromide gradients than linear or nicked DNAs (1.55 g/cc).
• The reaction of EtBr with DNA is reversible but takes a longer time (in days) to get dissociated. The dissociation can also be achieved by passing the solution through a cation exchange resin or by extraction with either isopropanol or n-butanol.
• Ethidium bromide also binds with RNA, heat-denatured or single-stranded DNA, and the cyclic-coil form of closed circular DNA.

5.4. FACTORS INFLUENCING THE MIGRATION OF DNA MOLECULES THROUGH AGAROSE GEL

DNA is a negatively charged molecule, and when an electric field is applied, it moves through an agarose matrix towards the positively charged anode. The electrophoretic migration rate of DNA through agarose gel depends on the following parameters: 1) size of the DNA molecule, 2) agarose concentration (Table **1**), 3) DNA conformation, 4) voltage applied, 5) presence of ethidium bromide, 6) type of agarose, and 7) electrophoresis [2, 3].

Table 1. Suitable agarose concentrations for making gel to separate DNA fragments of various sizes.

Concentrations of Agarose (%)	Size of DNA Fragments (Kb = 1000 bp)
0.5%	1 kb to 30 kb
0.7%	800 bp to 12 kb
1.0%	500 bp to 10 kb
1.2%	400 bp to 7 kb
1.5%	200 bp to 3 kb
2.0%	50 bp to 2 kb

- **Size of the DNA molecule:** DNA molecules, being negatively charged, migrate through an agarose matrix towards the anode at a rate that is inversely proportional to their molecular weight. A double-stranded DNA moves at a rate that is inversely proportional to the logarithm of the number of base pairs. Hence, smaller molecules travel faster than larger molecules in gel, which takes a longer time as they experience frictional drag [3, 4] while moving in the gel.
- **Agarose Concentration**: At different concentrations of agarose, the migration of a linear DNA fragment of a fixed size varies (Table **1**). A linear relationship exists between the logarithm of the electrophoretic mobility of the DNA (μ) and the gel concentration (τ) [3, 4]. This relation is mathematically expressed by the following equation:

$$\log \mu = \log \mu_0 - K_r\tau$$

where μ_0 is the free electrophoretic mobility of the DNA, and K_r is the retardation co-efficient (a constant related to the gelling properties of the agarose and the size and shape of the molecules migrating through the gel) [3].

- **DNA Confirmation**: DNA molecules *in vivo* and *in vitro* exist in divergent

topological forms that include super helical circular (form I), nicked circular (form II), and linear (form III) DNAs. The rate of migration of the different forms of DNA will depend on the concentration and type of agarose used. Interestingly, their migration is also governed by the strength of the applied current, the ionic strength of the electrophoresis buffer, and the density of superhelical twists in the form I DNA. Under many conditions, superhelical (form I) DNA migrates fastest, followed by the linear (form III) and nicked circular (form II) DNA [2 - 4].

- **Voltage applied:** The higher the voltage, the faster the DNA will travel through the gel. However, voltages that are too high can possibly melt the gel or cause smearing or distortion of DNA bands. At low voltage, the movement of linear DNA is proportional to the voltage applied. However, as the strength of the electric field is increased, the mobility of high-molecular-weight fragments does not follow linearity. DNA migration rate decreases when voltage is set constant, which in turn causes the current and wattage to decrease with a consequent increase in the resistance. If the resistance increases dramatically, the current and wattage will drop as the voltage cannot increase. The power supply will shut off unable to compensate in such a situation [3, 4].
- **Presence of ethidium bromide**: Ethidium bromide, which intercalates with DNA, can change the charge density and length, as well as the superhelicity of the DNA molecule; therefore, its presence in the gel during electrophoresis can affect its movement [3]. For example, the positive charge of ethidium bromide can reduce the DNA movement by 15%.
- **Type of agarose**: The migration rate of a DNA molecule decreases as the concentration of agarose in the gel increases. Researchers commonly adjust the agarose concentration to optimize the resolution of DNA molecules within a particular size range. Generally, two types of agarose are available: regular and low melting types. The voltage to be applied for low melting agarose should be low in order to avoid heat generation, in which case the gel will melt.
- **Electrophoresis buffer**: The mobility of DNA through the gel depends on the composition and the ionic strength of the buffer. Hence, DNA will not move or move very slowly if the buffer is replaced by water. The most common buffers used are Tris-acetate EDTA buffer (TAE) and Tris-borate EDTA buffer (TBE) [3]. However, the mobility of DNA in TBE buffer is approximately two times slower than in TAE buffer. This is due to the lower porosity of agarose gel when agarose polymerizes in the presence of borate ions. Varying the agarose concentration and/or buffer, approximately in the size range of 20-50 000 bp, double-stranded DNA can be separated reliably.

PROTOCOL 1: RESOLUTION OF DNA FRAGMENTS ON STANDARD AGAROSE GELS

Materials

Plasmid and genomic DNA sample.

Chemicals/Reagents

Electrophoresis Buffer: TAE/TBE buffer –50X (stock solution) working solution – 1X

(Tris, glacial acetic acid, EDTA, NaOH/ Tris, boric acid. EDTA) (See ***APPENDIX A.1***)

Molecular biology grade agarose powder

6X gel loading dye (See ***APPENDIX-A.1***)

Ethidium bromide solution (10 mg/mL and 0.5 mg/mL stock solutions) (See ***APPENDIX-A.1***)

DNA standard size marker (100 kb/1 kb ladder DNA)

Distilled water.

Equipment

Submarine gel electrophoretic system with DC power supply unit, gel casting platform/tray, gel casting comb, gel documentation system, UV trans-illuminator with face shield or goggles.

Glassware/Plasticware

Micropipettes (Accupipette T-20 or Gilson-P-20), tissue paper, parafilm, cello tape or gel casting tape, *etc.*

PROTOCOL 5.1.A. PREPARING THE AGAROSE GEL

Reagents

1X TAE, 1X TBE, or 0.5 X TBE electrophoresis buffer.

Agarose powder

Ethidium bromide (10 mg/mL stock) (See ***APPENDIX-A.1***)

Equipment

Microwave oven or hotplate, fine-weighing balance.

Procedure

- Pour 100 mL of 1X TAE or TBE buffer into a 250 mL Erlenmeyer flask. The buffer used for dissolving agarose should be the same as the running buffer.
- Weigh out an appropriate amount of agarose powder into the flask containing 1X TAE/TBE. Therefore, to prepare 100 mL of a 0.8% agarose solution, weigh out 0.8 g agarose into a glass beaker or flask that contains 1X electrophoresis buffer. Mix by swirling the flask.

Note: The volume of the buffer should not exceed 1/3 volume of the capacity of the flask. The concentration of agarose in a gel should be dependent on the ranges of sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2%.

- Dissolve the agarose by boiling the mixture either in the microwave or stirred on a hot plate until the solution becomes clear. At every 30-second interval, the flask is removed, and the contents are mixed well by swirling. Repeat until the agarose has completely dissolved. This is done so that any air bubbles generated during the heating process will be removed.

Note: Removal of bubbles is important since the presence of bubbles in a hot and viscous liquid leads to accidental bumping of the agarose solution. Removal of the air bubbles prevents the solution from bumping out.

- Allow the solution to cool down to about 55°C. Boiling will cause evaporation of the buffer, which will reduce the total initial volume, leading to an increase in the concentration of agarose and buffer components. Therefore, to restore the final concentration of the buffer, sterile water should be used to make up the volume to 100 mL.
- Add ethidium bromide (EtBr) 0.5 mg/mL (1000X stock) to a final concentration of 0.5 μg/mL to the agarose solution. For 100 mL of 0.8% gel mix, add 100 μL of 0.5 mg/mL EtBr stock. Alternatively, the gel may also be stained after electrophoresis in a running buffer containing 0.5 μg/mL EtBr for 15-30 min, followed by destaining the gel in 250 mL of distilled water for an equal length of time (see below).

NOTE: CAUTION: Ethidium bromide should be handled very carefully as it is a mutagen and potential carcinogen. Appropriate clothing like lab coats and gloves should be worn while working with it.

- Allow the agarose to cool either on the benchtop or by incubation in a 55°C water bath. If the agarose solution is too hot, it may distort the gel tray.

NOTE: Before pouring onto the gel platform, melted agarose must be cooled to 55°C in a water bath. This can otherwise cause the gel apparatus to become distorted/warped. Remember to keep in mind that the volume of the sample wells will be determined by both the thickness of the gel and the size of the gel comb.

Precautions

- Always wear eye protection, either a face shield or goggles.
- EtBr is a carcinogen and must be properly disposed of per institution regulations.
- Gloves should always be worn when handling gels, buffers, or any solutions containing EtBr. Although alternative DNA staining dyes are available, due to their low cost and sensitivity, EtBr is the most popular and is widely used.
- The beaker containing the agarose and buffer is heated in the microwave oven on high power until bubbles appear and may become superheated and foamy when agitated. Care should be taken while handling this solution to prevent burns. Always use heat-resistant gloves when handling a hot flask containing agarose gel.

PROTOCOL 2: CASTING A HORIZONTAL AGAROSE GEL AND SEPARATION OF DNA FRAGMENTS

Typically, 3-4 mm thick horizontal gels are routinely cast for optimal resolution. As mentioned above, the concentration of the agarose to be chosen will depend on the size range of the DNA fragments to be determined. The amount of the gel solution required can be determined by multiplying the surface area of the casting chamber and the gel thickness.

Materials

Agarose solution (prepared as described in ***PROTOCOL 5.1.A***.)

Electrophoresis buffer (TAE or TBE)

Equipment

Horizontal electrophoresis apparatus plus accessories (*e.g.*, power supply, gel plate, comb)

Tape for sealing

Gel tray leveler

Procedure

Setting up of Gel Apparatus

- Clean the gel tray and comb it with a dilute solution of phosphate-free detergent, followed by thoroughly rinsing and cleaning with distilled water. Check the teeth of the comb for the presence of a thin film of residual dried agarose that will cause the wells to be deformed. Dried agarose can be removed by scrubbing the comb with a lint-free tissue soaked in hot distilled water.
- Prepare the gel tray by carefully sealing the two open edges of the tray to create a mold. Alternatively, place the gel tray into the casting apparatus that is equipped with a sealing rubber gasket, which will automatically seal the two open edges.

NOTE: Most gel trays are sealed by taping the open ends with adhesive tape. To prevent leakage, apply a small amount of melted agarose either with a pipet tip or a Pasteur pipet at the edges of the tray along the edges of the adhesive tape and allow it to harden. This will prevent leakage when the bulk volume of the agarose is poured on the gel casting tray.

- Take an appropriate-sized comb and place it into the gel tray perpendicular to the longer arm of the tray at a place away from 1 cm from one end. Pay attention during placement of the comb so that the teeth of the comb remain vertical and the bottom edge of the teeth of the comb remains 1-2 mm above the surface of the tray and should not touch the surface.

NOTE: Most gel platforms are designed in such a way that a minimum of 0.5 to 1 mm space remains between the comb and the gel casting tray. This space is filled with agarose, which in turn confirms that the wells are completely sealed and also prevents the tearing of the wells while the comb is removed. Agarose gels are set at 4°C as they are made with low melting temperatures. Being very soft, this temperature helps prevent the tearing of the gel and, at the same time, provides rigidity.

- Level the gel tray with a bubble leveler and pour the agarose solution cooled to ~ 50°C into the gel tray. Allow 20 minutes for the agarose to solidify at room temperature to form a semi-solid gel slab.

NOTE I: The initial volume of the agarose should be chosen in such a way that the final thickness of the final agarose slab gel varies between 0.3 and 0.5 cm thick. It is important to remember that the capacity of the sample wells that hold the final DNA sample will depend both on the thickness of the gel and the comb used.

NOTE II: Since gels made from Low-melting-temperature and intermediate-melting-temperature agarose are easily broken, an additional 30 mins at 4°C is required for their gelling. This not only increases the rigidity but also can be handled easily.

- Remove the comb by pulling it vertically upward sharply but gently. Failing to remove the comb gently may cause distorted well formation and may lead to leakage of the sample from the bottom. Remove the tape used for sealing the two open edges. Now, the gel is ready to be electrophoresed. At this stage, the gel may be stored at 4°C by wrapping the gel with a plastic/Saran wrap until used.
- In the electrophoresis chamber, lay down the gel casting tray containing the casted gel with the wells facing the cathode.
- Fill the electrophoresis tank with the buffer (1X TAE or TBE) until that level is reached when the surface of the buffer covers the surface of the gel and the wells are submerged. Use the same buffer that has been used to prepare the agarose.
- Prior to loading the samples, it is important to remove any loose gel fragments from the wells by flushing out the wells with electrophoresis buffer gently using either a Pasteur pipette or a micro-tip fitted to a micropipette.
- Close the chamber by tightly fitting the lid of the gel box. Attach the red and black leads to the power supply. Turn on the power supply and electrophorese briefly (This procedure is known as pre-run). This is done to make sure that both the gel box and power supply are working.

NOTE: The resolution of the gel depends on the thickness of the comb used while casting the gel. Sharper DNA bands are observed when a thin comb (1 mm thick) is used. Whereas DNA bands appear broader when using a thicker comb. However, a greater volume of DNA samples can be loaded in the well when using a thicker comb.

Separation of DNA Fragments

- Prepare the DNA samples for electrophoresis: Mix 1-2 μL of 6X gel loading dye with 5-10 μL of DNA solution on a piece of parafilm and mix well by pipetting up and down several times. 5-12 μL of DNA plus loading dye sample is loaded in every well (for mini-gel).
- A sample of agarose gel stained with ethidium bromide showing the different DNA fragments that appeared as fluorescent bands. Lane 1: Undigested plasmid DNA samples. Lanes 2-3 and 5-7: same DNA digested with different restriction enzymes, Lane 4: Standard DNA Marker 1 kb ladder.
- Close the lid of the gel box. The cathode (black leads) should be closer to the wells than the anode (red leads). Double check that the electrodes are plugged

into the correct slots in the power supply.
- Electrophorese at 50-150 volts (5-8 volts per centimeter) for 60 to 90 minutes or until the bromophenol blue dye marker from the loading dye migrated an appropriate distance, depending on the size of DNA to be visualized or the tracking dye covers more than ¾ distance in the gel.

NOTE: To prevent electrical shocks, the gel apparatus should always be covered and kept away from heavily used work spaces.

Observing Separated DNA Fragments

- The power supply is turned off when electrophoresis is completed; the lid of the gel box is then removed.
- Remove the gel tray from the gel box. Lift the gel along with the gel-casting tray by draining off excess buffer from the surface of the gel. Place on paper towels to absorb any extra running buffer if necessary.
- Place the gel on the quartz surface of a UV-trans-illuminator to expose the DNA sample in the gel to short-wave UV light if the DNA sample loaded in the gel will not be used further for any downstream application. If, however, the sample

  DNA will be excised from the gel for purification and will be used further, please use a hand-held, long-wave UV light.

NOTE: If the gel is not stained with ethidium during the run, stain the gel in 0.5 μg/mL ethidium bromide until the DNA has taken up the dye. The gel can be stained post-electrophoresis procedure by placing the gel in the staining container containing a dilute solution of ethidium bromide (final concentration 0.5 μg/mL in water) and gently agitating for 10 to 30 minutes. If necessary, the gel can also be destained by shaking in water for an additional 30 min. This serves to remove excess ethidium bromide, which causes background fluorescence and makes visualization of small quantities of DNA difficult. DNA bands should finally appear as orange fluorescent bands. Take a photograph of the gel.

- Properly dispose of the gel and running buffer as per institution regulations.

Representative Results

DNA fragments are visible as clearly defined bands whose size can be determined by comparing them with the standard DNA ladder (Refer to Fig. **3**).

Hence, the DNA standard or ladder should be separated to a degree that allows for the useful determination of the sizes of sample bands.

PROTOCOL 3. STAINING DNA IN AGAROSE GELS WITH ETHIDIUM BROMIDE

Reagents

Ethidium bromide stock and working solutions (10 mg/ mL stock solutions and 0.5 mg/mL working solutions) (See ***APPENDIX I***).

Equipment

Staining vessels larger than the gel, UV trans-illuminator.

Procedure for Post-Staining Gels

- In a tray, prepare the staining solution. Add ethidium bromide to a final concentration of 0.5-1.0 mg/mL in distilled water or electrophoresis buffer. The volume should be enough to cover the surface of the agarose gel.
- Remove the gel from the gel casting tray.
- Stain the gel by placing it in the staining vessel containing ethidium bromide solution for 20 minutes under moderate shaking (50 rpm) condition.
- Destain the gel for 20 min by placing it in deionized water in a new container filled under moderate shaking (50 rpm) condition.
- Repeat step 4 using fresh deionized water.
- Visualize the DNA bands in the gel either with a hand-held or table top UV emitter.

Procedure for Inclusion of Ethidium Bromide in the Agarose Gel

- Prepare the agarose gel solution as described in **Protocol 5.1.A**.
- When the agarose solution has cooled down, add ethidium bromide to a final concentration of 0.1 to 0.5 μg/mL. Slowly, in a swirling motion, mix the solution and then cast the gel as described in **Protocol 5.1.B**.
- View the gel with either a hand-held or a tabletop UV emitter during or after electrophoresis,

Precaution

- Ethidium bromide is a powerful mutagen. Hence, proper precaution is needed while handling solutions of this dye. Gloves should be worn when working with stained gels.
- Appropriate protective clothing like gloves, lab coats, and shields for the eyes and skin should be worn when observing ethidium bromide-stained gels on a UV trans-illuminator.

5.5. SOME IMPORTANT CONSIDERATIONS

The choice between TAE and TBE

Using TAE or TBE buffer at a standard concentration, DNA fragments about 12-15 kb that do not need to be eluted from the gel can be visualized [2 - 4]. At a field strength of 1-2 V/cm, TAE-formed gels form larger pores. Longer electrophoretic runs reduce the tendency of the DNA to smear. On the other hand, TBE is used for small DNA molecules less than 1 kb. Agarose gel prepared with this buffer forms small pores resulting from interaction with agarose. In such a case, the DNA bands yielded are less broad due to less dispersion and diffusion in this tighter gel [3].

No matter what buffer is used during electrophoresis, the amount of buffer should be such that it should be 3-5 mm higher than the surface of the gel in a horizontal electrophoretic system to prevent the gel from drying out. On the other hand, too much buffer can result in excessive heating within the system, a decrease in DNA mobility, and band distortion [3].

Buffer Depletion

If the buffer gets depleted, then gel melting, overheating and/or DNA smearing are the indicators. The buffer and its buffering capacity determine how fast the buffer will get depleted [3, 4]. As the pKa of borate is closer to the initial pH of the buffer, 0.5X TBE buffer has a greater buffering capacity than a 1X TAE buffer at the pH used. The manufacturer specifications have to be consulted for different-sized electrophoresis chambers in regard to buffer depletion. For example, an electrophoresis chamber of 15 X 30 cm can withstand 40-50 W-h, whereas mini-electrophoresis chambers can withstand 10-13 W-h. If electrophoresis is done for a longer period of time or if the buffering capacity of the buffer used is low, then recirculating the buffer can reduce the effects of buffer depletion and also the development of a pH gradient [3].

Other Buffering Systems

Apart from TAE and TBE buffers, Tris-phosphate buffer (TPE), having a high buffering capacity, is another buffer that can also be used for DNA electrophoresis. One of the disadvantages of using this buffer is that phosphate ions may contaminate the recovered DNA samples, which might potentially further interfere with the downstream enzymatic reaction [3, 4].

Loading and Running DNA in an Agarose Gel

A critical factor in running a gel lies in the DNA quantity of the bands of interest, and therefore, DNA of variable amounts can be loaded per well. The following factors need consideration.

- Well volume.
- DNA fragment size—the capacity of the gel drops sharply as the fragment size increases, even over a few kb.
- Distribution of fragment sizes.
- Voltage gradient—higher voltage gradients are better suited for DNA fragments less than 1 kb, whereas lower voltage gradients are better suited for fragments greater than 1 kb [3, 4].

Ethidium bromide-containing gels can detect a minimum amount of 10 ng of DNA. The minimum amount that can be detected is 60 pg with a special stain such as GelStar nucleic acid stain (BioWhittaker Molecular Applications) or SYBR Green I stain (BioWhittaker Molecular Applications). 100 ng is the maximum amount of DNA that can be loaded. If the amount of DNA is overloaded, then it might result in trailing and smearing. This causes a problem when working with a larger size of the DNA [3, 4].

Loading Buffers

Gel loading buffers serve three purposes in DNA electrophoresis:

- The density of the sample increases, ensuring that the DNA settles evenly into the well.
- Simplify loading by adding color to the sample.
- The electrophoretic process can be monitored as the dyes migrate in an electric field toward the anode at predictable rates.

Loading buffers commonly used for agarose gel electrophoresis are usually prepared as 6X or 10X stock solutions [3, 4].

5.6. RECOVERY OF DNA FROM AGAROSE GELS

5.6.1. Introduction

Isolating a specific DNA fragment in pure form from a mixture of DNA fragments is important because the purified fragment is routinely used in multiple applications in molecular biology that include cloning, transfection, sequencing, *etc.* Generally, DNA fragments are obtained either by restriction enzyme digestion or by PCR amplification, which needs further purification. The typical

purification method involves the separation of DNA fragments either in agarose or polyacrylamide gels that result from restriction digestion and PCR amplification followed by excision of the desired DNA band from the gel matrix and liberation of the DNA from the matrix into the solution. Applications such as cloning, labeling, and sequencing of DNA frequently require purified DNA fragments that can be accomplished from agarose gels. Purification of a specific DNA fragment improves efficiency in subsequent downstream reactions.

5.6.2. Recovery of DNA from Agarose Gels

Identification of the specific DNA band to be purified is the foremost step towards extraction, recovery, and purification from the gel. The desired band is identified in the agarose gel after illuminating under UV light and then carefully excising a little piece of agarose gel containing the specific DNA fragment with a scalpel blade. In the next step, the DNA is extracted from the gel matrix, which can be accomplished by several methods [1, 3]. The method that uses the DEAE-cellulose membrane is rapid and effective, which is placed into a slit made in the gel. The DNA band is then transferred from the gel to the paper by applying an electric current. DNA is recovered from the paper by washing and precipitation with ethanol [3]. For recovering larger DNA fragments, electroelution that makes use of a dialysis bag is also a good method [1]. In electro-elution, the gel containing the desired DNA band is excised and placed into a dialysis bag with electrophoresis buffer. The bag containing the gel piece is then placed into a gel box containing a buffer. An electric current is then applied, which helps in the transfer of the DNA band from the gel matrix to the buffer. The DNA is then recovered from the buffer following the extraction of phenol followed by its precipitation from the solution by salt and ethanol. The phenol freeze-thaw method provides another very simple and easy way of gel extraction that yields good quality and quantity of DNA [3]. Finally, a widely used method for DNA purification makes use of low melting point agarose. This type of agarose melts at a lower temperature than standard agarose [3]. Notably, at this temperature, double-stranded DNA remains intact and does not denature. However, the commercially available regular agarose is of high quality, so this agarose can be used for purifying DNA fragments at 0.8%-1% concentration. To extract DNA fragments from agarose gel, it is advisable to use a gel that is buffered with TAE rather than TBE. This is because the purification methods are interfered with in the presence of borate [3]. Nowadays, laboratories make use of several kits designed to elute DNA from the gels.

5.6.3. Excision of the DNA Band

The correct DNA band/fragment is identified from a sample containing a mixture of DNA fragments. It is then excised. Precise excision of the right DNA band from the agarose gel is an art, and the skill should be mastered through practice. Once the required band is identified on a UV light-box (a trans-illuminator) in an ethidium bromide-stained gel, a fresh sterile 1.5 mL microfuge tube is taken, and the trans-illuminator is switched on to long-wavelength UV (consisting of lower-energy UV). The band is excised from the gel as quickly as possible during this step while the long-wave UV trans-illuminator is still on [1, 3, 4]. Note that it is extremely crucial to minimize the exposure time of UV to the DNA because the UV frequently mutagenizes the DNA at a measurable rate [3]. The UV trans-illuminator is switched off immediately after the excision, and the white light is switched on. The band is carefully removed from the gel and placed on the glass. Since it is a good practice to trim off and remove as much empty agarose as possible, it is necessary to go back to the UV illuminator briefly for this step. Finally, the excised agarose piece containing the DNA band is transferred to a 1.5 mL microfuge tube.

Precaution

- Exposure to UV can have drastic effects. Hence, appropriate clothing like gloves, long-sleeve lab coats, and face protection is necessary.
- Always wear a UV protective shield to protect the eyes and skin.

5.6.4. Important considerations

- Using a lower concentration of agarose gels ranging between 0.7%-0.8% is the most efficient for gel purification of DNA with respect to yields. Therefore, if possible, stay in this range of agarose concentration during gel purification.
- Nice crisp bands can be achieved by using a wider gel comb, running the gel at a lower voltage, and loading less amount of DNA in the well.
- There should be enough space on both sides of each band during the excision step. Keeping a wider space between the DNA bands from two consecutive lanes is important to avoid the interference of other DNA from nearby lanes and subsequently contaminating the sample. In this case, it is best to skip lanes between samples and between the ladder and the nearest sample.
- The UV exposure of the DNA should be limited. Long-wavelength UV is used to cut out the bands. The exposure time should be as short as possible [3]. This is performed to minimize the risk of UV-induced DNA damage. Therefore, it is a bad idea to use a gel imager to take a picture of the gel before cutting out the bands.
- In the case of similarly sized bands that run so close that it becomes almost

impossible to separate them, one needs to adjust the agarose gel percentage for good separation of bands. A higher percentage of agarose gel will help resolve smaller bands from each other and a lower percentage of gel will help separate larger bands.

- 10% Rule: It is wise to prepare 10% more volume of the sample than needed because several microliters can be lost in pipetting. For example, if you want to load 1.0 µg in 10 µL, make 1.1µg in 11µL.
- To protect the UV box, it is a good idea to place the gel on a glass plate or on a saran wrap. This will prevent the top surface of the trans-illuminator from being cut by the razor blade. Unlike the plastic tray, a glass plate or saran wrap will not hinder or significantly reduce the UV but will protect the UV box.
- The band should be excised in such a way that little excess gel should remain around it. Following the initial cut, the excised band is laid down on the UV trans-illuminator box and trimmed from the top, bottom, and sides with the razor blade. This is especially important during the DNA purification step, as many kits cannot handle more than a certain total volume of gel per reaction.

PROTOCOL 5.4: EXTRACTION AND PURIFICATION OF DNA BY PHENOL FREEZE METHOD

Materials Required

Biological sample: Desired DNA band in agarose gel piece

Reagents

Agarose

1X TAE Buffer (See ***APPENDIX A.1***)

TE saturated Phenol

Chloroform

3M Sodium Acetate, pH 5.2 (See ***APPENDIX A.1***)

Ethanol (Chilled)

70% Ethanol (Chilled)

Sterile distilled water

10 mM Tris-HCl, 1 mM EDTA pH-8.0 (See ***APPENDIX A.1***)

Equipment

Electrophoresis apparatus, power pack, UV trans-illuminator, microcentrifuge, -70°C freezer.

Miscellaneous

Scalpel blade, micropipettes, micropipette tips, microcentrifuge tubes.

Procedure

- Electrophorese the desired DNA (or mixtures of DNA fragments) resulting from either restriction enzyme digestion or PCR amplification on a 0.7% agarose gel at 100V.
- Using a sterile blade, excise the desired band from the gel. Remove most of the gel matrix from the sides and bottom of the gel slice, as stated above.
- Weigh out the gel piece by subtraction. First, weigh out the empty microcentrifuge tube. Then, weigh the tube again after transferring the gel slice into it. Find out the weight of the gel piece by subtraction.
- Add an equal volume (relative to the weight of the gel slice) of TE-saturated phenol.
- Store the tube at -70°C for at least 30 minutes. To obtain a good result, leave the tube overnight at -70°C.
- Centrifuge at 12,000 rpm for 15-20 minutes at 4°C. Two layers will be formed- a top aqueous layer and a bottom organic layer. The disrupted gel matrix partitions to the organic layer.
- Take the aqueous layer by avoiding the interface.
- Add an equal volume of chloroform to the aqueous layer.
- Vortex or mix vigorously for 5 minutes.
- Centrifuge at 12,000 rpm at 4°C for 10 minutes.
- Take the aqueous layer in another fresh microcentrifuge tube.
- Precipitate DNA by adding 1/10th volume of 3M sodium acetate and 2.5 volumes of chilled absolute ethanol. Keep the tube at -70°C overnight for precipitation.
- Spin the tube in a centrifuge for 15 minutes at 12000 rpm at 4°C.
- Wash the pellet with chilled 70% ethanol.
- Collect the pellet by spinning the tube at 4°C for 5 minutes.
- Save the pellet, dry it at 37°C or leave it for air drying for 10-15 minutes at room temperature.
- Add 10 µl of sterile distilled water or TE to the pellet and resuspend it.
- Check for the purity/homogeneity and quantity of the eluted DNA by running it in an agarose gel.

Note: A clean and crisp band following the gel purification is a signature of a DNA band with high purity, whereas the presence of multiple bands in the sample indicates that the preparation is unclean.

PROTOCOL 5.5: EXTRACTION AND PURIFICATION OF DNA FROM LOW-MELTING AGAROSE GELS

Rationale for Purification of DNA from Low Melting Agarose Gels

The low-melting-point (LMP) agarose, at a lower temperature, melts in contrast to the regular agarose that melts at a higher temperature. Hence, the name LMP is an apt one. Although difficult to handle at concentrations less than 1.0%, this type of agarose has opened the door to a fast way of isolating nucleic acids from gels without the need for electroelution, enzymes, or other methods [3]. In fact, in some cases (for example, ligations and random primed oligo-labeling procedures), the reactions can be done right in the LMP gel without the requirement for prior purification and hence has greatly facilitated many procedures in molecular biology.

Materials

Desired DNA band in agarose gel piece

Chemicals/Reagents

Elution buffer [20 mM Tris-HCl Buffer (pH 8.0) and 1 mM EDTA (pH 8.0)],

Low melting point agarose powder

6X Gel Loading Dye (See ***APPENDIX A.1***)

Ethidium Bromide (10 mg/mL stock)

TE buffer

Chloroform

Phenol equilibrated to pH 8.0

Phenol: Chloroform (1:1) v/v

10 M Ammonium Acetate (See ***APPENDIX A.1***)

70% ethanol (Chilled)

95% ethanol (Chilled)

Equipment

Electrophoresis apparatus, power pack, UV trans-illuminator, dry/water bath incubator, centrifuge, freezer, -70 °C.

Miscellaneous

Scalpel blade or sterile razor blade, micropipettes, micropipette tips, micro centrifuge tubes.

Procedure

- After electrophoresis, the low melting point gel is placed on an open UV trans-illuminator. The gel is carefully removed by gentle sliding from the tray. In order to protect the surface of the trans-illuminator from getting scratched by the scalpel, a saran wrap is placed on it, the gel is transferred on the saran wrap, and the desired DNA band is identified.

NOTE: Wear gloves when handling the agarose gel to avoid direct contact of the ethidium bromide-containing gel with the skin. Also, use appropriate UV protection devices such as face shields or goggles - especially for your eyes!

- Using a clean, sterile razor blade, carefully cut around the desired DNA fragment from the gel.
- Weigh out an empty, fresh, clean microfuge tube and label it. Note the weight.
- Take the labeled microfuge tube and transfer the excised gel piece into it. Weigh the tube again. By subtraction, determine the weight of the gel slice.
- The gel slice is submerged in an elution buffer. To the microfuge tube, add the elution buffer up to the point just above the level of the gel slice.
- Leave the tube in a water bath maintained at 65°C or in a dry bath until the gel slice melts completely. However, the lid of the tube should be wrapped with parafilm to prevent any evaporation of the buffer.
- Bring the gel solution to room temperature by cooling it down. Add an equal volume of equilibrated phenol. Vortex for 20 seconds.
- Centrifuge at 4000Xg (5800 rpm in Sorvall SS-34 rotor) for 10 min at room temperature and transfer the aqueous phase into a new microcentrifuge tube. The interface contains the agarose.
- Extract the aqueous phase with phenol:chloroform and once with chloroform.
- Add 0.2 volumes of 10 M ammonium acetate and 2 volumes of absolute ethanol kept at 4°C and mix thoroughly. Incubate at room temperature for 10 minutes.
- Recover the DNA by centrifugation 5000Xg (6500rpm in Sorvall SS-34 rotor)

for 20 minutes at 4°C.
- Wash the pellet with 200 µL of 70% ethanol.
- Centrifuge for 5 minutes and discard the supernatant again.
- Allow the pellets to dry well.
- Suspend the pellets in an appropriate volume of TE buffer. (If you want to confirm the recovered DNA, run 1µL of dissolved DNA sample on a gel.
- The recovered DNA can be stored at -20°C freezer or used for cloning purposes.

Result

The DNA that is extracted can be measured or estimated in the next step by taking an A_{260} reading in a nano spectrophotometer.

Precautions

- Wear proper clothing like gloves and lab coat while handling the mutagen EtBr to avoid contact.
- Take care to protect the eyes from exposure to UV by wearing eye protection glasses/ face shields. This is particularly required while visualizing agarose gel and cutting the DNA band under a UV trans-illuminator.

PROTOCOL 6: EXTRACTION AND PURIFICATION OF DNA BY ELECTROELUTION USING DIALYSIS TUBING

Rationale for Purification of DNA by Electroelution

This method of purification involves the use of dialysis tubing into which the agarose slice containing the required DNA band is placed along with the running buffer and is again subjected to electrophoresis. During this electrophoresis process, the DNA fragment migrates out of the gel slice into the buffer. The buffer containing the dilute solution of DNA is then extracted with phenol:chloroform, followed by recovery of DNA by precipitation. This method is good for purifying DNA ranging from 50 to 20,000 bp sizes [3].

Materials

Desired DNA band in agarose gel piece

Chemicals/Reagents

Ethidium bromide solution (10 mg/mL) (See ***APPENDIX A.1***)

TAE buffer (See ***APPENDIX A.1***)

100% and 70% ethanol

TE buffer, pH 8.0

Phenol:Chloroform:Isoamyl Alcohol (25:24:1) (See ***APPENDIX A.1***)

Chloroform:Isoamyl Alcohol (24:1)

3M Sodium Acetate, pH-5.2 (See ***APPENDIX A.1***)

Equipment

Electrophoresis apparatus, power pack, UV-trans-illuminator, dialysis tubing (Sigma D9777), small syringe (5-mL) or Pasteur pipette, dialysis clips (Sigma Z37096-7), micropipettes, microfuge tube, microtips.

Preparation of Dialysis Tubing

Dialysis tubing (Sigma D9777) is typically available in rolls (dried) cut into 5 cm lengths. They are semi-permeable membranes prepared by boiling them in distilled water and rinsing them in the same buffer used for the gel (1X TAE or TBE). They are stored submerged in the same buffer in a small beaker at 4°C.

Procedure

- Following electrophoresis, the DNA band is excised from the gel slab using a scalpel blade and hand-held UV trans-illuminator. For easier handling of the gel slice, it can be frozen at -20°C for 30 minutes.
- Take a piece of pre-treated dialysis tubing that has been prepared in the appropriate buffer as stated above. Seal one end of the tubing with a dialysis clip.
- Insert the gel-slice into the tubing and fill the tubing with enough TAE/ TBE buffer until it expands almost completely. With a second dialysis clip, the tubing is closed tightly at the open end. Use the same gel running buffer in dialysis tubing along with the gel slice.
- Transfer the sealed dialysis bag containing the gel slice to the horizontal gel electrophoresis apparatus so that the orientation of the gel slice/DNA band remains parallel to the electrodes. Fill the apparatus with 1X TAE/TBE buffer. Make sure that the dialysis bag remains submerged in the buffer.
- Electrophorese at 5V/cm electric field for 15 minutes. The DNA from the gel piece will migrate toward the positive electrode into the buffer in the dialysis tubing. It will be retained within the dialysis tubing.

NOTE I: The migration of the DNA band can be visualized by shinning long-wavelength UV. Complete migration requires about 10–15 minutes.

NOTE II: Elution of the DNA from the gel slice to the dialysis buffer requires electrophoresis at a constant voltage of ~2 V/cm at different times for different target fragments. For example, if the target fragment is 50- to 500-bp, 30 to 45 minutes of electrophoresis are required. Whereas, for a 500- to 2000-bp fragment and 2000- to 4000-bp fragment, 2 hours and 4 hrs are required, respectively. For larger fragments, overnight electroelution is done at 1 V/cm.

- Remove the dialysis tubing from the electrophoresis chamber. Unseal the tube by opening the clip at one end. Using a Pasteur pipet suck out the buffer from the dialysis tubing into a 1.5mL microfuge tube. In this way, transfer all the buffer to the tube for the next step.
- Measure the volume of the buffer. Add an equal volume of phenol/chloroform/isoamyl alcohol, extract the buffer containing the diluted DNA once, and then repeat the extraction procedure with phenol/chloroform mixtures.
- Carefully remove the aqueous layer and transfer it into a fresh 1.5 mL microfuge tube. Measure the volume of the recovered aqueous layer. Precipitate the DNA by mixing with 0.1 volume of 3M Na-acetate and 2.5 volumes of chilled ethanol. Keep the tube at -70 degree C for 30 minutes or -20 degree C ON. Precipitate the DNA by centrifuging for 20 minutes at 4 degree C. Take pellet.
- Wash twice with 70% chilled ethanol followed by centrifugation at 12,000 rpm for 5 minutes at 4°C.
- Dry the DNA pellet (which may not always be visible). Add an appropriate volume of water or TE buffer to re-dissolve the DNA pellet.

NOTE: The yield for DNA fragments varies with their size. For fragments >1 kb, a yield of 80% to 90% is expected after electroelution and purification. Meanwhile, for smaller fragments, yield typically varies between 50% and 60%.

Precaution

Wear safety goggles or a full face mask while visualizing the DNA in a UV transilluminator. UV rays harm the cornea, causing cornea burning, which is extremely painful and can result in infections and loss of vision. A brief exposure to UV is enough to cause this burning if the eyes are left unprotected. Exposed sensitive skin, such as the face and hands, can cause “sunburn” under a strong UV source in <1 min.

PROTOCOL 7: EXTRACTION AND PURIFICATION OF DNA BY SPIN-COLUMNS (NUCLEIC ACID PURIFICATION COLUMNS)

Rationale for Purification of DNA Using Spin Column

This method makes use of spin columns that contain silica-based membranes, which are known to bind DNA strongly in the presence of chaotropic salts. After the excision of the gel piece, it is solubilized in the presence of a chaotropic agent. The melted gel piece is then passed through the column using a high salt buffer by centrifugation. Under these conditions, the solubilized DNA adsorbs onto the silica membrane, and other contaminants flow through the silica matrix and are eventually washed off. Washing the column with low salt buffer finally causes the DNA to be eluted out [3, 4]. Excellent kits are available in the market for extracting DNA from reliable manufacturers like Qiagen Inc., Novagen Inc., Sigma-Aldrich Ltd., and Promega Inc. Note that in the presence of chaotropic salts, DNA binds to the silica membrane or resin in any form after dissolution of the gel. The silica membrane can be available in a hard matrix/membrane form in a spin column or used as a loose silica resin. All the high and low salt buffers, other necessary reagents, spin-columns, and microfuge tubes are provided in the kit, along with a user-friendly protocol. This method of purification is routinely used when DNA is to be purified from a regular, high-melting-point agarose gel and is less frequently used for purification from low-melting agarose gels. Though this procedure is faster than the organic extraction or electroelution methods, the method is expensive, and the yield of purified DNA is not up to the mark.

Materials

6.0 M NaI solution (filter through filter paper following preparation, store up to 3 months in the dark at 4°C) (See ***APPENDIX A.1***)

Binding buffer (See ***RECIPE*** at the end of the chapter)

Wash buffer (See ***RECIPE*** at the end of the chapter)

TE buffer (See ***APPENDIX A.1***), pH 8.0 or nuclease-free H_2O.

Equipment

Electrophoresis apparatus, power pack, UV-trans-illuminator, 1.5-mL microcentrifuge tubes, 45° to 50°C water bath, silica membrane spin columns (*e.g.*, Qiagen, Promega, Invitrogen, Novagen).

Procedure

- Electrophorese the DNA sample through a 1% agarose gel containing ethidium bromide for an appropriate time. Briefly check under a hand-held UV torch to ensure appropriate separation and resolution.
- With a clean scalpel or razor blade, carefully excise the target band and transfer it into a 1.5-mL microcentrifuge tube (step 4).
- Weigh out an empty, fresh, clean microfuge tube. Note the weight.
- Take the labeled microfuge tube and transfer the excised gel piece into it. Weigh the tube again. By subtraction, determine the weight of the gel slice.
- Dissolve the gel-slice in 3 volumes (relative to the weight of the gel slice) of chaotropic agent, assuming the volume of the agarose (μL) is equal to its weight in grams. For example, 100 mg of gel slice is considered to have a volume equal to 100 μL, and 250 to 300 μL 6.0 M sodium iodide is added to it.

NOTE: In the presence of the chaotropic salt-NaI, DNA binding to the silica membrane is reduced if TBE is used for electrophoresis. This binding may be inhibited in the presence of TBE. As a result, the recovery of DNA is reduced. The addition of 1/10 volume of sodium phosphate with pH 6 can resolve this problem. When using sodium perchlorate or guanidine isothiocyanate as chaotropic salts to dissolve the agarose gel, one should keep in mind that the pH of the gel/salt solution should be at pH 6.5. This will, in turn, help the DNA binding to the silica membrane efficiently.

- To dissolve the agarose, leave the tube in a water bath for 10 minutes at 50°C. At intervals, tap the tube to mix and homogenize the contents once or twice. After the stipulated time, if all of the agarose is not melted, take the tube back to the water bath and incubate for 1 to 2 min or a few minutes until all the agarose clumps dissolve completely.
- Cool the contents of the tube to room temperature by leaving it on the benchtop.
- Add 2 gel volumes (700 μL to 800 μL) of binding buffer to the tube and mix the contents.
- Place a spin column inside its collection tube. Pour the entire solution into it with a microtip.
- The spin column is centrifuged for 1 minute at maximum speed. (DNA remains bound to the silica matrix of the column). The flow through gets collected in the collection tube. Discard flow-through by removing the spin column from the collection tube.
- Place the spin column into the collection tube again.
- Add 750 μL of wash buffer to the spin column matrix and spin for 1 min at maximum speed. The flow through gets collected in the collection tube. Discard flow-through by removing the spin column from the collection tube.

- Reinsert the spin column in the collection tube again. To remove any residual wash buffer (ethanol) from the column, centrifuge the column for an additional 1 minute at full speed.
- Take a fresh sterile 1.5-mL microcentrifuge tube. Transfer the spin column to it. To the center of the silica membrane, add 75 to 100 µL nuclease-free water or TE buffer, pH 8.5. Store for 2 to 10 mins (at 37°C if necessary).
- Spin the column with microfuge in the centrifuge for one min at maximum speed. DNA is finally eluted out from the column and gets collected in the microfuge tube. Store the eluted sample at 4°C until use.

NOTE: This method typically yields DNA ranging from 60% to 80%, but it yields lower amounts of DNA for smaller fragments (e.g., ~50% for a fragment <500 bp).

5.7. SEPARATION AND PURIFICATION OF DNA FRAGMENTS IN NON-DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

5.7.1. Separation of DNA Fragment from Polyacrylamide Gels

Notably, agarose gel electrophoresis cannot resolve well a mixture of relatively smaller DNA molecules differing by only a single or a few base pairs in size. These DNA molecules from a mixture can be better resolved and separated in a non-denaturing polyacrylamide gel electrophoresis, which provides a method of very high resolution of DNA molecules ranging from 10–3,000 bp in size [3, 5]. Interestingly, single-stranded DNA is most effectively separated by polyacrylamide gel electrophoresis in the presence of 7 M urea. The rationale behind this separation lies in the fact that single-stranded molecules are prevented from base pairing due to the presence of a denaturing agent, 7M urea. Therefore, they separate as discreet bands with a resolution of a single nucleotide. For separation of double-stranded DNA of 20-1000 bp, non-denaturing polyacrylamide gels of 2-10% are often used.

Polyacrylamide gels are routinely used for separation of smaller double-stranded DNA fragments as low as 5 bp. Like the migration in the agarose gel, the distance (D) migrated by double-stranded DNA through a non-denaturing polyacrylamide gel is also inversely proportional to the log of its molecular weight, *i.e.*, $D \alpha -1/\log (MW)$ [3, 5]. It can be calculated which concentration of acrylamide is to be used for the desired DNA fragments to migrate approximately one-half to three-fourths of the way through the gel when the loading dye has reached the bottom of the gel, as shown in Table **2**.

Table 2. Concentrations of acrylamide giving maximum resolution of DNA fragments.

(%)Acrylamide	Size of Fragments Separated (bp)	Migration of Bromophenol Blue Marker (bp)	Migration of Xylene Cyanol Marker (bp)
3.5	100 to 1000	100	460
5.0	100 to 500	65	260
8.0	60 to 400	45	160
12.0	50 to 200	20	70
20.0	5 to 100	12	45

Unlike agarose gels, non-denaturing polyacrylamide gels consist of vertical gels that are typically poured in a space formed between two parallel sealed glass plates. A variety of plates of different sizes are available for gel casting. Typical preparative gels consist of about 200 X 200 mm with a gel thickness of 2.0 mm. After the gel is set by polymerization of acrylamide and bis-acrylamide, the sealing spacer from the bottom of the glass plates is removed, and the glass-plates holding the gel are set vertically in a vertical electrophoresis apparatus that consists of upper and lower buffer chambers. Electrophoresis buffers are subsequently poured into these two chambers to establish electrical continuity and are subjected to electrophoresis for 15-20 minutes without any DNA sample (pre-run). The DNA samples are then loaded onto different vertical lanes, and electrophoresis is conducted for a specific time at a specific voltage/current. After electrophoresis, the gel is dismantled from the glass plates and subsequently transferred to a UV trans-illuminator to visualize the DNA [3]. In this section, the methods involved in gel setup, sample loading, and electrophoresis will be described.

PROTOCOL 8: SEPARATION AND RESOLUTION OF DNA FRAGMENTS IN NON-DENATURING POLYACRYLAMIDE GELS

Materials Required

Plasmid and Genomic DNA sample

Chemicals/Reagents

10X and 1X TBE electrophoresis buffer, pH 8.0

29:1 (w/w) acrylamide/bisacrylamide (see ***RECIPE*** and ***APPENDIX A.1***)

TEMED (*N, N, N′*, *N′*-tetramethylethylenediamine; store at 4°C)

10% (w/v) ammonium persulfate (APS) in water (store ≤1 month at 4°C) (See

APPENDIX A.1)

5X loading buffer (see ***RECIPE*****)**

DNA-molecular-weight markers: *e.g.*, pBR322 DNA restriction digested with *Hin*fI or M13digested with *Hpa*II

0.5 µg/mL ethidium bromide (See ***APPENDIX A.1***)

Equipment

Glass plates, spacers, and combs for casting vertical gels, vertical gel electrophoresis apparatus.

DC power supply, longwave UV trans-illuminator, centrifuge with Beckman JA-20 rotor or equivalent, pipet tips for gel loading.

Gel Composition for 12 mL and 60 mL Gel Materials

Typically, 12 mL of acrylamide/bis-acrylamide gel mix is sufficient for pouring two glass plates that fit in one Hoefer minigel electrophoresis unit of 1 mm thickness. In contrast, 60 mL of gel material is required to run a bigger gel of the dimension 20 cm × 16 cm ×1.6 mm. Depending on the concentration of the gel to be used and the size of the apparatus (*i.e.*, whether minigel or large gel), various volumes of acrylamide solutions and water are required along with constant volumes of 1X TBE buffer, APS, and TEMED according to Table **3** [3, 5].

Table 3. Recipe of preparation of various concentrations of acrylamide gels (Final Volume 12 mL).

Gel %	30% 29:1Acrylamide (mL)	H_2O (mL)	5X TBE (mL)	10% APS (µl)	TEMED (µl)
8%	3.2 mL	6.4	2.4	200	10
10%	4.0 mL	5.6	2.4	200	10
12%	4.8 mL	4.8	2.4	200	10
Recipe of preparation of various concentrations of acrylamide gels (Final Volume 60 mL).					
Gel %	30% 29:1Acrylamide (mL)	H_2O (mL)	5X TBE (mL)	10% APS (mL)	TEMED (µl)
8%	16 mL	31	12	1	50
10%	20 mL	27	12	1	50
12%	24 mL	23	12	1	50

Procedure

Preparation of the Gel

- Use warm, soapy water to remove dirt from the glass plates and spacers. Wash them thoroughly first by rinsing them in distilled water. Finally, the plates are cleaned with ethanol or acetone, dried and set aside.

NOTE: Wear gloves while handling the glass plates so that oils from the hands do not get deposited on the working surfaces of the plates. The glass plates must be free of grease spots to prevent air bubbles from forming in the gel during pouring and casting. If the plates are particularly dirty or any residual nucleic acids are not completely removed, then the plates may be soaked in 0.1 M NaOH for 30 min prior to washing them.

- The glass plates with spacers are assembled in the gel casting apparatus in the gel caster. Leakage needs to be avoided. The gel casting systems have been so developed so as to avoid leakage.

NOTE: The modern gel apparatus has been developed in such a way so that leakage does not take place through the spacer. However, applying grease to the side and/or bottom spacers or pouring an agarose plug provides additional protection toward leakage. If necessary care is exercised while ensuring that the bottom of the plate assembly is completely sealed, then agarose plugs or grease is not a necessity.

- Prepare the gel solution in a wide-mouth flask with a spout for easy pouring. Appropriate acrylamide concentrations are selected to resolve DNA fragments of different sizes. Prepare the gel solution with the desired polyacrylamide concentration either for minigel or for the large gel according to Table **5.3** above based on your requirement. The selection of the correct concentration of acrylamide gives the appropriate amount of each component required to make the gel. In the case of running acrylamide gels in 0.5X TBE, the volumes of 5X TBE and deionized H_2O to be added are adjusted accordingly. Apart from 29:1 (% w/v) acrylamide: bis-acrylamide stock solutions, other ratios can also be used to cast polyacrylamide gels.
- Mix the solution vigorously using a magnetic stirrer for complete mixing of the components.
- Add 250 μL of 10% (w/v) APS and mix well. This is followed by the addition of 34 μL TEMED, and the flask is swirled gently but thoroughly to ensure complete mixing. Work quickly after the addition of TEMED.
- Pour the acrylamide solution between the two gel plates gently, avoiding bubble formation, and insert the comb carefully so that no air bubble gets trapped

underneath the teeth. With bulldog paper clips, clamp the comb on the top of the gel. As the acrylamide polymerizes, the tendency of the gel to get separated from the plates is prevented by the use of the bulldog clips. If required, the remaining acrylamide gel solution is used to fill the gel mold completely, making sure that there are no leaks. In about 20-30 min time, the gel polymerizes.

NOTE: Polymerization begins as soon as TEMED is added to the mixture, and hence, the following steps must be performed promptly. Whereas TEMED may be stored indefinitely at 4°C, a new APS stock should be kept for a month at 4°C. APS induces free radical-induced acrylamide polymerization. However, with time in storage, its ability gradually decreases. Hence, there is the need to prepare a new stock.

- After polymerization is complete, remove the comb, and any bottom spacers are removed from the gel. The gel plates are cleaned of any spilled acrylamide, and it is made sure that the spacers are properly seated. At this stage, the gel can be stored at 4°C by first wrapping the comb with wet paper towels that have been soaked in 1X TBE, and then the whole assembly is wrapped in a Saran wrap or plastic bag.
- To go ahead with electrophoresis, the gel assembly is very carefully removed from the gel caster. Take the electrophoresis tank and fix the gel plates at an angle into the lower reservoir in such a way as to avoid the formation of air bubbles between the plates and the gel bottom. Finally, clamp the gel plates to the top of the electrophoresis tank and then fill both the lower and upper chambers with 1X TBE buffer. Make sure that the buffer covers the wells.
- Use a micro tip (specially designed for vertical gel loading) or a Pasteur pipette to flush out the wells with 1X TBE. Make sure to check for bubbles, if any, which may disrupt the current flow during electrophoresis. Allow a pre-run at 5 V/cm for at least 30 min (constant voltage) to warm up the gel.
- Prepare the DNA sample by adding 5X loading buffer to DNA samples and molecular-weight markers (to make 1X final concentration of the loading buffer) and load them on to specific wells of the gel. For good resolution and optimum visibility using acrylamide gel electrophoresis, 25 μg of DNA sample should be loaded per 2 cm X 2 cm X 1.6 mm well.

NOTE: 15 ng of DNA per band is the detection limit with ethidium bromide staining for acrylamide gels.

- Electrophorese the gel at ~5 V/cm (2 to 10 V/cm range is acceptable), and take care to avoid excessive heating. The length of the run will depend on the desired resolution needed, as determined empirically from Table **5.2**. Electrophoresis

time can be reduced when a higher voltage is used to run the gel. In this case, one of the potential problems arises in the warming up of the gel, in which case the run can be conducted in the cold room. If the gel gets noticeably warm to the touch, the sample loaded in the middle will run faster or may even be denatured. Hence, the length of the run depends on the denaturation temperature of the sample.

- Turn off the power supply when the run is complete. Remove gel plates from the electrophoresis apparatus and lay the plates flat on the lab bench. With the help of a spacer or plastic wedge, lift the corner of the upper glass plate slowly and gently by keeping the gel still attached to the lower plate; the upper plate is lifted very slowly and carefully. Remove the spacers.
- Stain the gel with EtBr or SYBR gold. Alternatively, dry the gel in a gel dryer and expose it to X-ray film or storage phosphor imager screen.
  - For samples ≥2 μg, and if using a TLC plate with a fluorescent indicator, the DNA can be visualized with UV shadowing.
  - Alternatively, use EtBr to stain the gel. Submerge the plate with the gel attached in a tray containing 0.5 μg/mL ethidium bromide solution for 5 to 10 min. If necessary, soak the gel and plate in deionized water for 10 to 30 min after the staining step to lower the background absorption.
- In order to visualize the DNA bands in a UV trans-illuminator, prepare the gel and plate by wrapping with plastic wrap. Then, it is inverted on the surface of the transilluminator and finally photographed. Since longwave UV light transmits through plastic wrap, the gel can also be put directly on the trans-illuminator. For preparative gel, long UV exposure should be avoided as it will damage the nucleic acids. Unpolymerized acrylamide absorbs strongly at 211 nm and may also cause shadowing that is confined to the edges and wells of the gel.

Precautions

- Extreme care is required while handling the neurotoxin – Acrylamide. Always wear proper clothing like gloves, safety glasses, and a surgical mask when working with acrylamide powder [3, 5].
- Polyacrylamide solutions, which are commercially available, have long shelf lives. They are highly recommended, as manipulating with the neurotoxic acrylamide powder, especially during its preparation, can be avoided.
- Extreme precaution is recommended while pouring the gel, as unpolymerized acrylamide is more dangerous than when polymerized. Safety glasses should be worn while handling it.
- Methods need to be adapted for pouring the gel. The acrylamide should be poured directly from the mixing flask for thick gels. But for thinner ones a

syringe fitted with a needle can be useful. The first step toward pouring the gel is to hold the plates at 45° relative to the bench top by tilting the gel plate. Pour the gel mix starting from one corner, avoiding bubbles while the plates are still tilted. Once filled up, lay the gel flat on the table to avoid undesirable hydrostatic pressure on the gel bottom [3, 5].

5.7.2. Recovery of DNA Fragment from Polyacrylamide Gels

Both single-stranded and double-stranded DNA can be purified from polyacrylamide gels. The methods generally used for this purpose involve passive diffusion and electroelution [5]. The former method is time-consuming while the latter one is much faster. The yields of the DNA recovery are more or less comparable in both methods. The methods describe specifications for small and large DNA fragments to be recovered. Following are the two protocols for purifying DNA fragments (both small and large) from polyacrylamide gel [5].

PROTOCOL 9: RECOVERY OF DNA FROM POLYACRYLAMIDE GELS USING THE CRUSHING METHOD

Materials

Desired DNA band in agarose gel piece

Reagents and Chemicals

10X and 1X TBE electrophoresis buffer, pH 8.0 (See ***RECIPE*** and ***APPENDIX A.1***)

29:1 (w/w) acrylamide/bisacrylamide (see ***RECIPE***)

TEMED (*N, N, N′*, *N′*-tetramethylethylenediamine; store at 4°C)

10% (w/v) ammonium persulfate (APS) in water (store ≤1 month at 4°C) (see ***APPENDIX A.1***)

5X loading buffer

DNA-molecular-weight markers: *e.g.*, pBR322 DNA restriction digested with *Hin*fI orM13digested with *Hpa*II

0.5 μg/mL ethidium bromide (see ***APPENDIX A.1***)

Elution buffer, pH 7.5 (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0)

100% and 70% ethanol

TE buffer, pH 7.5

3 M sodium acetate, pH 5.2 (see ***APPENDIX A.1***)

Equipment

Glass plates, spacers, and combs for casting vertical gels, Vertical gel electrophoresis apparatus.

DC power supply, Longwave UV trans-illuminator, Centrifuge with Beckman JA-20 rotor or equivalent, Pipet tips for gel loading, scalpel or razor blade, Longwave UV trans-illuminator.

3-mL capacity small-bore disposable syringe, syringe equipped with siliconized glass wool plug or 2-μm filter.

Procedure

- The desired DNA band is excised with a scalpel or razor blade,
- Crush the gel slice by passing it repeatedly through a 3-mL capacity small-bore disposable syringe.

NOTE: Passaging of the gel slice through the syringe allows the slice to convert into many fine pieces which helps the DNA fragment diffuse from the gel matrix.

- All the pieces are collected in a fresh microcentrifuge tube.
- Estimate the rough volume of the crushed gel pieces by briefly centrifuging the microfuge tube at 1000 rpm for 10 seconds, followed by an eye estimation. Add 2 volumes of elution buffer. Incubate the slices in the elution buffer either under a shaking condition or in a rotary wheel at room temperature for 5 hours overnight.

NOTE: Elution efficiency increases with the increase in the volume of elution buffer used. Note that the elution of the DNA molecule from the crushed gel pieces is a diffusion-assisted process. Larger DNAs will thus take longer to come out from the gel pieces into the solution than smaller fragments. By raising the temperature of incubation to 37°C, the process of diffusion can be enhanced. The time of recovery depends on the size of the DNA fragments i.e., for DNA fragments that are not too large, enough recovery results from a few hours of incubation. Repeated elutions may improve the yield. For smaller fragments (<300 bp), elution for 4 hours is sufficient in contrast to the larger fragments (>750 bp) that require overnight elution.

- Depending on elution volume, centrifuge the tube either in a tabletop centrifuge or microcentrifuge for 10 minutes and 1 minute, respectively, at room temperature. The pellet will contain the gel fragments.
- Take supernatant carefully, avoiding the polyacrylamide pieces, and transfer the soup to a fresh microcentrifuge tube.
- Any trace of DNA remaining within the polyacrylamide gel can be extracted by repeating steps 4 to 6 using a lesser volume of elution buffer. Recentrifuge, if necessary, sucks out the supernatant from the second elution and combine the supernatant solutions from the two elutions.

NOTE: If acrylamide pieces are still present, then filter the supernatant using a syringe, which is equipped with a disposable 0.2 µm filter. If the volume of the elution buffer is too large, it can be concentrated by successive extractions against equal volumes of butanol. For every volume of butanol used, about 1/5th of the volume of the aqueous layer is extracted into the organic butanol layer. If water is completely extracted into butanol when too much butanol is added, then the simplest way is to add water and then concentrate again.

- The DNA is precipitated with salt and ethanol. Add 0.1 volume of 3M Na-acetate and 2.5 volume of chilled 100% ethanol. Incubate the tube at -20°C overnight or at -70°C for 30 min.

- Centrifuge the microfuge tube for 10 min at 12,000X g. DNA precipitates and is collected.

NOTE: Depending on the application, sometimes 10 µg of tRNA or glycogen that acts as a carrier is used to aid precipitation. However, the small acrylamide polymers, which are generated from the gel slice, are sufficient, in which case a carrier is not required.

- Add 100 µL TE buffer, pH 7.5, to the DNA pellet. Resuspend it. Re-precipitation can be done by adding 10 µL of 3 M sodium acetate, pH 5.2, and 200 µL of 100% chilled ethanol when required by taking the DNA in a microfuge tube. Incubate the tube at -20°C overnight or for 30 min at -70°C. Precipitate the DNA by centrifugation as in step 9.
- Wash the pellet with chilled 70% ethanol. This is done twice. Air dry the pellet and dissolve the pellet in TE buffer, pH 7.5, if appropriate.

PROTOCOL 10: RECOVERY OF DNA BY ELECTROELUTION OF SMALL DNA FRAGMENTS FROM POLYACRYLAMIDE GELS

This method involves elution using dialysis bags and works best for small DNA fragments (<300 bp). Unlike the passive diffusion process, which requires a

longer time, this method is much faster. Moreover, the recovery and yield of the DNA fragments are comparable in both methods [3, 5].

Materials

Desired DNA band in agarose gel piece

Reagents and Chemicals

10X and 1X TBE electrophoresis buffer, pH 8.0 (See ***RECIPE*** and ***APPENDIX A.1***)

29:1 (w/w) acrylamide/bisacrylamide (see ***RECIPE***)

TEMED (*N, N, N′*, *N′*-tetramethylethylenediamine; store at 4°C)

10% (w/v) ammonium persulfate (APS) in water (store ≤1 month at 4°C) (see ***APPENDIX A.1***)

5X loading buffer

DNA-molecular-weight markers: *e.g.*, pBR322 DNA restriction digested with *Hin*fI or M13digested with *Hpa*II

0.5 µg/mL ethidium bromide (see ***APPENDIX A.1***)

Elution buffer, pH 7.5 (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0)

100% and 70% ethanol

TE buffer, pH 7.5

3 M sodium acetate, pH 5.2 (see ***APPENDIX A.1***)

Equipment

Glass plates, spacers, and combs for casting vertical gels, Vertical gel electrophoresis apparatus.

DC power supply, Longwave UV trans-illuminator, Centrifuge with Beckman JA-20 rotor or equivalent, Pipet tips for gel loading, scalpel or razor blade, Longwave UV trans-illuminator.

Small dialysis bag.

Procedure

- The DNA band of interest is sliced out. However, in this procedure, the gel slice is kept intact and is not crushed.

NOTE: Since electroelution of DNA molecules embedded in acrylamide gel involves their expulsion from the gel matrix, which is facilitated by electrophoresis, keeping the gel slice intact will speed up the elution of the DNA instead of crushing it.

- Take a small piece (about 5 cm long) of pre-treated dialysis tubing that has been prepared in the appropriate buffer as stated above. Seal one end of the tubing with a dialysis clip.
- Insert the gel slice into the tubing and fill the tubing with enough 0.5X TBE buffer until it expands almost completely. Seal the open end of the tubing with a second dialysis clip.
- Place the dialysis bag in a small horizontal electrophoresis apparatus containing 0.5X TBE buffer.
- Electrophorese the DNA sample using an electric field of ~4 V/cm. The DNA sample will slowly move out from the polyacrylamide gel matrix into the buffer present in the dialysis bag. Typically, for complete transfer, the time taken for DNAs ranging around <300 bp or longer is about 2 hours and 6 hours, respectively.

NOTE: The DNA gel should be UV shadowed and stained again after the transfer process. The absence of DNA in the gel will confirm that it is completely transferred. The presence of any trace amount of DNA will call for the elution process to be continued.

- After the stipulated time, ensure the complete transfer of the DNA fragment from the gel matrix to the buffer inside the dialysis bag, turn off the power pack, and take out the dialysis bag from the apparatus.
- Open the clip on one end of the dialysis bag, and the buffer containing the eluted DNA is recovered using a Pasteur pipet. Rinse the inner surface of the bag with some more buffer to ensure complete recovery of any remaining DNA.
- Precipitate DNA by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol. Store the tube at -70°C for 30 mins or at -20°C overnight.
- Centrifuge the tube at top speed for 20 minutes at 4°C. Discard the supernatant and save the pellet containing the DNA.
- Wash the pellet with chilled 70% ethanol twice. Finally, dry the DNA pellet and dissolve it in 50 to 100 μL of TE buffer.

NOTE: If needed, a second ethanol precipitation can be done.

5.8. SPECTROPHOTOMETRIC ESTIMATION OF ISOLATED DNA

5.8.1. Introduction

Once an isolated plasmid or genomic DNA sample has been analyzed by agarose gel electrophoresis and if no abnormal banding or smearing has been observed under UV, the DNA sample needs to be checked for its quality and quantity for further downstream processes. It is extremely crucial for applications like restriction digestion of DNA samples or PCR amplification of target DNA. The amount of nucleic acid present in a preparation can be quantified by gel electrophoresis and spectrophotometric analysis, the two most commonly used methods. A simple and accurate estimation of the concentration of the nucleic acid in a sample is determined by analyzing the UV absorption by the nitrogenous bases present in either DNA or RNA. All nitrogenous bases absorb electromagnetic radiations around 260 nm, as exemplified by the absorption by dATP at 259 nm, dCTP at 272 nm, dGTP at 254 nm, and dTTP at 247 nm, provided these base samples are pure and are neither contaminated with proteins nor any organic solvents [3, 5]. The limitations of this method lie in both the quantity and purity of the DNA of the preparation. If DNA sample preparation is contaminated with RNA, then a contribution of absorption will also be embedded in the resultant spectrophotometric reading of that sample, and the determination of the DNA concentration from such reading will be erroneous. Inaccuracy in spectrophotometric readings results from the presence of contaminants like proteins, RNA, *etc.*, or if very little amount of DNA is present in the sample. Typically, the purity of the sample is determined by the ratio of A_{260}/A_{280} (A stands for Absorption at either 260 or 280 nm). Some standards readings have been set empirically [3, 5].

- A ratio of ≈ 1.8 denotes that the absorption in the UV range is principally due to DNA and is considered to be reasonably pure.
- A ratio lower than 1.8 (≈1.6 to 1.8) indicates the presence of proteins, phenol and/or other UV absorbers in the sample.
- A ratio higher than 1.8 indicates the presence of RNA in the DNA sample.

5.8.2. Principle

The method of determination of DNA concentration by absorption spectroscopy has long been used to measure the amount of DNA or RNA in a solution. The principle of absorption spectroscopy lies in the fact that it is the conjugated double bonds present in the nitrogenous bases of the DNA (purines and pyrimidines) that absorb the UV light [3, 5]. Their collective absorbance maxima lie at wavelengths of 260 nm. Absorption at 280 nm indicates the presence of protein and phenols because the phenolic ring and aromatic amino acids (tryptophan, tyrosine, and

phenylalanine) absorb strongly at 280 nm. An estimate of the purity of the nucleic acid is thus obtained from the ratio of the absorbance readings made at 260 nm and 280 nm. A_{260}/A_{280} value of 1.8 indicates reasonably pure preparation of DNA. A value less than 1.8 indicates the presence of protein or phenol in the sample, and then accurate quantification of nucleic acid will not be possible. As a measure of the purity of isolated nucleic acids, their absorption ratio at 260 nm and 280 nm is being used. Since the extinction coefficient of proteins is much less than that of nucleic acids at 260 nm and 280 nm, the reverse is not true, and hence, A_{260}/A_{280} of nucleic acid does not greatly change after significant contamination with proteins [3, 5].

The Beer-Lambert Law (also called Beer's Law) describes a relationship between the attenuation of light through a substance and the properties of that substance. The Beer-Lambert law is a linear relationship between the absorbance and the concentration of the chromophore (a substance displays the absorption property such as light/UV ray), molar absorption coefficient (ε), and optical path length (l) in a solution of the chromophore through which the light is passed. If the absorption at a specific wavelength is described by $A_{,\lambda}$ then it is given by the following formula:

$$\boldsymbol{A_{\lambda}} = \boldsymbol{\varepsilon} \times \boldsymbol{c} \times \boldsymbol{l}$$

Note that the molar absorption coefficient (ε) is an inherent property of the chromophore (DNA, RNA, *etc.*) and is a measure of how strong a given chromophore absorbs the specific wavelength of light/UV. The concentration is simply the moles L^{-1} (M) of the sample dissolved in the solution, and the optical path length is the width of the cuvette (the container used for the absorbance measurement), which is typically kept at 1 cm as a standard [3, 5].

Thus, the Beer-Lambert law states that a linear relationship exists between the concentration and the absorbance of the solution. So, measuring the absorbance of the sample enables the concentration of a solution to be calculated. Based on the extinction coefficient of DNA (which is a constant), an A_{260} of 1.0 determined at 260 nm corresponds to approximately 50 μg/mL of double-stranded DNA, 40 μg/mL of single-stranded DNA/RNA, and 33 μg/mL of oligonucleotides [3, 5].

Standard spectrophotometric conversions for various Nucleic Acids:

1 A_{260} of double-stranded DNA = 50 μg/mL

1 A_{260} of single-stranded RNA = 40 μg/mL

1 A_{260} of single-stranded oligonucleotides = 33 μg/mL

PROTOCOL 11: ESTIMATING THE QUANTITY AND PURITY OF THE DNA SAMPLE BY USING UV-VIS SPECTROSCOPY

Materials

DNA sample to be estimated.

Chemicals/Reagents

TE buffer (10 mM Tris-HCl pH-8.0, 1 mM EDTA) (see ***APPENDIX A.1***).

Equipment

Double beam UV-Vis Spectrophotometer, Quartz cuvette.

Glassware/Plasticware

Test tubes, Microcentrifuge tubes, Microtips.

Procedure

- Prepare an appropriate dilution of the sample DNA either with TE buffer or with sterile deionized water (anything between 1:50 and 1:250 dilution).
- Standardize the spectrophotometer using TE buffer/deionized water as blank.
- Measure the absorbance of the sample at both 260 and 280 nm.
- Calculate the DNA concentration of the sample using the following formula:

Concentration of DNA (μg/mL) = A_{260} X 50 X dilution factor

or

Concentration of DNA (μg/μL) = A_{260} X 50 X dilution factor/1000

Precautions

- For accurate measurement, the UV –VIS Spectrophotometer is calibrated prior to use.
- Prior to taking readings, the spectrophotometer is stabilized by switching it on for 15 min.

Trouble Shooting

Common problems encountered in Spectrophotometric estimation of DNA are described below, along with several possible causes:

- Protein Contamination: If the A_{260} /A_{280} ratio is less than 1.8, then traces of

phenol or protein contamination are present in the DNA sample.

- RNA Contamination: If the A_{260} /A_{280} ratio is greater than 1.8, then RNA is present in the DNA sample as a contaminant.
- Amount of Salt in the DNA Sample: A_{260} /A_{280} ratio less than 1.5 leads to the presence of salt in the sample due to their strong absorbance around 230 nm. If the A_{260} /A_{280} value is 1.5 or lower, it indicates the presence of organic compounds or chaotropic salts in the DNA sample.
- Non-nucleic acid contamination: A_{320} reading indicates the presence of other possible contaminants in the DNA sample.

5.9. Recipes Of Reagents And Solutions

Tris-Acetate (TAE) Buffer 50X (Stock Solution)

50 X TAE solution is prepared by dissolving

Tris base 242 gm

0.5 M EDTA (pH 8.0) 100 mL

Glacial Acetic acid 57.1 mL

Double distilled water to 1000 mL

***Tris-Acetate (TAE) 1X (working solution)*:** A working solution is prepared by following the method of $V_1S_1=V_2S_2$ that is making 1:49 dilution

50X TAE 20 mL (0.04 M Tris-Acetate 0.001 M EDTA)

double distilled water 980 mL

Tris-Borate (TBE) 10X (Stock Solution)

(Half-Strength Formula)

Tris base 54 g

boric acid 27.5 g

0.5 M EDTA (pH 8.0) 20 mL

Tris-Borate (TBE)1X (working solution): A working solution is prepared by following the method of $V_1S_1=V_2S_2$

10X TBE 100mL (0.045 M Tris-borate0.001 M EDTA)

double distilled water 900mL

Ethidium Bromide Solution: 10 mg/ml (Stock)

Dissolve 1g ethidium bromide in 100 ml H_2O

Mix well and store at 4°C in dark

Add 1g ethidium bromide to 100 mL distilled water and stir on a magnetic stirrer for several hours. Transfer the distilled water solution to a dark bottle.

CAUTION: *Ethidium bromide is a mutagen and must be handled carefully.*

Working Solution: 0.5 µg/mL

Dilute stock for gels or stain solution

Protect from light

TE Buffer

10 mM Tris-HCl pH-8.0

1 mM EDTA

10X Gel Loading Buffer: Ficoll Based

20% Ficoll 400

0.1 M disodium EDTA, pH 8

1.0% sodium dodecyl sulphate

0.25% bromophenol blue

0.25% xylene cyanol (optional; runs ~50% as fast as bromophenol blue and can interfere with visualization of bands of moderate molecular weight, but can be helpful for monitoring very long runs)

Gel loading Buffer, 6X

1X TAE buffer containing:

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol

30% (v/v) glycerol

Store up to 1 year at 4°C

Binding Buffer (6 M Guanidine HCl)

Add 0.75 g Na_2SO_3 and 57.3 g guanidine HCl (Sigma) to 35 mL H_2O and stir until dissolved. Adjust volume to 100 mL with water, sterilize, and store for 3 to 4 months in the dark (in aluminum foil) at 4°C. Discard if precipitate is observed.

Wash Buffer

1 part 10 mM Tris-HCl, pH 7.5

1 part 100 mM NaCl

4 parts 100% ethanol (final 80%)

Store indefinitely at room temperature

Preparation of DNA Sample for Loading

DNA sample 5 μL

6X Gel Loading dye 1 μL

29:1 (w/w) Acrylamide/Bis-Acrylamide

acrylamide 29 grams

N, N′-methylene-bis- acrylamide 1 g

Distilled deionized water to 100 mL

Heat the solution slightly (approximately 37°C) and stir until the acrylamide and bis-acrylamide are dissolved. Store at ≤1 month at 4°C.

CAUTION: Acrylamide is a neurotoxin. Always wear gloves when working with the solutions or powder of unpolymerized monomer.

Loading Buffer For Non-Denaturing PAGE, 5X

50 mM EDTA, pH 8.0

50 mM Tris-HCl, pH 8.0

50% (v/v) glycerol

10% (w/v) ammonium persulfate (APS) in water (store ≤1 month at 4°C) (see Appendix I)

Elution Buffer, pH 7.5

10 mM Tris-HCl, pH 7.5

50 mM NaCl

1 mM EDTA, pH 8.0

CONCLUSION

The know-how of DNA sample analysis, including the rationale of agarose and polyacrylamide gel electrophoresis explained in this chapter, will provide the new students beginning with molecular biology experiments with an insight into critical parameters associated with these procedures. The expertise required to carry out such procedures may be gained from the step-by-step protocol for running agarose and polyacrylamide gels mentioned here. The extraction of DNA samples from the agarose and acrylamide gels following their separation has also been elaborated vividly for the newcomers in the laboratory. Along with this, potential experimenters are made familiar with the hazards associated with the use of ethidium bromide and other critical parameters. At the end of the chapter, we included the procedures to quantify DNA involving spectrophotometric methods. Overall, the information from this chapter will equip us with all the technical details regarding analyzing DNA.

FURTHER READING

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CHAPTER 6

Construction of Recombinant DNA Molecules

Abstract: The strategies and methods of construction of recombinant DNA molecules, which is the central objective of this book, are elaborated in this chapter. A specific strategy associated with a particular molecular cloning procedure is extremely crucial for its successful execution. Various cloning strategies and procedures, which are successfully used in different laboratories, are described at the beginning of the chapter. Various steps of cloning are described in detail, including the methods of preparation of the vector and insert DNA samples either by digestion with a restriction enzyme or by polymerase chain reaction, modification of vectors and insert DNA, ligation, and transformation. Moreover, the inclusion of the properties and utilities of various end modification enzymes like Klenow, alkaline phosphatases, *etc.*, used in making both the vector and insert molecules compatible for ligation (essential to create successful recombinant molecules) fulfills the goal of the chapter. The process of cutting and joining, though it may seem simple, can pose problems in reality, leading to unsuccessful ligation for which a rigorous troubleshooting guide is provided. The process of making a strain of bacteria 'competent' to uptake the ligated DNA by transformation (protocol included) is also included in this chapter. The buffers and solutions required for the entire process can be found at the end of the chapter.

Keywords: Blunt and staggard ends, Cloning, Gateway cloning, Insert, Ligation, PCR, Restriction and modification enzymes, Vectors.

1. INTRODUCTION

Recombinant DNA technology involves the creation of a recombinant DNA molecule that consists of an exogenous DNA (called insert) inserted at a unique site of a vehicle DNA (called a vector) by employing a wide array of molecular tools such as restriction and modification enzymes. Once the recombinant DNA molecule is generated, it is inserted into a suitable host bacterium that allows multiple replication events of the recombinant DNA molecule using the bacterial replication machinery to create millions of clones of the insert DNA [1 - 5]. In this process, the vector component of the recombinant molecule aids in the ferrying process of the exogenous DNA into a host bacterial cell. Plasmid and bacteriophage DNAs are generally used as common cloning vehicles or vectors [4, 5]. The creation of a recombinant DNA molecule (commonly dubbed as cloning) is crucial for studying the structure and function of a gene of interest

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from an exogenous source organism. Thus, the goal of recombinant DNA technology is to create, maintain, and propagate an exogenous DNA inside a host bacterium as a part of the vector molecule. The ability to stably maintain the exogenous DNA inside the host bacterium aids in the investigation of the sequence and structure (such as the protein-coding part, different *cis*-acting regulatory elements, *i.e.*, promoter, terminator, *etc.*) of the exogenous gene of interest and its expression profile. The data obtained from such cloning experiments reveal fundamental information about the nature and function of the gene that is essential for understanding the function of the genes and genome. In this chapter, the essentials of molecular cloning and protocols involved in creating recombinant DNA molecules will be described in detail.

6.2. MOLECULAR CLONING ESSENTIALS

Molecular cloning is the process by which recombinant DNA molecules are generated and further propagated by introducing them into a host organism, where they replicate over generations using the replication machinery of the host [1 - 5]. The entire process of molecular cloning is essentially comprised of the following major steps:

- Preparation of the linear insert DNA fragment to be cloned.
- Preparation of the linear plasmid DNA to be used as a vector.
- Ligation (gluing) of the vector and insert to form recombinant DNA.
- Introduction of the products of ligation reaction containing the mixture of the self-ligated vectors, self-ligated inserts, and recombinant DNA molecules into a suitable bacterial host, followed by its propagation.
- Screening of the correct clone of recombinant DNA constituting the appropriate insert DNA.

Preparation of the insert or DNA of interest, such as a gene, regulatory element(s), operon, *etc.*, from a foreign source is the first step toward cloning. This is done by either excising it out of the source DNA using restriction enzymes, selectively amplifying it from the genome using polymerase chain reaction (PCR, See ***UNIT 6.4.2***), or assembling it from individual oligonucleotides. In parallel, a plasmid vector is prepared in a linear form (having two free ends) using restriction enzymes (REs) or polymerase chain reaction (PCR). The two free ends of the linear insert and the plasmid vector are then physically joined through phosphodiester bonds by a process called ligation [3, 4] (Fig. **1A**). Sometimes, the ends of the insert and the vector generated by any of the processes above may not yield compatible ends. There are several strategies to make them compatible for joining through the action of a DNA ligase. In this case, the ends of both the insert and vector are further modified, which is accomplished by utilizing enzymes such

as nucleases, phosphatases, kinases and/or ligases [2 - 5]. Once joined, the insert DNA becomes part of the new recombinant plasmid, which is now capable of replicating when introduced inside the host. The DNA of interest is subsequently replicated multiple times within the host to yield millions of clones of the original recombinant DNA. Studies on protein expression, gene expression studies, and functional analysis of biomolecules have become possible due to the advent of the molecular cloning process. Many cloning methodologies and, more recently, kits have been developed to simplify and standardize these processes.

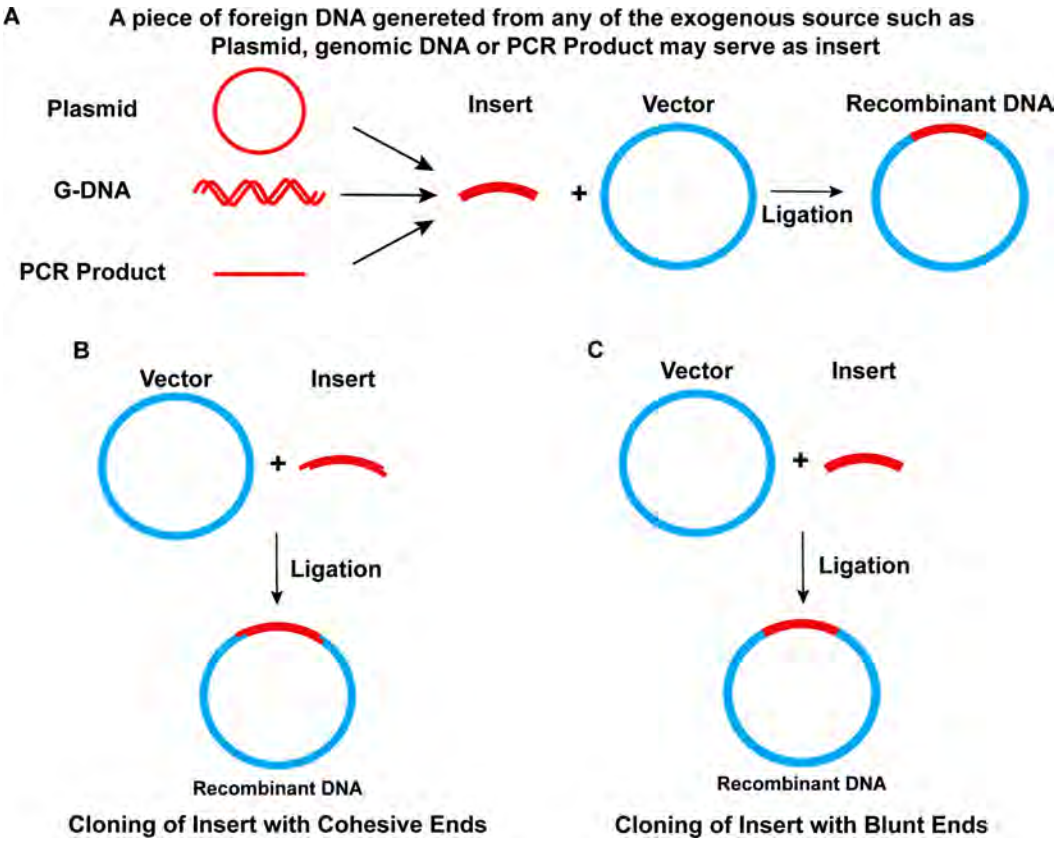

Fig. (1). (**A**) Outline of the Cloning Process Showing the Basic Steps Involved in Cloning. (**B-C**) Rationale of Cloning of DNA Fragment with Cohesive (**B**) and Blunt (**C**) Termini. See text for details.

6.3. VARIOUS CLONING STRATEGIES

The exogenous DNA (insert) constructs are typically generated by several methods, of which two methods are described below:

6.3.1. Traditional Cloning

Traditional cloning involves preparations of both the DNA fragment to be cloned (insert) and a self-replicating DNA plasmid (vector) by cleaving with the same set(s) (either one or two different) of restriction enzymes to generate DNA fragments with ligation-compatible complementary end sequences. These ends are subsequently subjected to join/glue together with a modification enzyme called DNA ligase prior to transformation (Fig. **1B-C**). Notably, all currently available plasmid/vector DNAs were engineered in such a way that circular vector DNA harbor the cleavage sites of several commonly used restriction endonucleases in tandem, often called the multiple cloning site (MCS) or polylinker site. The linear insert DNAs are inserted into the MCS of the vector that is linearized with one or two of the restriction endonucleases (REs). Ideally, the same set(s) of restriction endonuclease(s) are used to generate both the linear vector and insert. If a single RE is used to generate the linear vector and insert, then the two free ends of both the vector and insert molecules have compatible cohesive ends, all of which are compatible for ligation [1, 2, 4, 5] (Fig. **1B-C**). Consequently, ligation of such vectors and inserts yields three different types of ligated products: self-ligated vector, self-ligated insert, and ligated vector-insert (recombinant) molecule. Note that the former two products are undesired products of the ligation, and the self-ligated vectors will be transformed and propagated within the host along with recombinant DNA, thereby contributing to the background of the desired recombinant clone. To prevent self-ligation, the vector needs to be de-phosphorylated (involves removal of 5′-phosphate group from both ends), which prevents ligation of the two free ends of the vector molecule. In addition, the insert in this type of cloning may be ligated in both orientations with respect to specific landmarks of the vector molecule and is consequently called **non-directional cloning** (Fig. **2A-B)**) [3-5]. The bacterial clones generated in the non-directional cloning process subsequently need to be screened to determine the correct and desired insert orientation.

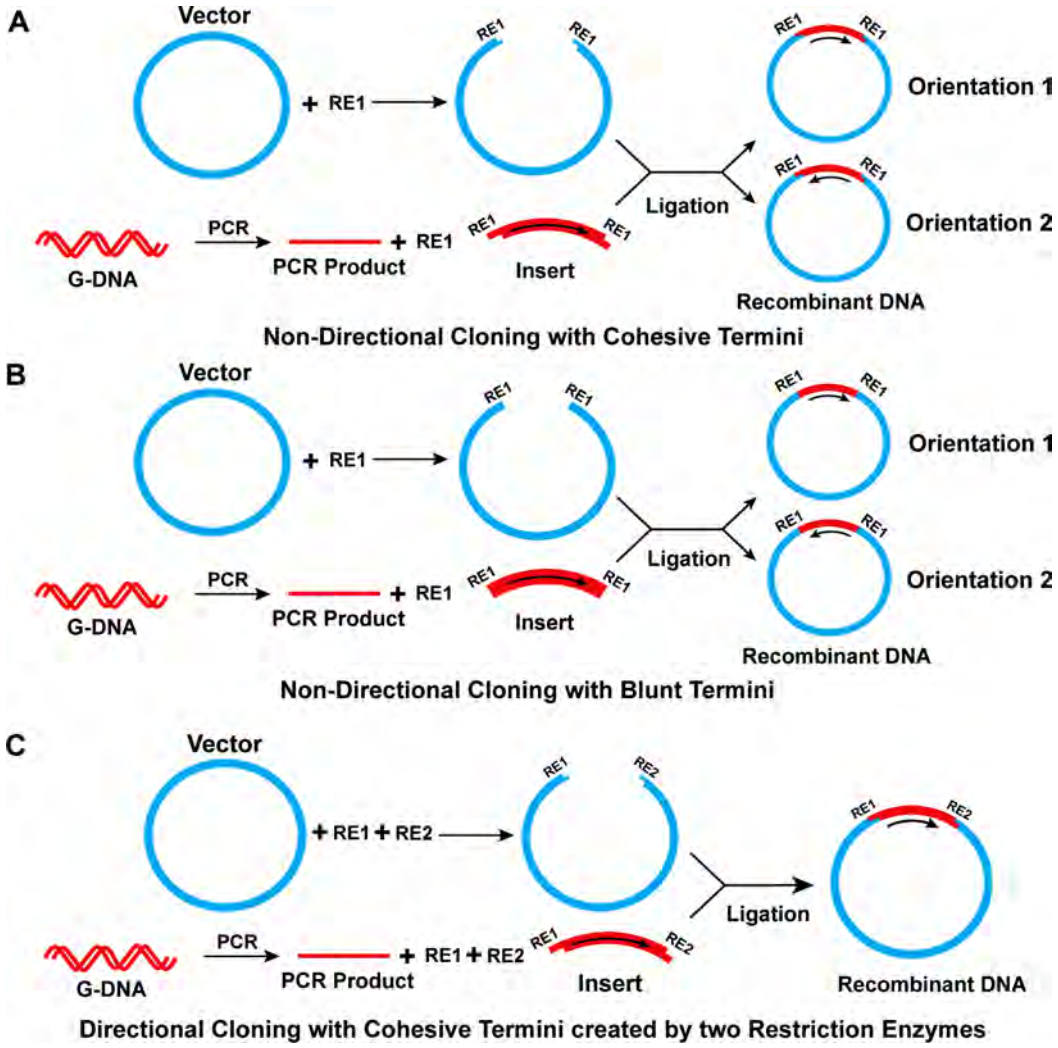

Fig. (2). Schematic diagram showing the essentials of directional and non-directional cloning.

In case two REs are used to generate the vectors/inserts, the two ends of either the vector or the insert DNA molecules become non-compatible for ligation that prevents the self-ligation of either the vector or insert without affecting the vector insert ligation. In this case, the insert that will be cloned/ligated is oriented in a specific direction with respect to the specific landmarks of the vector molecule. This type of cloning is called **Directional cloning** (Fig. **2C**) [1-2, 4-5], which is useful to create an expression clone that maintains the orientation of a potential open reading frame in the insert with respect to a promoter sequence present in

the vector and also lowers the transformation background of self-ligated vector alone.

Often, the polymerase chain reaction (PCR) is used as an upstream step in a cloning protocol to introduce a desired set of RE sites at both ends of the insert (the insert does not carry any of those internal RE sites) (see Fig. **1A**) using specially designed sense and antisense primers harboring the same sets of RE sites that allow directional cloning of the insert. This step is subsequently followed by the preparation of the vector and insert by digestion with the same sets of REs, followed by fragment purification, fragment ligation, and transformation into an *E. coli* strain for the propagation and amplification of the recombinant molecules [4, 5].

The ligated products containing recombinant DNA molecules that were generated are then introduced into a suitable bacterial host by a process called transformation [1, 4, 5]. Note that the vector component of the recombinant DNA carries an antibiotic-resistant gene (called selectable marker) that allows the transformed bacteria to become resistant to that specific antibiotic. Colonies of the transformed bacteria are initially selected from non-transformed bacteria by growing the entire transformation mixture (containing both transformed and non-transformed bacteria) on the antibiotic-containing solid plates. This selection permits only the transformed bacteria to grow on the antibiotic. In the next step, the multiple colonies of the transformed bacteria (that potentially harbor either the self-ligated vector or recombinant DNA molecule) are subjected to screening by several strategies, including colony PCR, blue-white screening, and/or isolation of resident plasmid DNA, followed by digestion with appropriate sets of REs for the presence of correct insert [4, 5] (see ***CHAPTER 7***). Finally, direct sequencing of the insert is often performed to verify the sequence integrity of the cloned fragment.

Previously, adaptors containing the desired restriction sites were used to clone fragments of genomic DNA into plasmid vectors prior to ligation. Genes or other DNA elements were swapped between vectors using compatible ends contained by both vectors.

Advantages of Traditional Cloning

- Low cost
- Versatile
- Many different vector choices
- Directional cloning can be easily done
- High efficiency, with dedicated vectors

Disadvantages

- Possible sequence constraints due to the presence and/or translation of restriction site.

6.3.2. PCR Cloning

PCR cloning typically involves the production of the insert from genomic/plasmid DNA source *via* selective amplification of the target sequence employing polymerase chain reaction (PCR) (see ***UNIT 6.4.2***). The amplified DNA fragment serves as the insert that is subsequently joined/ligated to the vector molecule *via* a blunt ligation or single-base overhang ligation prior to transformation. This method allows for the selective and successful cloning of DNA fragments that are typically present in relatively small amounts [4, 5]. Along with the insert, the vector can also be amplified by PCR and ligated together without the use of restriction enzymes. *Taq* DNA Polymerase is a temperature-resistant polymerase isolated from the thermotolerant bacterium *Thermus aquaticus* that is routinely used to amplify the target DNA sequence [4, 5]. Importantly, the resulting PCR product from such an amplification reaction contains a single adenine (A) residue as an overhang at the 3′-end, which is added by the *Taq* polymerase to the amplified DNA in a template-independent fashion. These "A-tailed" products are then ligated to a complementary T-tailed vector using the enzyme T4 DNA Ligase, followed by transformation into a suitable host (Fig. **3A**). Nowadays, commercially available blend/mix of various genetically engineered high-fidelity polymerases is routinely used to amplify DNA sequences that yield a PCR product with no 3′-extensions. The blunt-end fragments thus obtained using these blend enzymes are subsequently ligated to a blunt-ended linearized plasmid vector through a typical ligation reaction. However, PCR cloning with blunt end fragments is difficult and non-directional and hence requires a lot of screening procedures after the transformation of the ligated product [4, 5].

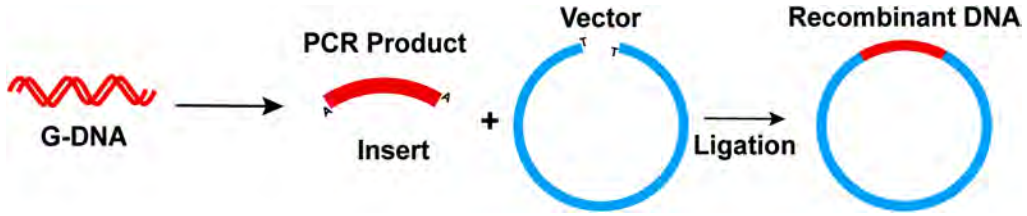

Fig. (3A). Schematic diagram showing the steps involved in the cloning of PCR products having a non-templated A residue.

Advantages of PCR-Based Cloning

- High efficiency, with dedicated vectors
- Amenable to high throughput

Disadvantages

- Limited vector choices
- Higher cost
- Lack of sequence control at the junction
- Multi-fragment cloning is not straightforward
- Directional cloning is difficult

6.4. PREPARATION OF VECTOR AND INSERT DNA SAMPLES

This is the first step in any of the cloning workflows. The plasmid DNA that would be used as a vector in the construction of recombinant DNA is usually prepared using one of the routinely utilized methods (see ***CHAPTER 3*** for a detailed description). This is followed by the linearization of this vector DNA using digestion with a specific restriction endonuclease (see next section), whose site has to be present in the multi-cloning site [4, 5]. In parallel, the insert DNA usually are procured in a number of ways, including the digestion of a previously existing piece of DNA as a clone that was screened from the genomic DNA library. In addition, the insert may also be obtained from a genomic DNA sample (see ***CHAPTER 4*** for a detailed description) *via* selective amplification of that specific fragment (Fig. **1A**). Both of these linearized vectors and insert fragments are then subjected to further purification from agarose or polyacrylamide gels (see ***CHAPTER 5*** for detailed information) [4, 5]. Once the purified vector and insert DNA are recovered, they are then quantified, ligated together to form recombinant DNA (Fig. **1**), and introduced into the bacterial host by transformation [1, 4, 5].

6.4.1. Preparation of the Vector and Insert DNA samples by Digestion with Restriction Enzymes

Digestion of the DNA using specialized enzymes (dubbed restriction endonucleases) constitutes a crucial step in the molecular cloning procedure, which is routinely employed to produce linearized vectors and insert DNA samples. Restriction endonucleases are site-specific endonucleases that cleave DNA at a very specific site. Their discovery led to a revolution in the history of molecular biology [4, 5]. The use of restriction endonucleases plays a crucial role in generating a physical map of the DNA and in the production of linearized vectors and insert DNA molecules that, in turn, facilitate the method of molecular cloning. The ability of REs to cleave DNA at a specific site enables them to be

used as sharp and precise molecular scissors, making them a powerful tool of modern-day molecular biology. This unique property of restriction endonucleases is conveniently used to generate a unique blend of very specific DNA fragments from a starting parent DNA molecule [4, 5]. The desired DNA fragment is then purified from this mixture of DNA fragments (see ***CHAPTER 5*** for details) for the subsequent creation of a hybrid molecule *via* joining with the vector by the process of cloning. Furthermore, a wide variety of enzymatic manipulations of these resultant DNA fragments are also done to carry out other downstream processes.

Three different types of restriction endonucleases are available, of which the type II restriction endonucleases are routinely employed in the molecular cloning processes because the site of recognition and site of cleavage of the type II enzymes in a piece of DNA molecule is identical [4, 5] (See **SPECIAL INFORMATION PANELS ON RESTRICTION ENDONUCLEASE** below). Type II endonucleases often recognize a short segment of DNA sequences (4-6 nucleotides long) as their recognition and cleavage site and subsequently cleave double-stranded DNA at those specific sites within the recognition sequences. A wide range of these restriction enzymes are currently available commercially, and each of them cleaves DNA at a particular sequence at their recognition/cleavage site. In doing so, some restriction enzymes may create a staggered (cohesive) end, whereas other enzymes generate blunt (non-cohesive, blunt, or flush) termini (Fig. **3B**). Three types of cuts are generated with blunt and sticky ends. Sticky ends can have 5′ or 3′ overhangs (Fig. **3B**). Typically, both the insert and the Vector DNAs are cleaved with either a single enzyme or a pair of different enzymes depending on the availability of the restriction enzyme site. The use of the same (when a single enzyme is used to prepare both vector and insert) or the same sets of two restriction endonucleases (when two different enzymes are used) to ensure that the termini created in both of them are compatible for ligation. All restriction enzymes usually come with a buffer, which is essential to carry out the digestion reaction *in vitro*. The temperature and any other requirements for the digestion reaction are also mentioned by the manufacturer in the datasheet. One needs to adhere to them when setting up a restriction digestion reaction (see below). If possible, restriction enzyme sites that are unique to both the insert and vector should be chosen. Unidirectional cloning is achieved using two different restriction enzymes, each with unique recognition sites at the end of the insert [4, 5] (Fig. **2C**). The protocols for a digestion reaction with a single restriction enzyme, with more than one enzyme, and digesting multiple DNA samples with the same enzyme are appended below.

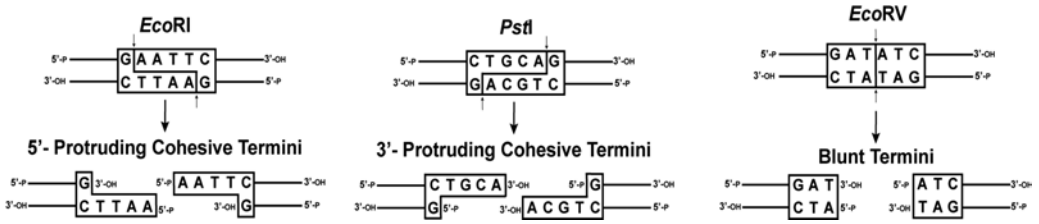

Fig. (3B). Various kinds of cohesive and blunt termini created by different restriction enzymes.

SPECIAL INFORMATION PANEL: RESTRICTION ENDONUCLEASES

Restriction endonucleases have been classified into three groups or types: Type I, Type II, and Type III, depending on where they cleave the double-stranded DNA. They bind specifically to a particular sequence known as the recognition sequence and cleave at specific sites either within or adjacent to this sequence. Neither Type I nor Type III restriction enzymes are widely used in molecular cloning because Type I enzymes bind to the recognition sequence but cleave at random sites when the DNA loops back to the bound enzyme. Further, Type III enzymes cut the DNA at the recognition site and then dissociate from the substrate. Both of them carry modification (methylation) and ATP-dependent restriction activities in the same protein [4, 5].

It is the Type II enzyme that has been widely used in molecular biology research, and a large number of Type II restriction endonucleases have been isolated, characterized, and cloned. Many Type II enzymes are extremely useful for cloning and other molecular manipulations. The main advantage is that these enzymes cleave at specific sites within the recognition sequence. They are composed of binary systems that consist of a restriction endonuclease that cleaves at a specific site and a separate methylase that modifies the same recognition sequence.

The recognition sequences for Type II enzymes are four, five, or six nucleotides in length, and they display twofold symmetry called palindromes. Longer sequences or sequences that are degenerate are recognized by a few enzymes. The cleavage pattern of each enzyme varies. Some cleave both strands exactly at the axis of symmetry, generating fragments of DNA that carry blunt ends, whereas others cleave each strand at similar locations on opposite sides of the axis of symmetry, creating fragments of DNA that carry protruding single-stranded termini like 5′ or 3′ overhangs (Fig. **3B**) [4, 5].

All the updated information about the restriction endonuclease is available in a publicly accessible database known as REBASE, which is maintained by New England Biolabs. This database contains a complete listing of all known

restriction endonucleases, including their recognition sequences, methylation sensitivity, commercial availability, and references. The database is available at rebase.ncb.com/rebase.

PROTOCOL 1: DIGESTING A DNA SAMPLE WITH A SINGLE RESTRICTION ENDONUCLEASE

Principle

Digestion of a DNA sample with one restriction endonuclease is achieved by incubating the given DNA sample with the preferred enzyme in the presence of a specific buffer under appropriate reaction conditions (Table **1**). The set of conditions used for the digestion of a DNA sample with a particular restriction enzyme may vary from the conditions required for digestion with another restriction enzyme. Hence, the amounts of enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of the reaction will vary widely depending on a specific restriction endonuclease used for the digestion reaction [4, 5].

Materials

DNA sample in H_2O or TE buffer

Reagents

10X restriction endonuclease activity buffers

Specific Restriction endonuclease

Equipment

Water Bath or Incubator, Tabletop or micro centrifuge, surgical gloves, plastic floater.

Glassware/Plasticware: Microfuge tubes, micropipettes, and microtips.

(NOTE: All plasticware must be sterilized.)

Procedure

Procedure for Setting up a Standard Digestion Reaction (20 µl Volume)

This protocol is aimed to set up a pilot digestion of DNA in a standard 20 µl reaction.

- Prepare the digestion reaction mixture in a clean and sterile microcentrifuge tube. Add the following into the microfuge tube for a standard 20 µl reaction.

Table 1. Small scale pilot digestion reaction.

DNA	0.1 to 4 µg DNA (in *x* µl)
10X buffer	2 µl (Final conc. 1X)
Restriction enzyme	1 µl (1 to 5 U/µl)
dH_2O	20-(*x*+2+1) µl
Total volume	20 µl
Incubation time	60 to 120 minutes
Incubation temperature	Enzyme dependent (in general, at 37°C)

- Start by adding water first in the tube, followed by 2 µl 10X buffer and the requisite volume of DNA sample. Note that the restriction enzyme should be added as the last component. The value of *x* will depend on the concentration of the DNA solution. Once all the components were added, mix the contents gently, either by tapping the tube or by pipetting up and down the entire reaction content several times.

NOTE: All restriction enzymes are stored in 50% glycerol to maintain the stability of these enzymes for the long term. When a small volume (1-2 µl) of restriction enzyme is added to the reaction, it immediately sinks to the bottom of the reaction owing to the higher specific gravity/viscosity. Therefore, it is very important that the mixture should be homogenized very gently without creating any frothing. Pipetting the entire content up and down seems to be the best way to homogenize the reaction mixture. Insufficient mixing may lead to incomplete digestion due to a lack of proper homogenization of the enzyme into the reaction that further prevents efficient DNA-enzyme collision in the medium, which is essential for effective cleavage.

- Make sure that the entire content is transferred completely to the reaction from the micro tip after mixing by pipetting. Briefly centrifuge the microfuge tube to transfer every drop of liquid from the inner side-wall of the tube to the bottom.
- Incubate reaction mix in 37°C (or other requisite temperature) water bath using a plastic floater for 1-2 hours.
- Place the microfuge tube in a 65°C water bath for 10 mins to stop the reaction after the stipulated time. Alternatively, chelate Mg^{2+} by adding 0.5 µl of 0.5 M EDTA (12.5 mM final concentration), which will stop the reaction.

NOTE I: The addition of EDTA should be avoided if the digested DNA is to be used in subsequent downstream enzymatic reactions (e.g., ligation or "filling-in" reactions).

NOTE II: Some enzymes are inactivated by incubating for 15 min at 75°C; they are partially or completely resistant to heat inactivation. DNA is subsequently purified using a silica matrix suspension or the reaction mixture extracted with phenol/chloroform extraction and is precipitated by ethanol in the presence of Na-acetate for those enzymes that are completely heat resistant.

- Store the reaction on ice.
- Analyze the digestion reaction by gel electrophoresis. To check the digested DNA sample in an agarose or in a polyacrylamide gel, mix 5 µl of the digestion reaction with 1 µl of 6X loading buffer (see ***CHAPTER 5***) so that the final concentration of the loading buffer becomes 1X.

NOTE I: To a regular user of the restriction enzymes, the concept of its Unit (U) needs to be clarified. 1U of restriction endonuclease completely digests 1 µg of purified DNA in 60 minutes using the recommended assay conditions. However, crude DNA preparations prepared by rapid procedures often require more enzymes and/or more time for complete digestion because that preparation typically contains various contaminants, some of which inhibit the activity of the restriction enzyme.

NOTE II: Glycerol is typically present in the enzyme storage buffer at the final concentration of 50%. Therefore, whenever a restriction enzyme is added to the reaction, some amount of glycerol will be carried over to the digestion reaction. Thus, a thumb rule that should be followed is that the volume of restriction endonuclease added to the reaction must not exceed 1/10th volume of the final volume of the reaction mixture. Exceeding the volume by more than 1/10th of the final volume will increase the final concentration of the glycerol by more than 5%, which may interfere with the digestion reaction.

NOTE III. A 20 µl reaction is generally set up as pilot digestion for initial analysis of the DNA by electrophoresis in polyacrylamide or agarose gels. The reaction volume can be increased by the addition of more DNA.

Procedure for Setting up a Medium-Scale Digestion Reaction (50 µl Volume)

This protocol is aimed to set up the digestion of a larger amount of DNA in a 50 µl reaction Table **2**.

Procedure

- In a sterile 1.5 ml microfuge tube, add the following reagents sequentially, starting with water to restriction enzyme as mentioned in the previous protocol. The value of *x* will depend on the concentration of the DNA solution.

Table 2. Medium scale digestion reaction.

DNA	Up to 1 µg (in *x* µl)
10X Buffer	5 µl (Final Conc. 1X)
Restriction Enzyme	2 µl (10 Units)*
dH_2O	50-(*x*+5+2) µl
Total Volume	50 µl
Incubation Time	60 to 120 minutes
Incubation Temperature	Usually 37C, but it is enzyme-dependent

*Sufficient to digest all types of DNAs.

- Finally, add l µl of the restriction enzyme (10 U/µl) to the reaction mixture. Mix gently with the pipette tip or by tapping the contents gently. Once all the components are added, mix the contents gently, either by tapping or by pipetting up and down several times.

NOTE: All restriction Enzymes are stored in 50% glycerol to maintain the stability of these enzymes for the long term. When a small volume (1-2 µl) of restriction enzyme is added to the reaction, it immediately sinks to the bottom of the reaction owing to the higher specific gravity/viscosity. Therefore, it is very important that the mixture should be homogenized very gently without creating any frothing. Pipetting the entire content up and down seems to be the best way to homogenize the reaction mixture. Insufficient mixing may lead to incomplete digestion due to a lack of proper homogenization of the enzyme into the reaction that further prevents efficient DNA-enzyme collision in the medium, which is essential for effective cleavage.

- Make sure that the entire content is transferred completely to the reaction from the micro tip after mixing by pipetting. Briefly centrifuge the microfuge tube to transfer every drop of liquid from the inner side wall of the tube to the bottom.
- Incubate in a water bath at the appropriate temperature for the desired length of time. The temperature and time depend on the enzyme and will be stated in the product guide provided by the supplier.
- Store the reaction on ice briefly before analyzing the digestion by gel electrophoresis.

NOTE 1: The buffers are provided by the manufacturers for each restriction enzyme. Alternatively, enzyme buffers can also be prepared following their recipes.

NOTE II. It is preferable to use 100-200 ng per digestion for easy detection of small DNA fragments, though concentrations of DNA as low as 10 ng can be visualized on an agarose gel.

NOTE III. Add about l unit of enzyme per µg of DNA. If necessary, the enzyme stock may be diluted with the storage buffer described in the manufacturer's product guide. Adding more than 1 unit/µg of DNA is acceptable, but large excesses should be avoided to avoid unnecessary wastage of the enzyme.

PROTOCOL 2: DIGESTING A DNA SAMPLE WITH TWO RESTRICTION ENDONUCLEASES

Principle

Different experimental procedures often require that the sample DNA (both the vector and insert) have to be cleaved with two different restriction enzymes generating two different termini. In such cases, sometimes it is possible that both the enzymes require the same buffer, *i.e.*, they have maximum activity in one single buffer. In such a situation, the digestion procedure is carried out by two enzymes simultaneously using a single buffer that displays optimal activity for both of them. In contrast, very often, the buffer required for the optimal activity of the two enzymes does not match *i.e.*, enzymes do not have the same buffer compatibility. In such a case, the digestion procedure has to be carried out separately and sequentially. The digestion procedure with the one enzyme has to be carried out first using the procedure mentioned above, followed by the analysis and recovery of the digested DNA. This digested DNA by enzyme 1 is then subjected to digestion by enzyme 2 as usual.

NOTE: Detailed information about the various physico-chemical conditions that are required for the optimum activity of various restriction enzymes, such as optimum temperature, the activity of a given enzyme in different restriction enzyme buffers, and much other useful information, is appended in the enzyme catalog/manual typically provided by the manufacturer.

Materials

DNA sample in H_2O or TE buffer

10X restriction endonuclease activity buffers

Restriction endonucleases

Equipment

Water Bath or Incubator, Tabletop or micro centrifuge, surgical gloves, plastic floater.

Glassware/Plasticware: Microfuge tubes, micropipettes and tips.

Procedure

- Choose the first restriction enzyme that requires the buffer with a lower salt concentration. Alternatively, the enzyme that will be used first may be selected arbitrarily. Set up the reaction in the usual way with this enzyme in a 50 µl volume following the procedure mentioned above.
- Incubate the tube at a suitable temperature (typically 37°C) for the desired length of time in a water bath. Once the time of incubation is over, place the reaction on ice while analyzing a small aliquot of the digested DNA sample by gel electrophoresis.
- Check if the DNA sample underwent complete digestion by the first enzyme from the gel analysis. If the digestion is not complete, incubate the reaction mixture again for an additional period of 1-2 hours. Alternatively, add another 5-10 U (1-2 µl) of the enzyme to the reaction, mix well as stated above, and subject the partially digested DNA sample to further digestion.
- Finally, withdraw a small aliquot and electrophorese for the final check. If the digestion by the first restriction enzyme is complete, incubate the microfuge tube at 65°C water bath for 10 mins to stop the reaction.
- Add 200 µl of dH_2O to increase the volume to 250 µl. Extract the mixture initially with 250 µl phenol:chloroform:isoamyl alcohol mix twice, followed by 250 µl chloroform:isoamyl alcohol mixture. Centrifuge the mixture at 10,000 rpm for 5 minutes.
- Recover the aqueous phase and discard the organic phase. Add 1/10 volume (25 µl) of Na-acetate and 2 volumes (1 ml) of chilled ethanol. Store at -20°C for overnight or at -70°C for 30 minutes.
- Recover the digested DNA by centrifugation at 12,000 rpm for 30 minutes, followed by washing the nucleic acid pellet with 70% ethanol and drying.
- Finally, resuspend the DNA pellet in 20-30 µl dH_2O.
- Set up the second digestion reaction in the usual way with the second enzyme in a 50 µl volume following procedures 6.1.2.5.
- Incubate the tube at a suitable temperature (typically 37°C) for the desired length of time in a water bath. Store the reaction on ice.
- Analyze a small aliquot of the digested DNA sample by gel electrophoresis.
- Verify the completeness in digestion by the second enzyme from the gel

analysis. As stated above, in case the digestion did not go to completeness, increase the incubation time as usual or add an additional 5-10 U (1-2 µl) of the enzyme to the reaction.

- Mix well as stated above and subject the partially digested DNA sample to further digestion.
- Check the completeness of the digestion by gel analysis using a small aliquot of the digestion mixture.
- Incubate the microfuge tube at 65°C water bath for 10 mins to stop the reaction.
- Based on the downstream application, proceed to the next step if it is possible to carry out the next step in the same reaction condition.
- In case the next step requires a completely different buffer, proceed with steps 5-8, as stated above, to bring the digested and purified DNA in the pure aqueous medium.

NOTE 1: In the majority of the cases, the difference in the buffer composition of the two enzymes is so large that a simple alteration of the salt concentration in the buffer of the first enzyme is not sufficient to bring about the compatibility to the second enzyme. Moreover, the first enzyme may lose its specificity of DNA cleavage and begin to cleave the DNA in a non-specific and unexpected manner in the reaction buffer of the second enzyme. In such a situation, the first enzyme needs to be completely inactivated after the reaction by incubation at 65°C for 10 min before continuing with the second reaction. If the enzyme is resistant to heat, then phenol extraction followed by ethanol precipitation is done to purify the DNA before setting up the next digestion with the second enzyme, as stated above.

*NOTE II: In the polylinker multicloning site in a plasmid, the restriction sites are adjacent or very close to one another. When digesting with two such enzymes, it is impossible to check for complete double digestion of DNA by simple gel electrophoresis from DNA cleaved by only one of the enzymes because the tiny stuffer fragment (ranges from 2-20 nucleotides in size) remains undetectable in the gel. In order to sort this problem out, it is recommended to carry out a simple ligation reaction (**PROTOCOL 6.14**) following the purification of the double-digested vector to ensure correct double digestion. If the product of the double digestion yields non-complementary termini, unlike a DNA fragment cleaved by one restriction enzyme, it cannot ligate to itself to form circular molecules. These differences are easily analyzed by transforming the single-digested and double-digested ligated samples in a suitable bacterial host. Always add a non-digested parent plasmid DNA as a control in the transformation reaction. The colonies are seen on the agar plate containing appropriate antibiotics for the whole plasmid, and none are to be expected for a completely digested product.*

PROTOCOL 3: DIGESTING MULTIPLE SAMPLES OF DNA WITH ONE OR TWO RESTRICTION ENDONUCLEASES

Principle

When carrying out the digestion of multiple samples of DNA with the same (one) or same sets (two) enzymes, it is wise to make a "Cocktail" or "Master mix". Typically, this mix contains all the reagents except the DNA samples. Notably, this procedure has several advantages and is always recommended. First, this procedure helps minimizing the potential for contamination of the stock vial of the restriction enzyme as the number of times the restriction enzyme is withdrawn from the stock vial is significantly reduced. Second, the total number of pipetting steps that would otherwise have been required in the case of setting up a single reaction separately is also greatly reduced, thereby minimizing errors and time to a great extent. This procedure is very useful when screening out a desired recombinant clone from a large number of plasmid DNA candidates isolated from multiple candidate clones.

Materials

DNA sample in H_2O or TE buffer

10X restriction endonuclease activity buffers

Restriction endonucleases

Equipment

Water Bath or Incubator, Tabletop or micro centrifuge, surgical gloves, plastic floater.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Dispense a constant volume (let us assume 1 µl for each sample) of each individual plasmid DNA sample in separate clean, sterile microfuge tubes.

NOTE: To prevent cross-contamination, different pipet tips must be used for withdrawing each DNA sample.

- Prepare the cocktail/master mix. In the first step, determine the total number of DNA samples. For example, if there are ten plasmid DNA samples to be screened, then it is prudent to prepare the master mix for one additional reaction *i.e.*, for a total of eleven samples Table **3**. Multiply the amount of 10X Buffer

and water required for a single 20 µl reaction by 11, following the volumes appended in the table below. This will contain enough mix for 10 reactions. Place solution on ice.

Table 3. Setting up Master Mix for Restriction Digestion Reaction.

10X Buffer	2 µl X 11= 22 µl
dH_2O	16.5 µl X 11 = 181.5 µl
Restriction Enzyme	0.5 µl (2.5 U) X 11= 5.5 µl
Total Volume	209 µl

- Add the appropriate volume (5.5 µl in this example) of restriction endonuclease(s) as the last reactant to the master mix. Mix quickly but gently, either by pipetting up and down or by flicking the tube, and replace on ice. If necessary, centrifuge briefly to bring down every drop of liquid from the inner walls of the tube to the bottom.

*NOTE: The solution to which the enzyme is added should not be more concentrated than 2*X *buffers.*

- Divide the master mix by eleven and then add the resultant amount of the master mix (which is 19 µl in this example) to each of the ten microfuge tubes containing equal volumes of DNA.
- Mix the master mix and plasmid DNA sample well by flicking. If necessary, centrifuge the microfuge tubes briefly to transfer the liquid to the bottom of the tube.
- Incubate each tube at the desired temperature for a specified time.

NOTE: Care should be taken when aliquoting the master mix in each tube containing DNA. The same pipet tip can be used to dispense the mix to multiple sample tubes if the tip does not come in direct contact with the DNA at the bottom of the tubes. For preparative purposes, it is advisable to use a different pipet tip for each sample.

- Stop the reactions by heating each sample at 65°C for 10 minutes, followed by their analysis by gel electrophoresis.

6.4.2. Preparation of the Insert DNA Samples by Polymerase Chain Reaction (PCR)

Principle of PCR Amplification

Cloning of a DNA fragment that is prepared by PCR amplification is often more

rapid than traditional cloning in which the DNA fragments are prepared by restriction endonuclease digestion. The latter procedure requires microgram quantities of DNA and relies on the restriction enzyme sites, which may or may not be present in a desirable manner on a given DNA fragment. In contrast, PCR-based cloning procedure makes it possible to ligate any two segments of DNA in any configuration and join any desired junction-point reading frame or restriction sites by incorporating additional non-homologous nucleotides within the PCR primers. Another additional advantage of this method involves that PCR reaction can efficiently amplify a target DNA that is present in a very low abundance or even degraded [4, 5]. Essentially, two oligonucleotide primers (approximately 25-30 nucleotides) are designed using the sequence that flanks the DNA to be amplified in such a way that the primers can act as anchors during the amplification reaction (Fig. **4**). In addition, restriction endonuclease recognition sites can be incorporated in the primers used for amplification.

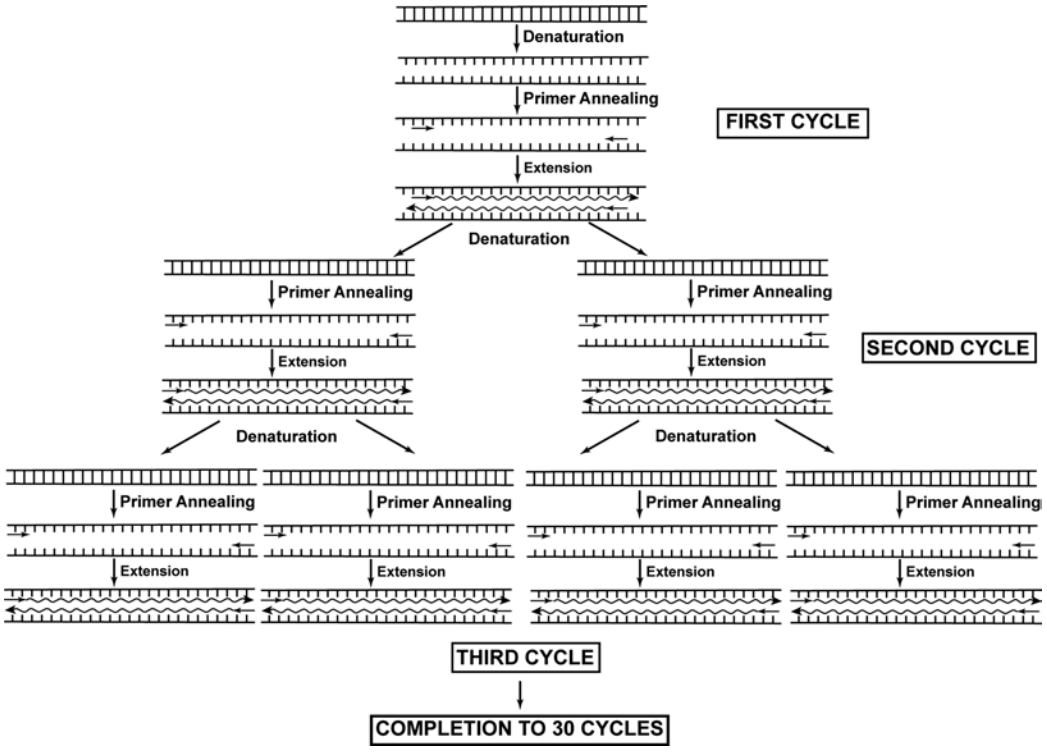

Fig. (4). Schematic diagram showing the outline of polymerase chain reaction (PCR). Note that the events involved and the progress of the reaction up to the third cycle are shown.

Amplification is the primary step for the PCR cloning procedure. Amplification can be performed to generate a blunt insert or to have a 1-base overhang,

depending on which polymerase is used in the amplification of the target DNA [4, 5] (Tables **4** and **5**). The insert prepared by amplification is digested with the specific restriction endonucleases that are now incorporated in the flanking region of PCR amplified insert *via* the primer that eventually generates cohesive termini. Alternatively, the insert can be directly cloned into the specially designed plasmid vector. This unit describes the construction of recombinant DNA molecules using the PCR amplified DNA as an insert [4, 5].

PROTOCOL 4: PREPARING INSERT DNA SAMPLES BY SELECTIVE AMPLIFICATION USING POLYMERASE CHAIN REACTION (PCR) WITH *TAQ* DNA POLYMERASE

Materials

Template DNA (Genomic or Plasmid DNA)

10 μM Primer Mix (10 μM each of forward and reverse primers)

10 mM dNTP Mixtures

Taq Standard 5X Reaction Buffer

Taq DNA Polymerase

Nuclease-free water

Equipment

Thermo Cycler, Tabletop or microcentrifuge, surgical gloves, ice pale.

Glassware/Plasticware: Small (0.5 ml) capacity microfuge tubes, micropipettes and tips.

Procedure

- In a clean 0.5 ml microfuge tube (also called PCR tube), add the following reagents as shown in the chart. Start with the addition of water and then the others. *Taq* DNA polymerase should be added to the reaction as the last component.
- Mix all the reagents by gently tapping or pipetting up and down.
- Centrifuge briefly to bring all of the liquid to the bottom of the tube.
- Set up a program in the Thermo Cycler Machine following the table given below.

Table 4. Polymerase chain reaction (PCR) with taq DNA polymerase.

Reaction Components	25 µl Reaction	50 µl Reaction	Final Concentration of each component
5X *Taq* Standard 5X Reaction Buffer*	5 µl	10 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM primers (forward and reverse)	0.5 µl	1 µl	0.2 µM
Template DNA	variable	variable	20ng-1 µg
Nuclease-free water	To 25 µl	To 50 µl	-
Taq DNA Polymerase**	0.125 µl	0.25 µl	1.25 units/50 µl reaction

*If 10X reaction buffer is used, the volume of the reaction buffer should be halved.
** The amount of polymerase added will depend on the polymerase used.

Table 5. A standard Program for PCR reaction.

-	Cycles	Temperature	Time
Initial Denaturation:	**1**	**94°C**	**30 seconds**
Denaturation	**30**	**94°C**	**30 seconds**
Annealing		**45–68°C***	**60 seconds**
Extension		**68°C/72°C**	**1 minute per kb**
Final extension	**1**	**68°C/72°C**	**10 minutes**
Hold	**1**	**4°C**	-

*Tm values of each of the primers should be determined using the available Tm calculator. Nowadays, all the manufactures mention the Tm value of each of the primer/oligonucleotides in the data sheet that comes with the purchase of custom-made oligonucleotides. Determination of the annealing temperature requires the information of Tm values of both the primers.

- Place the tubes in the holders and press START.
- Pour a small or medium 1% agarose gel, as mentioned in Chapter 5.
- Once the entire amplification cycle is over, take out the sample tubes from the slots of the Thermo-Cycler and store the tubes either in ice (if the amplification would be checked right away). Alternatively, the samples may be stored at 4°C for one to a few days.
- Check for the success of the amplification reaction by electrophoresing about 5 µl of the reaction sample in a 1% agarose gel.

NOTE: Calculate the size of the DNA that is amplified. A standard DNA sample close to the size of the amplified product or an appropriate standard DNA ladder in the agarose gel should be used in the gel analysis to confirm that the amplification reaction is successful.

PROTOCOL 5: PREPARING INSERT DNA SAMPLES BY SELECTIVE AMPLIFICATION USING STANDARD POLYMERASE CHAIN REACTION (PCR) WITH HIGH-FIDELITY Q5® POLYMERASE

Principle

Taq DNA Polymerase is error-prone, whose error rate is quite high (of the order of 10^{-3}), and hence, it loses its fidelity while amplifying. In other words, there is a high probability that one wrong/inappropriate nucleotide may be incorporated out of every one thousand nucleotide-long DNA sequence amplified. Hence, a DNA fragment that is amplified by *Taq* DNA polymerase may contain one or a few wrong nucleotides (based on the length of the amplified product). Consequently, if such a DNA fragment containing a few nucleotide mutations is cloned into the vector, that will not be identical to the original template sequence and may yield misleading results. Therefore, in order to clone a DNA fragment that is faithfully amplified and represents the original target DNA, different companies have come up with high-fidelity enzymes that are not error-prone and do the amplification faithfully [4, 5]. Q5® high-fidelity polymerase is one such enzyme from New England Biolabs (MA, USA). A typical reaction with Q5® follows (Tables **6** and **7**).

Materials

Template DNA (Plasmid or Genomic DNA)

5X Q5® Polymerase Reaction Buffer

10 mM dNTPs

10 μM primers (forward and reverse)

Nuclease-free water

Q5® High-Fidelity DNA Polymerase (NEB, MA, USA)

Equipment

Thermo Cycler, Tabletop or microcentrifuge, surgical gloves, ice pale.

Glassware/Plasticware: Small (0.5 ml) capacity Microfuge tubes, Micropipettes and tips.

Procedure

- In a clean 0.5ml microfuge tube (PCR tube) add the following reagents as shown in the chart. Start with the addition of water and then the others. Q5® High-Fidelity DNA polymerase should be added last.

Table 6. Setting up polymerase chain reaction with Q5® high-fidelity DNA polymerase.

-	25 µl Reaction	50 µl Reaction	Final Concentration
5X Q5® Reaction Buffer*	5 µl	10 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM primers (forward and reverse)	1.25 µl	12.5 µl	10.5 µM
Template DNA	variable	variable	20 ng-1 µg
Nuclease-free water	To 25 µl	To 50 µl	-
Q5® High-Fidelity DNA Polymerase**	0.25 µl	0.5 µl	0.05 units/50 µl reaction

**If 10X reaction buffer is used, the volume of the reaction buffer should be halved.*
***The amount of polymerase added will depend on the polymerase used. For amplicons > 6 kb, up to 2 units of the Q5®High-Fidelity Polymerase/50 µl reactions can be added.*

- Mix all the reagents by gently tapping or pipetting up and down.
- Centrifuge briefly to bring all of the liquid to the bottom of the tube.
- Set up a program in the Thermo Cycler Machine following the table given below.

Table 7. Program for PCR using Q5 Polymerase.

-	CYCLES	TEMP	TIME
Initial Denaturation	**1**	**98°C**	**30 seconds**
Denaturation	**30**	**98°C**	**30 seconds**
Annealing		**50–72°C***	**30 seconds**
Extension		**72°C**	**20-30 seconds per kb**
Final extension	**1**	**72°C**	**2 minutes**
Hold	**1**	**10°C**	-

* Tm values should be determined using the available Tm calculator. Annealing temperature will depend on the Tm values of the primers.

- Place the tubes in the holders and press START.
- Pour a small or medium 1% agarose gel, as mentioned in Chapter 5.
- Once the entire amplification cycle is over, take out the sample tubes from the slots of the Thermo-Cycler and store the tubes either in ice (if the amplification

would be checked right away). Alternatively, the samples may be stored at 4°C for one to a few days.
- Check for the success of the amplification reaction by electrophoresing about 5 µl of the reaction sample in a 1% agarose gel.

NOTE: Calculate the size of the DNA that is amplified. A standard DNA sample close to the size of the amplified product or an appropriate standard DNA ladder in the agarose gel should be used in the gel analysis to confirm that the amplification reaction is successful.

6.4.3. Tips for Successful Amplification Reaction by Polymerase Chain Reaction

Tips about DNA Template

- Use only high-quality, purified DNA templates whenever and wherever possible.
- For low-complexity templates (*i.e.*, plasmid DNA), use only 1 pg–10 ng of DNA per 50 µl reaction.
- For higher complexity templates (*i.e.*, genomic DNA), use 1 ng–1 µg of DNA per 50 µl reaction.
- Higher DNA concentrations tend to decrease amplicon specificity, particularly for higher numbers of cycles.

Tips about Primers

- Primers should typically be 20–40 nucleotides in length, with 40–60% GC content.
- Primer Tm values should be determined with a T_m calculator like Oligo DT or Oligo Analyzer.
- Primer pairs should have their T_m values that are within 5°C.
- Avoid secondary structure (*i.e.*, hairpins, self-dimer, *etc.*) within each primer and potential dimerization between the primers.
- Higher than recommended primer concentrations may decrease specificity.
- When engineering restriction sites onto the end of primers, at least 6 nucleotides should be added 5′ to the site of incorporation.

Tips about Enzyme Concentration

- The optimal concentration is the concentration that is specific to each polymerase. Follow the guidelines by the manufacturer for the optimum enzyme concentration that should be used
- Master mix formulations already contain optimal enzyme concentrations for most applications

Tips about Magnesium Concentration

- Most PCR buffers provided by NEB, Thermo Fisher Scientific, or other manufacturers already contain optimum levels of Mg^{2+} at 1X concentrations.
- Excess Mg^{2+} may lead to spurious amplification; insufficient Mg^{2+} concentrations may cause reaction failure.

Tips about Deoxynucleotides

- Ideal dNTP concentration is typically 200 μM each
- Use *Taq* DNA Polymerases when uracil is either present in the primer, template, or deoxy nucleotide mix.

Tips about Starting Reactions

- Unless using a hot start enzyme, assemble all reaction components on ice.
- Add the polymerase last, whenever possible.
- Transfer reactions to a thermocycler that has been pre-heated to the denaturation temperature. Pre-heating the thermocycler is not necessary when using a hot start enzyme.

Tips about Denaturation

- Avoid longer or higher temperature incubations unless required due to the high GC content of the template.

Tips about Annealing

- Primer T_m values should be determined using the NEB T_m Calculator, Oligo DT, or Oligo Analyzer.
- Non-specific product formation can often be avoided by optimizing the annealing temperature or by switching to a hot start enzyme (*e.g.*, Q5® Hot Start High-Fidelity DNA Polymerase or One *Taq* Hot Start DNA Polymerase).

Tips about Extension

- Extension rates are specific to each PCR polymerase. In general, extension rates range from 15–60 s/kb.
- Longer than recommended extension times can result in higher error rates, spurious banding patterns and/or reduction of amplicon yields.

Tips about Switching from Taq DNA Polymerase

When switching from a *Taq* product to a high-fidelity polymerase to Q5® High-Fidelity DNA Polymerase, remember to use:

- Higher annealing temps.
- Higher denaturation temps – particularly beneficial for difficult templates.
- Higher primer concentrations.
- Shorter cycling protocols.

6.5. MODIFICATION OF THE VECTOR AND INSERT DNA SAMPLES

The final goal of a cloning procedure is to glue or ligate the vector and insert DNA molecules together to generate recombinant DNA. In order to join the termini of the vector DNA to that of the insert, the two ends must be compatible. Compatibility requires that either the two ends be blunt/flush or they should have cohesive termini that are complementary to each other. It should be noted here that, in reality, such compatible termini are not always obtained due to a number of reasons. For example, a given insert may not have the sites for the same restriction enzymes that were used to prepare the vector. As a result, the vector and insert do not have the compatible termini because they were produced by different sets of restriction sites. As a result, these molecules cannot be ligated directly unless the ends/termini of one or both of them are modified in some way to convert these ends as compatible [4, 5].

Second, if the vector DNA molecule is linearized using a single restriction enzyme, the two ends of the vector are compatible for ligation, leading to the self-ligation or recircularization of the vector molecules during the ligation events even in the presence of insert. Such religated vector molecules are not desirable because they contribute to the background of the cloning process. Note that, during ligation *in vitro*, DNA ligase catalyzes the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide carries a 5′-phosphate residue and the other nucleotide carries a 3′-hydroxyl terminus. Recircularization of plasmid DNA can, therefore, be significantly minimized if the 5′-phosphate residues from both termini of the plasmid/vector DNA are removed with alkaline phosphatase (Fig. **5**). Moreover, a foreign DNA segment with intact 5′-terminal phosphate residues can be ligated efficiently *in vitro* to the dephosphorylated plasmid DNA to generate an open circular molecule containing two nicks. Once these open circular DNA molecules are transformed into E. coli, most of these nicks are repaired by the host machinery, leading to the formation of supercoiled recombinant DNA (vector ligated to insert) molecules. End modifications, therefore, improve the efficiency of the cloning process, ensuring that the ends to be joined are compatible and optimizing the positioning of regulatory and translated sequences.

6.5.1. Commonly used DNA End Modification Reactions

6.5.1.1. Phosphorylation of the PCR Products

Principle

As stated above, ligation between the two compatible termini requires a 5′-phosphate residue in one of the participating nucleotides and a 3′-hydroxyl terminus in the neighboring nucleotide [4, 5]. Notably, PCR primers are synthetic oligonucleotides, and they are typically synthesized with a 5′-hydroxyl end and do not carry a phosphate at the 5′end. As a result, the amplification products that are produced after the PCR reaction using these non-phosphorylated primers also lack the 5′-phosphate. Therefore, it becomes imperative that either these oligonucleotides or the PCR products prepared with the non-phosphorylated primers must be phosphorylated before the ligation reaction and consequently need to be treated with a kinase in presence of donor adenosine triphosphate (ATP) (Table **8**). Alternatively, primers for PCR can also be procured with 5′-phosphate to avoid the need to separately phosphorylate either the oligonucleotides or the PCR products with a kinase.

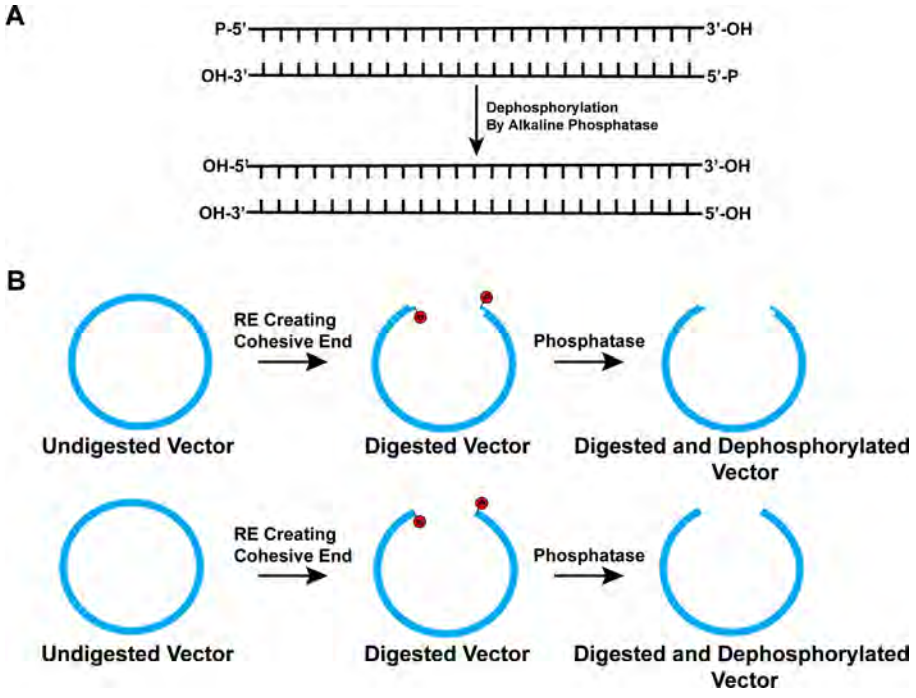

Fig. (5). Dephosphorylation of the 5′-phosphate present in both ends of a linear vector DNA, which can be removed by the treatment of the DNA with various alkaline phosphatases (see **UNIT 6.5.1.2**).

PROTOCOL 6: PHOSPHORYLATION OF THE PCR PRODUCTS WITH T4 POLYNUCLEOTIDE KINASE

Materials

DNA sample to be phosphorylated (PCR product)

10X Polynucleotide Kinase Buffer

10 mM Adenosine 5′-Triphosphate (ATP)

T4 Polynucleotide Kinase (PNK)

Nuclease-free water

Equipment

Water Bath, Tabletop or microcentrifuge, surgical gloves, ice pale.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Add all the reagents mentioned below in the table, starting with water and ending with the T4 Polynucleotide Kinase Enzyme.

Table 8. Phosphorylation reaction with T4 polynucleotide kinase.

DNA to be phosphorylated	1-2 µg
10X Polynucleotide Kinase Buffer	5 µl
10 mM Adenosine 5′-Triphosphate (ATP)	5 µl (1mM final Concentration)
T4 Polynucleotide Kinase (PNK)	1 µl (10 units)
Nuclease-free water	to 50 µl
Incubation	37°C, 30 minutes

- Mix the contents very gently by tapping. As mentioned before, all the modification enzymes, including T4 PNK, are stored in 50% glycerol. Consequently, the enzyme will sink immediately to the bottom of the reaction. Make sure the entire content of the reaction is properly homogenized after the addition of the enzyme. If needed, pipet up and down the entire content gently several times after the addition of the enzyme.
- Centrifuge the tube briefly to bring the last drop of liquid to the bottom of the tube.

- Incubate at 37°C for 30 minutes, followed by heating the reaction at 65°C for 10 minutes to stop the reaction.

6.5.1.2. Dephosphorylation of Vector DNA

Principle

As stated above (section 6.5), dephosphorylation is a very crucial and widely used method to suppress the self-circularization of the vector DNA to reduce the background noise. The most commonly used phosphatases used in carrying out dephosphorylation are Bacterial alkaline phosphatase (BAP) from *E. coli* and calf intestine phosphatase (CIP) from veal [4, 5]. Both enzymes catalyze the hydrolysis of 5′-phosphate residues from the free end of vector/linear DNA and require Zn^{2+} for their activity (Tables **9** and **10**). BAP is more stable than CIP. BAP is difficult to inactivate following the dephosphorylation reaction since it is resistant to both heat and phenol extraction. On the other hand, CIP is readily inactivated by heating to 70°C for 10 min and/or extraction with phenol. Furthermore, CIP has a 10- to 20-fold higher specific activity than BAP [4, 5]. Therefore, most of the research laboratories involved in molecular biology research opt for CIP to dephosphorylate the 5′-phosphate of linear DNA over BAP. Shrimp alkaline phosphatase is another choice for dephosphorylation besides BAP and CIP (Table **11**), and it is sold by many companies.

PROTOCOL 7: DEPHOSPHORYLATION OF THE LINEAR VECTOR DNA WITH BACTERIAL ALKALINE PHOSPHATASE (BAP)

Materials

10 x BAP Reaction Buffer:(500 mM Tris-HCl, pH 8.0, 10 mM $ZnCl_2$)

Linearized Vector DNA

BAP (150 U/µl)

Proteinase K (100 µg/ml)

SDS: (1%)

Equipment

Water Bath, Tabletop or microcentrifuge, surgical gloves, ice pale.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Prepare a working stock of 1U/µl of the stock enzyme using the diluent buffer that comes with the enzyme.
- Purify the vector DNA to be dephosphorylated by digestion and ethanol precipitation and resuspend the pellet in dH_2O.
- Add the following reagents to a clean microfuge tube.

Table 9. Dephosphorylation reaction with Bacterial Alkaline Phosphatase (BAP).

Linearized Vector DNA	40 µl DNA (containing 1 to 20 pmol final concentration)*
10X BAP Dephosphorylation Buffer	5 µl
BAP (1 U/µl) Stock**	5 µl
Incubation	60°C, 30 minutes

*1µg of 1,000bp DNA = 1.52pmol DNA = 3.03pmol of ends.
**BAP is commercially available at a concentration of 150U/µl. It is convenient to prepare a 1U/µl working stock right before using the storage buffer as the diluent.

- Incubate the tube at 60°C for 30 min.

NOTE: This high temperature is recommended as any residual exonucleases in the enzyme preparation are readily suppressed.

- Add 0.1% SDS (from 1% stock) and 100 µg/ml proteinase K (from 1 mg/ml stock) to stop the reaction. Incubate at 37°C for 30 min.
- Extract twice with phenol and once with chloroform and precipitate the DNA with ethanol. Further purifications, including extra phenol extractions or silica binding of the DNA, are often performed before ethanol precipitation, considering the stability of the enzyme and being carried over in an experiment.

PROTOCOL 8: DEPHOSPHORYLATION OF THE LINEAR VECTOR DNA WITH CALF INTESTINAL ALKALINE PHOSPHATASE (CIAP/CIP)

Materials

10mM Tris-HCl (pH 8.0)

10X CIP Buffer (200 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM $ZnCl_2$)

Linearized Vector DNA

CIP: (l U/ µl)

CIAP stop buffer

TE-saturated phenol:chloroform

chloroform:isoamyl alcohol (24:1)

7.5M ammonium acetate (pH 5.5)

Ethanol, 100% and 70%

Equipment

Water Bath, Tabletop or microcentrifuge, surgical gloves, ice pale.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Dilute sufficient CIAP for immediate use in CIAP 1X Reaction Buffer to a final concentration of 0.01U/µl. Each picomole of DNA ends will require 0.01U CIAP (1µg of 1,000bp DNA = 1.52pmol DNA = 3.03pmol of ends).
- Purify the vector DNA to be dephosphorylated by digestion and ethanol precipitation, and resuspend the pellet in 40 µl of 10 mM Tris-HCl (pH 8.0).
- In a clean sterile tube, add the reagents as described in the following table.

Table 10. Dephosphorylation reaction with calf intestinal alkaline phosphatase.

Linearized Vector DNA	40 µl DNA (containing 1 to 20 picomole final concentration) **
10X CIAP Dephosphorylation Buffer	5 µl
CIAP (0.01 U/µl) Stock*	5 µl
Incubation	60°C, 30 minutes

*CIAP is commercially available at a concentration of 1 U/µl. It is convenient to prepare a 0.01 U/µl working stock right before using the storage buffer as the diluent.

- Incubate at 37°C for 30 min. Add another aliquot of diluted CIAP (equivalent to the amount used in Step 2), and continue incubation at 37°C for an additional 30 minutes.
- Place the tube at 75°C for 10 min to stop the reaction. Alternatively, add 300µl of CIAP stop buffer to stop the reaction, followed by extracting with phenol, and then precipitate with ethanol and 0.5M ammonium acetate (pH 5.5).

NOTE 1: 10 min at 75°C effectively inactivates CIP as CIP is heat labile, unlike BAP (It is recommended that before purifying the phosphatase-treated DNA by phenol extraction and ethanol precipitation, CIP should be heat-inactivated.

NOTE II: Usually, right after restriction digestion, phosphatase treatment can be done directly in the same reaction buffer. In this case, the buffer compatibility must be taken into account. The dephosphorylating enzyme should be active in the buffer used for digestion. This is supplied in the supplier's manual.

NOTE III: A useful conversion factor is that 1.0 µg of a 1.0 kb linear DNA contains about 3 pmol, and a 3-kb linear DNA contains 1 pmol of 5′ termini.

PROTOCOL 9: DEPHOSPHORYLATION OF THE LINEAR VECTOR DNA WITH SHRIMP ALKALINE PHOSPHATASE (SAP)

SAP is more heat-labile than CIP and is often used in reactions where absolutely no carry-over of inactivated phosphatase can be tolerated.

Materials

10X SAP buffer (20 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$)

Linearized Vector DNA

SAP (1 unit/ µl)

Equipment

Water Bath, Tabletop or microcentrifuge, surgical gloves, ice pale.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Purify the vector DNA to be dephosphorylated by digestion and ethanol precipitation, and resuspend the pellet in 10 µl of dH_2O.
- In a clean Eppendorf tube, add the following reagents; the enzyme is added last.

Table 11. Dephosphorylation reaction with Shrimp Alkaline Phosphatase (SAP).

DNA	10 µl (1 picomole of DNA ends)
10X SAP Reaction Buffer	2 µl (1X)
Alkaline Phosphatase (SAP)(1unit/µl)*	1 µl (I U)
Nuclease free water	7 µl
Incubation	37°C, 30 minutes
Heat Inactivation	65°C, 15 minutes

*SAP is commercially available at a concentration of 1 U/µl.

- Incubate the tube at 37°C for 30 minutes.
- Heat inactivate the reaction at 65°C for 15 minutes.

NOTE: Depending upon the individual application, the volume of reaction and concentration of DNA will vary.

Tips for Optimization

Dephosphorylation reaction can be done right after the restriction enzyme digestion reaction if the restriction enzyme(s) used can be inactivated by heat. Then, cleaning up the digested DNA is not required, and the dephosphorylated enzyme can be added directly to the same reaction tube. But if the restriction enzyme(s) used are not heat-inactivated, then a DNA clean step by phenol extraction followed by ethanol precipitation is recommended prior to the dephosphorylation step. The final concentration of a specific phosphatase and the time of dephosphorylation reaction can be chosen from the following (Table **12**) based on the nature of termini.

Table 12. Nature of termini, dephosphorylation enzyme and time/temperature.

Nature of Termini	Enzyme (Units per mole of DNA termini)	Incubation Temperature/Time
5′-Protruding	0.01 U CIAP*	37°C/30 minutes
-	0.1 U SAP	37°C/30 minutes
3′-Protruding	0.1-0.5 U CIAP**	37°C/15 minutes and then
-	-	55°C/45 minutes
-	0.5 U SAP	37°C/60 minutes
Blunt	0.1-0.5 U CIAP**	37°C/15 minutes and then
-	-	55°C/45 minutes
-	0.2 U SAP	37°C/60 minutes

*After an initial incubation for 30 minutes, the addition of a second aliquot of enzyme followed by incubation at 37°C for 30 minutes is recommended

**The addition of a second aliquot of CIAP is recommended right before the incubation step at 55°C.

Applications

Dephosphorylation plays a pivotal role in the preparation of vector DNA of optimum quality during the cloning process. The entire idea is to obtain optimum amounts of recombinant DNA molecules and prevent the unproductive ligation of vector molecules. The ends of the vector generated by a single restriction enzyme or cut with two enzymes with compatible ends can potentially self-circularize, resulting in unproductive ligation products. This is suppressed by modifying the vector ends by using alkaline phosphatase that catalyzes the hydrolysis of 5′-

Phosphate of the DNA. The hydroxyl groups present at both the 3′- and 5′-ends in the dephosphorylated vector DNA prevent this intramolecular ligation [4, 5]. This, in turn, reduces the background during subsequent transformation. It is essential to ensure that the insert contains a 5′-phosphate to allow ligation to proceed if the vector is dephosphorylated. Each double-strand break requires that one intact phosphodiester bond be created before transformation (and *in vivo* repair).

6.5.1.3. Converting Non-Compatible Cohesive Termini into Blunt Termini by 'Filling in' or by 'End-repair'

The process of blunting is usually done when either the vector or insert (or both) harbors noncompatible ends principally due to the absence of appropriate restriction sites in either or both of them [4, 5]. As stated above, the presence of the non-compatible ends cannot be joined or ligated to form a new circular recombinant plasmid. The key strategy to overcome this problem is to convert both the non-compatible cohesive termini into the blunt termini so that they can be conveniently ligated. The method involved in converting a given cohesive termini is known as 'blunting', which is accomplished either by "filling-in" or by "chewing-back". [4, 5] In the "filling-in" process, the single-stranded overhang that is earlier created by the previous restriction digestion step is filled in by adding nucleotides on the complementary strand using the overhang as a template for polymerization (Fig. **6**). In the "chewing-back" approach, the 3′→5′ exonuclease activity is utilized to processively degrade the 3′-protruding strand (Fig. **6**). To repair overhanging 5′ ends, 5′→3′ polymerase activity of either Klenow fragment of *E. coli* DNA polymerase I or T4 DNA Polymerase is generally used whereas overhanging 3′ ends are repaired or chewed back using either Klenow or T4 DNA polymerase, both of which have a 3′→5′ exonuclease activity. Removal of a 5′ overhang can also be accomplished with a nuclease, such as Mung Bean Nuclease [4, 5]. This section describes the standard protocols involved in converting the cohesive termini (with both 5′ and 3′ extensions) into blunt termini using different kinds of enzymes.

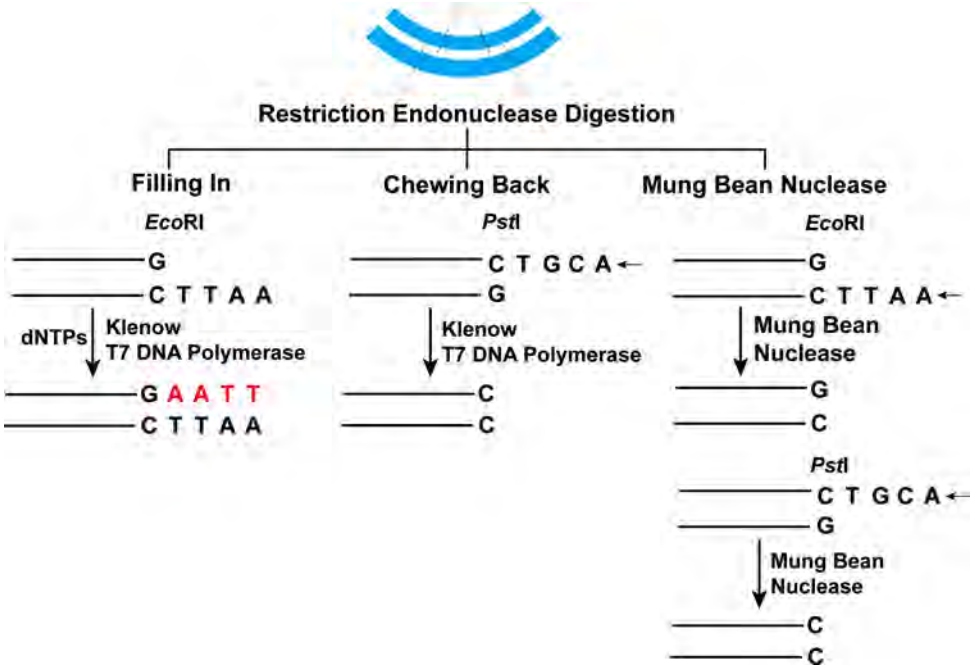

Fig. (6). Schematic diagram showing the various strategies to convert the non-compatible cohesive termini (both 5′-extended and 3′-extended) into ligation-compatible blunt termini using Klenow, T4 DNA Polymerase and Mung-Bean Nuclease.

PROTOCOL 10: CONVERSION OF NON-COMPATIBLE COHESIVE TERMINI INTO BLUNT TERMINI BY T4 DNA POLYMERASE

Principle

T4 DNA polymerase is composed of a single polypeptide chain of molecular weight 112,000. Apart from being a DNA-dependent DNA polymerase, this enzyme is equipped with a single-stranded and double-stranded 3′→5′ exonuclease activity but lacks a 5′→3′ exonuclease function. This enzyme is utilized in both the chewing-up process of 3′ overhangs using its 3′→5′ exonuclease activity as well as the fill-in process using its DNA polymerase activity. The combined action of both of these activities converts the 3′-overhang into a blunt termini and is (Table **13**), therefore, extensively useful in the cloning process.

Materials

T4 DNA polymerase (3 U/µl)

10X T4 polymerase reaction buffer (500mM Tris-HC1, pH 8.8, 50 mM $MgCl_2$. 50 mM DTT)

DNA to be blunted (2 µg)

1 mM dNTP mix

10 U T4 DNA polymerase

0.5 M EDTA

Equipment

Water bath, tabletop or micro centrifuge, surgical gloves, ice pale.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Purify the RE digested vector/insert DNA to be blunted by ethanol precipitation, and resuspend the pellet in 15 µl of dH_2O.
- In a clean microfuge tube, add the following reagents; the enzyme is added last.

Table 13. Reaction with T4 DNA polymerase to form blunt ends.

DNA in nuclease-free water	15 µl (containing 0.5 - 2 µg)
10X polymerase reaction buffer	2.5 µl
1 mM dNTP Mix	2.5 µl
T4 DNA polymerase (3 U/µl)	1 µl
Nuclease free water	4 µl
Incubation	Room temperature or at15°C for 15 min for RE-digested DNA 30 min for sheared/nebulized DNA or PCR products*
Heat Inactivation	70°C, 10 minutes or 2 µl of 0.5 M EDTA

*PCR-generated DNA must be purified before blunting using a commercial purification kit, phenol extraction/ethanol precipitation, or gel electrophoresis.

- Incubate at 15°C for 15-30 min.
- Stop the reaction by adding 2 µl of 0.5 M EDTA or by heating to 75 °C for 10 min.

NOTE: The volume of the reaction, the concentration of 4 dNTPs, and the temperature of the reaction will vary, depending upon the individual application.

PROTOCOL 11: CONVERSION OF 5′-OVERHANGING TERMINI INTO BLUNT TERMINI USING KLENOW FRAGMENT OF *ESCHERICHIA COLI* DNA POLYMERASE I

Principle

It is the Klenow fragment that is responsible for filling in 5′ overhangs (Table **14**), which is generated by the restriction enzyme. The Klenow fragment consists of the C-terminal 70% moiety of *E.coli* DNA polymerase I generated from mild proteolytic cleavage and retains its 3′→5′ exonuclease activity but lacks the 5′→3′ exonuclease activity.

Materials

Klenow DNA polymerase

10X Klenow reaction buffer (500 mM Tris-HCI, pH7.5, 100 mM $MgCl_2$, 10 mM DTT)

dNTP mix (1 mM Stock)

Equipment

Water Bath, tabletop or micro centrifuge, ice pale.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Digest 0.1 to 4 µg DNA with a restriction endonuclease in a total volume of 20 µl reaction. (Optional: Purify the digested DNA by phenol extraction followed by ethanol precipitation).

NOTE: The Klenow fragment works nearly in all restriction buffers and, hence, is added to the reaction directly; therefore, it is unnecessary to inactivate the restriction endonuclease, change buffers, or purify the DNA.

- From a stock of 0.5 mM dNTP (containing dATP, dCTP, dTTP, and dGTP), add 1µl each (total 4 µl) in the tube.

Table 14. Conversion reaction with klenow fragment of E.coli to form blunt ends.

DNA (restriction digested)	0.1 to 4 µg
10X Klenow Reaction buffer	2 µl
1 mM dNTP Mix	2 µl

(Table 14) cont.....

Klenow fragment	1 to 5 U
Nuclease free water	To 20 µl
Incubation	15 min for 30ºC RE-digested DNA
Heat Inactivation	75ºC, 10 minutes /add 1µl of 0.5 M EDTA

- Add 1 to 5 U of the Klenow fragment and incubate at 30°C for 15 min.

NOTE: Since the Klenow fragment lacks the 5′→3′ exonuclease activity, it cannot carry out the extensive repair of overhanging 3′ ends, which is usually carried out by T4 DNA polymerase (a much more expensive enzyme) or native T7 DNA polymerase.

- Stop the reaction by heating to 75°C for 10 min or by adding 1 µl of 0.5 M EDTA.

NOTE 1: If the DNA needs to be digested with more than one restriction enzyme that does not have a buffer compatibility, then the procedure needs a little modification. After digesting with the first enzyme, the ends are repaired using the Klenow fragment, which is then inactivated by heat (75°C for 10 min) before digesting with the second enzyme.

NOTE II: The progress of the reaction can be monitored by a simple ligation experiment, followed by gel electrophoresis. DNA that originally had complementary termini should not be ligated by low amounts of T4 ligase. Instead, ten times the amount of ligase should be required to achieve the necessary blunt-end ligation. To monitor the progress of the blunting reaction, an aliquot of the Klenow reaction mix may be transformed into the E. coli host. The expectation is that there will be no or only a few transformed colonies if the blunting reaction reaches completion.

DNA can be treated with exonucleases such as Bal 31, λ exonuclease, exonuclease III, or endonucleases such as S1 or mung bean nuclease if desired.

PROTOCOL 12: CONVERSION OF 5′-OVERHANGING TERMINI INTO BLUNT TERMINI USING MUNG BEAN NUCLEASE

Principle

This enzyme is extracted from sprouts of mung bean, which is a highly specific single-stranded endonuclease. It has been demonstrated that as the pH is reduced below 5.0, its activity increases and significantly decreases at NaCl concentrations more than 200 mM using the double-stranded DNA as substrate (Table **15**). One unit of the enzyme is defined as the amount of enzyme that produces one µg of

acid-soluble material in 1 min at 37°C using single-stranded salmon sperm DNA as the substrate.

Materials

Restriction Digested DNA to be blunted (1 to 10 µg at a concentration of 0.5 µg/µl)

Mung Bean Nuclease (10 U/µl)

10X Mung Bean Nuclease Reaction Buffer (300 mM Na-Acetate, pH-5.0, 1 M NaCl, 10 mM Zn-acetate. 50% glycerol)

EDTA (0.5 M Stock)

Equipment

Water Bath, Tabletop or microcentrifuge, ice pale.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Purify the RE digested vector/insert DNA to be blunted by ethanol precipitation, and resuspend the pellet in 20 µl of dH_2O in a clean microfuge tube.
- Add the following reagents to the DNA, the enzyme being added as the last component.

Table 15. Reaction with mung bean nuclease to form blunt ends.

DNA (restriction digested)	20 µl (10 µg)
10X Mung Bean Nuclease Reaction Buffer	3 µl
Mung Bean Nuclease (10 U/µl)	1 µl (10 U)
Nuclease free water	6 µl
Incubation	30 min for 37°C
Heat Inactivation	add 1µl of 0.5 M EDTA

- Place the tube containing the reaction in a 37°C water bath for 30 min.
- Add 1 µl of 0.5 M EDTA to stop the reaction.
- Extract the reaction with phenol:chloroform extraction followed by precipitation and recovery with ethanol precipitation.

NOTE: The volume of reaction, amount of DNA, units of enzyme, temperature, and time of reaction will vary depending on the application.

PROTOCOL 13: ADDITION OF NON-TEMPLATED T AND A RESIDUE TO THE BLUNT TERMINI USING KLENOW FRAGMENT (3′→5′ EXO$^-$) (A-TAILING)

Principle

Tailing is an enzymatic method in which non-templated nucleotides (such as A or T) are added to the 3′ end of a blunt, double-stranded DNA molecule. Tailing is usually recommended to construct a T-vector for use in TA cloning or to add an A-tail of a PCR product yielded by a high-fidelity polymerase (but not *Taq* DNA Polymerase) (Table **16**) for use in TA cloning system (Fig. **3A**). Notably, TA cloning is a fast and convenient method of cloning PCR products that utilize stabilization of the single-base extension (adenosine) produced by *Taq* DNA polymerase by the complementary T (thymidine) of the T-vector before they are ligated and transformed. This technique does not call for the use of restriction enzymes, and PCR products can be used directly without modification. Additionally, PCR primers do not need to be designed with restriction sites, thereby making the entire cloning process less complicated and more convenient. One demerit of this process is that it yields non-directional cloning because the insert can be ligated to the vector in both orientations.

Materials

Purified blunt DNA to be tailed with T or A

10X Reaction Buffer (10 mM Tris-HCl, pH-7.9, 10 mM $MgCl_2$, 50 mM NaCl, 100 µg/ml BSA)

dATP(1mM)

Klenow Fragment (3′-5′ *exo*$^-$)

Nuclease free water

Equipment

Water bath, tabletop or microcentrifuge, ice pale.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Purify the RE digested vector/insert DNA to be A-tailed by ethanol precipitation, and resuspend the pellet in 30 µl of dH_2O in a clean microfuge tube.

- Add the following reagents to the DNA, the enzyme being added as the last component.

Table 16. Tailing reaction with klenow fragment (3'-5' exo-).

Purified blunt DNA	30 µl (1-5 µg DNA)
10X Reaction Buffer	5 µl
dATP (1mM)	0.5 µl (0.1mM final)
Klenow Fragment (3′-5′ *exo*-)	3 µl
dH_2O	11.5 µl
Incubation	37°C, 30 minutes

- Mix the contents of the tube gently by tapping.
- Incubate the reaction at 37°C for 30 minutes.
- Inactivate the reaction by incubating the reaction at 75°C for 20 minutes.

6.5.1.. A Note on DNA End Modification Process

Vectors and inserts are often "blunted" to allow non-compatible ends to be converted to compatible ends before ligation. A little sequence of information is often lost or distorted by this method, and a detailed understanding of the modification should be considered before performing this procedure. Please note that as long as the altered/lost/distorted sequence is not part of a translated region or a critical regulatory element, the consequence of creating blunt ends is truly negligible. Blunting a region of the translated coding sequence, however, usually creates a shift in the reading frame. DNA polymerases, such as the Klenow Fragment of DNA Polymerase I and T4 DNA Polymerase, are often used to fill in (5′→3′) and chew back (3′→5′). In cases where blunting or modification may lead to information loss, it should be avoided [4, 5].

6.6. JOINING OF THE VECTOR AND INSERT DNA BY LIGATION REACTION

6.6.1. DNA Ligation

The most critical step in cloning workflows is the sealing of nicks between adjacent residues of a single-strand break on a double-strand substrate and the joining of double-strand breaks. It is the catalytic activity of the enzyme DNA ligases that catalyzes this ligation reaction of DNA (Refer to Fig. **7**). DNA ligase catalyzes the joining of 3′-OH to the 5′-phosphate *via* a three-step mechanism. Initially, the ligase is self-adenylated by reaction with free ATP. The second step involves AMP nucleotide, which is attached to a lysine residue in the enzyme's

active site and is transferred to the 5′-phosphate. Finally, the AMP-phosphate bond is attacked by the 3′-OH, forming the covalent bond and releasing AMP. To allow the enzyme to carry out further reactions, the AMP in the enzyme's active site must be replenished by ATP [4, 5]. DNA ligases play critical roles in DNA replication and repair in living organisms.

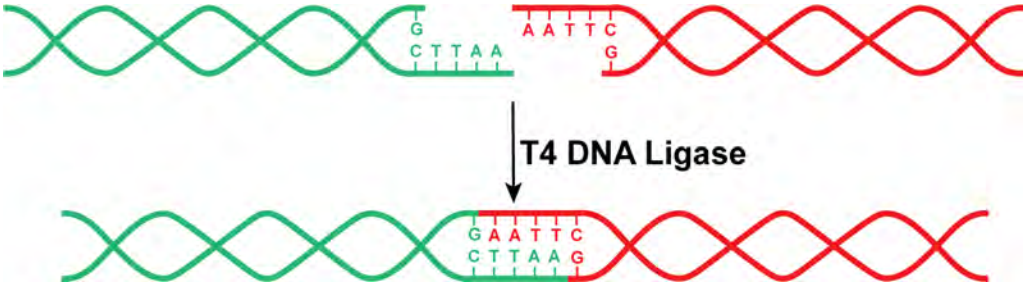

Fig. (7). Ligation of two cohesive termini catalyzed by T4 DNA Ligase

In the laboratory, DNA ligation is performed for both cloning and non-cloning applications. The goal of molecular cloning involves the construction of a new circular molecule termed "recombinant DNA molecule", which can replicate autonomously as an extra-chromosomal circular plasmid DNA within a microbial host. The ligation reaction physically joins the DNA vector to a sequence of interest. Both the vector and insert are prepared by restriction digestion, generating either blunt or cohesive ends, and contain at least one monophosphate group on its 5′ends. The formation of a phosphodiester bond is subsequently catalyzed by the DNA ligase. In this context, it should be noted that if the linear vector DNA is used in the ligation reaction and is prepared employing only a single restriction endonuclease without dephosphorylation event, the linear vector molecules have a strong propensity of undergoing self-circularization (see ***UNIT 6.5***). Note that this self-ligated product is capable of undergoing transformation and thus contributes to the background of the recombinant clone (vector and insert ligation). In addition, the linearized insert molecules, if prepared by single restriction endonuclease digestion, are also subjected to the ligation reaction to generate circularized ligated product. Thus, three different classes of products are generated in a ligation reaction if a single digested vector and insert DNA samples are used in a ligation reaction, which involves a religated vector, religated insert, and vector ligated to insert (desired). The entire ligated products that are created by the ligation reaction are introduced into a host bacterial strain by a process called transformation [1, 4, 5]. Once inside the host cell, the religated circular insert molecules are unable to propagate because they lack replication origin. The religated vector and recombinant clones are capable of propagation because both of them have the antibiotic resistance gene as well as the origin of replication.

Thus, both of these molecules can be maintained and amplified using a suitable selection pressure created by the proper antibiotics. The transformants containing the correct recombinant plasmid, therefore, need to be identified using smart screening strategies from transformants carrying religated vector molecules (***CHAPTER 7***) [4, 5]. Once screened out, the recombinant clone will be available for further downstream applications such as DNA sequencing, protein expression, or gene expression/functional analysis.

SPECIAL INFORMATION PANEL: DNA LIGASES

• DNA ligases ligated two DNA molecules by catalyzing a phosphodiester bond between the 5' phosphoryl termini of one molecule and 3' of another molecule and are considered to be the most important enzymes used in molecular cloning. Apart from DNA, it also acts on RNA. This enzyme can either be of bacterial origin or be bacteriophage-encoded [4, 5].
• In case of eubacterial origin (either thermophilic or mesophilic), the ligation reaction is NAD + dependent where the diphosphate linkage of NAD + is used as a phosphoanhydride, and the adenyl group is transferred to the ε-amino group of a lysine residue.
• In the case of eukaryotes and bacteriophages, ligases encode for the ATP-dependent ligation reaction involving hydrolysis of αβ pyrophosphate in ATP, followed by the transfer of the adenyl group to a lysine residue and formation of a high-energy enzyme intermediate.
• The two types of ligases then follow the same mechanism in the formation of phosphodiester bonds. First, the adenyl residue is transferred to the 5' -phosphate at the terminus of one DNA molecule. In the second step, it is then open to nucleophilic attack by a hydroxyl group at the 3' terminus of an immediately adjacent DNA molecule, forming a covalent bond with the elimination of AMP.
• *In vivo*, DNA ligases are required during the replication of DNA for enzymatic completion of lagging-strand synthesis, in genetic recombination, and in DNA repair.
• *In vitro*, recombinant molecules are created by using DNA ligases by stitching an insert DNA with a vector DNA, creating novel combinations of nucleic acid molecules. During cDNA synthesis, the sealing of nicks in the second strand, the amplification of DNA segments that lie outside the boundaries of known DNA sequences (the inverse PCR), the detection of nicks in DNA by the release of AMP and, more recently, the detection of point mutations in DNA by the ligase chain reaction (also known as the ligase amplification reaction) are the specialized uses of this enzyme.

Bacteriophage T4 DNA Ligase

- T4 DNA ligase is a monomeric protein of 487 amino acids (calculated M, = 55,230) and is encoded by gene 30 of bacteriophage T4. Commercially, this enzyme is purified from overproducing strains of *E.coli.* and has a Km of 6 X 10^{-7} M for cohesive termini, 5 X 10^{-5} M for blunt ends, and 1.9 x10^{-9} for nicks. The Km of the enzyme for ATP is -5 X 10^{-5} M.
- This enzyme catalyzes the ligation of cohesive termini, oligodeoxynucleotides, or oligoribonucleotides in RNA-DNA hybrids and promotes the end-to-end joining of two duplex molecules with fully base paired termini [4, 5].
- High concentrations (> 100 mM) of monovalent cations such as Na+ and K+ inhibit the activity of T4 DNA ligase. However, in the presence of 10%PEG, the monovalent cations have a paradoxical effect and stimulate the activity of the enzyme.
- Units of Ligase Activity.

A Weiss unit (Weiss *et al.* 1968b) is defined as the amount of ligase that catalyzes the exchange of 1 nmole of ^{32}P from inorganic pyrophosphate to ATP in 20 minutes at 37°C.

6.6.2. A Note on Vector Insert Ratio in the Ligation Reaction

After the vector and insert DNA have been prepared for ligation, determine the concentration of each one either by a spectrophotometer or by agarose gel against a known amount of standard DNA. Test various vector: insert DNA ratios in order to find the optimum ratio for a particular vector and insert. In most cases, either a 1:1 to a 1:3 molar ratio of vector: insert works well. The following example illustrates the conversion of molar ratios to mass ratios for a 3.0 kb vector and a 500bp DNA insert:

$$\frac{\text{Amount of vector (in ng) X Size of the insert (in kb)}}{\text{Size of the Vector (in kb)}} \text{ X Molar Ratio of the Insert/Vector}$$

Example: How much 500 bp insert DNA needs to be added to 100 ng of 3.0 kb vector in a ligation reaction for a desired vector: insert ratio of 1:3?

$$\frac{\text{100 ng (Amount of Vector) X 0.5 bp (size of the insert in kb)}}{\text{Size of the Vector (in kb)}} \text{ X } \frac{3}{1} = \text{50 ng of Insert}$$

For all the ligation procedures, follow the manufacturer's recommendations. Remember to include controls (Table **17**).

Table 17. Ligation reactions and controls.

-	Ligation 1	Ligation 2	Ligation 3	Ligation 4
Vector	√	√	–	√
Insert	√	–	√	√
Buffer	√	√	√	√
T4 ligase	√	√	√	–

Also, for the transformation, include an additional control for your plate solution (bacteria alone).

PROTOCOL 14: LIGATION OF VECTOR AND INSERT DNA USING T4 DNA LIGASE

Materials

10X T4 DNA Ligation buffer (0.66 M Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP)

T4 DNA ligase (20 U/µl)

Digested and modified (if any) Vector DNA

Digested and modified (if any) Insert DNA

Nuclease-free water

Equipment

Dry heating-cooling block, tabletop or microcentrifuge, ice pale.

Glassware/Plasticware: Microfuge tubes, micropipettes and tips.

Procedure

- Set up a 20 µl ligation reaction with DNA at <10 ng/ µl for circular ligation products following the volumes of different components given in the (Table **18**) below. The volume of the ligation mixture and the DNA concentration depends on the type of ligation experiment.

Table 18. Setting up ligation reaction.

Vector (4 kb)	50 ng (0.02 pmol)
Insert (1 kb)	37 ng (0.060 picomole)
T4 DNA Ligase Reaction Buffer (5X)	4 µl
Ligase	1 µl
Nuclease-free water	To 20 µl
Incubation	According to the supplier's manual, it is typically 16°Cl for 30 minutes to 2 hours.

- Add T4 DNA ligase. For a cohesive-end ligation, add 0.25 units enzyme/µg of DNA (**Fig. 7**), and for a blunt-end ligation, add 2.5 units/µg.
- Gently mix the reaction by pipetting up and down and microfuge briefly.
- For cohesive (sticky) ends, incubate at 16°C overnight or at room temperature for 10 minutes.
- For blunt ends or single base overhangs, incubate at 16°C overnight or at room temperature for at least 2 hours (alternatively, high-concentration T4 DNA ligase can be used in a 10-minute ligation as stated above in step 2).

NOTE: Simple cohesive-end ligations are usually complete in 1-2 h.

- Heat inactivate the ligation reaction for 10 minutes at 65°C.
- Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.
- Analyze the transformants for the presence of the recombinant clones after growing on the appropriate antibiotic plates.

6.6.3. Use of Linkers and Adapters in the Vector Insert Ligations

The linkers and adapters are essentially a stretch of oligonucleotides synthesized artificially. The concept of these molecules arose when it was observed that the blunt end ligations, though they may appear easy, in reality pose a lot of problems and difficulty in accomplishing. Consequently, strategies have been developed to convert the DNA with blunt ends artificially to possess cohesive ends since it is much easier to ligate DNA molecules with cohesive termini. This is accomplished by ligating specific oligonucleotide harboring restriction sites to both ends of blunt-ended fragment, which, when digested, will yield cohesive ends with the blunt-ended molecules. These oligonucleotides are of two types: linkers and adapters.

Linkers are complementary oligomers that form small double-stranded DNA fragments, including a restriction enzyme site. They are ligated to blunt-end DNA by DNA ligase. When digested with the appropriate enzymes, cohesive termini

are generated. So, both the blunt vector and insert are converted to DNA fragments containing cohesive ends, which makes it easy to ligate [4, 5].

Adapters are not perfectly double-stranded, unlike the linkers. They are a stretch of oligonucleotides, one end of which is ligated to the blunt target DNA, and the other end is synthesized to present cohesive termini without any digestion. In this way, the target DNA is ready for ligation to the complementary restriction enzyme termini of the vector.

SPECIAL INFORMATION PANEL: ADAPTORS

- Adaptors are synthesized as short, double-stranded oligonucleotides, either DNA or RNA.
- The main function of the adapters is to bridge or connect two different molecules. Adaptors usually have complementary regions that can hybridize with target sequences on two separate molecules. They allow for the joining of fragments or the attachment of specific sequences to the ends of DNA or RNA molecules [4, 5].
- Adapters become useful during blunt end ligation of a vector with insert. Adaptors find applications in techniques like RNA sequencing library preparation, DNA sequencing library preparation, and PCR-based fragment joining, where they are involved in linking different molecules or fragments.
- The adaptor molecule has one blunt end with a 5′ phosphate group and a cohesive end that is not phosphorylated. The blunt end contains normal 5′phosphate and 3′ OH, whereas the cohesive end contains 3′ OH and 5′ OH instead of a phosphate group. The phosphate group has been modified by the treatment with alkaline phosphatase. The formation of polymers of adapter molecules is hindered because of the absence of 5′P at the cohesive end.
- After the adaptors have been attached, the abnormal 5′OH terminus is converted to the natural 5′P form by the enzyme polynucleotide kinase. Now, the adaptors have sticky-ended fragments, which can be inserted into an appropriate vector.
- The foreign DNA and adaptors are ligated into the vector.

PROTOCOL 15: LIGATION OF LINKERS TO BLUNT-ENDED DNA MOLECULES USING T4 DNA LIGASE FOLLOWED BY THE CREATION OF COHESIVE TERMINI BY DIGESTION WITH RESTRICTION ENZYME

Materials

T4 DNA ligase

10X T4 DNA Ligation buffer (0.66 M Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP)

Restriction enzymes

10X Appropriate Restriction Enzyme Buffer

Bio-Gel A-50 m column (Bio-Rad)

Equipment

Dry Heating-Cooling Block, Water Bath, Tabletop or micro centrifuge, Ice pale.

Glassware/Plasticware: Microfuge tubes, Micropipettes and tips.

Procedure

- Ligate the DNA and the linkers in a total volume of 50 µl in the ratio of DNA to linkers as 1:1 (*e.g.*, 1 µg DNA to 1 ng linkers). This ensures a large molar excess of linkers relative to the blunt-ended DNA.
- Set up the ligation reaction following the recommended volumes of various components in the (Table **19**) below. To the reaction mixture, add 5 units of T4 DNA ligase.

Table 19. Ligation reaction of linkers to blunt ended molecules.

DNA	1 µg
Linker	1 ng
10X ligation buffer	5 µl
T4 DNA ligase	5 Units
Nuclease free water	To 50 µl

- Incubate for 16 hours at 15°C.
- Stop the reaction by heating at 70°C for l0min.
- Add 10 µl of the appropriate 10X restriction enzyme buffer and 40 µl water,

thereby increasing the volume of the ligation mixture to 100 µl.
- Add 100 units of restriction enzyme.
- Incubate at 37°C for 12 h.
- Inactivate the restriction enzyme by heating at 70°C for l0 min.
- Separate the large DNA with linkers at its termini from unligated free linkers by size fractionation on a Bio-Gel A-50 m column.
- Purify and concentrate the large DNA by ethanol precipitation.

Notes I: If the DNA contains internal restriction sites for the enzyme being used to cleave the ligated linkers, then these sites must first be blocked by treatment with the appropriate DNA methylase. Set up a 20 µ1 reaction containing DNA methylase, the appropriate buffer (obtained from the supplier of the methylase), and 80 µM S-adenosylmethionine. Incubate at 37°C (for most methylases) for 1 h, and then recover the DNA by phenol extraction and ethanol precipitation.

NOTE II: Adapters are becoming more favored as a method of adding complementary termini to blunt-end DNA molecules. The procedure is similar to that of linkers. However, no restriction enzyme digestion is required, thereby also removing the need to methylate the target DNA. The adapters containing single-strand complementary ends are ligated on to the DNA (step 1) and phosphorylated with T4 polynucleotide kinase directly in the ligation mixture.

6.7. INTRODUCTION OF THE LIGATED RECOMBINANT MOLECULES BY TRANSFORMATION

Transformation is the process by which the bacterial cells uptake exogenous DNA. The uptake and incorporation of naked DNA can either take place naturally from the cell's natural environment or be introduced artificially into the host [1]. The natural transformation applies to a few species of bacteria and is very rare. In the laboratory, the bacteria are made competent in artificial means to uptake exogenous DNA, and there is a wide array of methods of artificial transformation for triggering uptake [1, 4, 5]. In all cloning protocols, artificial means of transformation are used in ferrying the recombinant DNA formed in the ligation reaction into the host. Hence, the bacterial cells first need to be prepared to become competent to be able to uptake the foreign DNA [4, 5].

The most common method to increase the permeability of the bacterium's membrane involves the use of divalent cations (*e.g.*, Calcium chloride). This treatment makes the bacterial membrane porous and eventually becomes "chemically competent", thereby increasing the capacity of DNA acquisition by the bacteria [4, 5]. Another artificial method of transformation is electroporation, which makes use of high electric potential (voltage) to create holes/pores in the bacterial membrane, producing a cell shock. The porous membrane allows the

transport of the foreign DNA to enter into the cytosol of the bacterium [1, 4, 5]. Regardless of which method of transformation is used, the outgrowth of the bacterial cells following transformation allows repair of the bacterial surface and selection of recombinant cells if the newly acquired DNA conveys antibiotic resistance to the transformed cells [1, 4-5].

PROTOCOL 16: PREPARATION AND TRANSFORMATION OF COMPETENT *ESCHERICHIA COLI* USING CALCIUM CHLORIDE

Principle

Competent cell preparation was first developed as a simple treatment of soaking the bacterial cells in cold $CaCl_2$. To date, it is not known why this treatment is effective, but calcium ions (ii) mask the negative charge (owing to the presence of negatively charged phospholipids) of the membrane, permit efficient binding of the negatively charged DNA before uptake, and (ii) confer bacterial cells to become competent and be able to take up DNA. They initially started their research with λ DNA and later found that this method could be applied to plasmid (3) and chromosomal (4) DNA samples as well [4, 5].

Competent cell preparation is a simple procedure that involves the growth of *E. coli* cells to the early-mid-log phase, harvesting, and resuspension of the cell pellet in a solution containing ice-cold calcium chloride. Once the cell pellet/suspension is chilled at 0°C, all the solutions, plasticware, glassware, and cell suspensions are strictly maintained on ice till the cells feel the heat only during the heat shock step that allows the DNA to efficiently enter the cells. Plasmid DNA is mixed with the chilled cells once the cells are placed on ice for 20-30 minutes, followed by the heat shock. After the heat shock step, the cell suspension, along with the DNA, is brought back to the ice, followed by the addition of a pre-warmed growth medium and outgrowth in a nonselective medium. This step allows the repairing of the bacterial membrane and permits the plasmid to grow and synthesize plasmid-encoded antibiotic resistance proteins. Finally, an aliquot of this transformation mixture is plated on selective plates to identify plasmid-bearing colonies [4, 5].

Materials

A single colony of *E. coli* cells.

5 mL LB liquid medium in a 25 mL culture tube for initial overnight growth of the *E. coli* cells.

30-50 mL LB liquid medium in 500 mL conical flask for growth of the *E. coli*

cells.

1 mL LB medium for outgrowth.

LB Solid Plate with ampicillin.

Ice-cold, sterile 100 mM $CaCl_2$.

Plasmid or Ligated DNA.

Equipment

Incubator Shaker, Beckman J-6M centrifuge (or equivalent), Centrifuge Rotors JS-5.2 rotor or equivalent, 42°C/37°C water baths.

Glassware/Plasticware

25 mL Culture Tubes, 250 mL conical flasks, Chilled 50-ml polypropylene tubes, Chilled Glass Pipettes, Chilled Microfuge Tubes, Chilled Microtips.

NOTE: All materials and reagents must be sterile.

Procedure

Preparation of competent cells

- Grow an overnight culture by inoculating 5 mL of LB medium with a single colony of freshly grown *E. coli*. Grow the cells at 37°C overnight with shaking.
- Inoculate 30 mL fresh LB medium in a 500 ml flask with an aliquot of 300 µl of the overnight culture. Grow the culture vigorously in an incubator shaker at 37°C, with shaking at a speed of 250 rpm until the OD_{600} reaches 0.4-0.5. Approximately 2-3 hours are required to attain this OD_{600}.

NOTE: In order for the cells to achieve an OD of 0.4-0.5, they need to be grown vigorously. Vigorous growth requires sufficient air or oxygen, and therefore, for a 30 ml culture, a 500 ml flask should be used. The growth stage that the cells have reached is critical. If the culture is overgrown (beyond OD_{600} of 0.4), the quality of competent cells is found to be poor, and the yield of transformation efficiency decreases. There will be insufficient cells for transformation at less than OD_{600}=0.4-0.5.

- Chill the entire flask and the culture in an ice for 10 minutes. Harvest the cells in a pre-cooled rotor by centrifuging the culture at 6000 X *g* for 10 min at 4°C.

NOTE: From this point onwards, the cells are not allowed to be warmed up and should be maintained in cold for all subsequent steps until heat shock. The microtips and microfuge tubes, glass pipettes, and centrifuge tubes that will be used to transfer and handle the culture and cell suspension should all be maintained at a chilled temperature.

- Take the pellet and discard the supernatant. Place the tube back on ice immediately. Add 15 mL ice-cold, sterile 100 mM $CaCl_2$ and resuspend the cell pellet by gentle tapping/flickering. Avoid vortexing. Incubate on ice for 15 min with occasional shaking.

NOTE: The cells should always be kept on ice and the contents should be very gently tapped for resuspension.

- Centrifuge the cells in a pre-cooled rotor at 6000 X *g* for 10 min at 4°C, as in step 3, and discard the supernatant consisting of $CaCl_2$.
- Save the pellet and add 3 ml ice-cold, sterile 100 mM $CaCl_2$ to it. Tap very gently, keeping the tube on ice. Be sure to resuspend all the cells in the pellet so that no cell clumps remain. The cells are now competent and are ready to transform.

NOTE I: The tube containing competent cells can be frozen immediately or can be left on ice for several days. It is important to add sterile 50% glycerol to the cells to a final concentration of 10% before freezing, mix well and dispense in 100-200 µl aliquot in pre-chilled microfuge tubes, snap freeze the tubes with liquid nitrogen and store at -80°C. *Competency, on the other hand, increases with increasing storage time on ice and reaches a maximum of 12 to 24 hr. for many strains (e.g.*, *DH1*).

NOTE II: It is important to note that whatever the volume of the starting culture, as a rule of thumb, half the volume of $CaCl_2$ (relative to the original culture volume) should be added initially to resuspend the pellet. The pellet should always be resuspended in one-tenth volume of $CaCl_2$. For example, if the starting cell volume is 1 liter, the first pellet will be dissolved in half the volume, that is, 500 ml of $CaCl_2$. The pellet should be resuspended in 0.1 volume of $CaCl_2$, i.e. 100 mL.

Assess the Competency of Cells

- Dispense an aliquot of 100 µl of competent cells in a pre-chilled microfuge tube using a pre-chilled microtip.
- Add 10 ng of freshly prepared and analyzed supercoiled pUC19 DNA and transform the DNA following the steps mentioned in Section C of the protocol.

- Spread 1, 10, and 25 µl aliquots of transformation mixture separately on three different LB/ampicillin plates. Incubate the plates upside down in a 37°C incubator overnight.
- In the following morning, count the number of colonies that appeared on each plate. Calculate the number of transformant colonies that appeared per aliquot volume (µl) × 10^5: this is equal to the number of transformants per microgram of DNA.

Transformation efficiencies of 10^7 to 10^8 and 10^6 to 10^7 are obtained for E. coli MC1061 and DH1, respectively. The competency of strains decreases very slowly over months of storage time.

Uptake of DNA by Competent Cells

- Use a freshly prepared aliquot or frozen competent cell stock. If using frozen competent cells, thaw an aliquot of 100 µl of competent cells on ice. Add DNA to be transformed (whole plasmid or ligation reactions) to the 100 µl thawed or freshly prepared competent cells as described in the following (Table **20**).

Table 20. Protocol for setting up a standard transformation reaction.

DNA	1-10 µl containing 1pg-100 ng of plasmid or ligated DNA
Competent *E.coli*	100 µl
Incubation	On ice for 30 minutes after the addition of DNA
Heat Shock	At 42°C for exactly 90 seconds
Post Heat Shock	On ice for 5 minutes
Incubation/Outgrowth	Add 950 µl pre-warmed LB medium Grow at 37°C for 60 minutes with shaking

NOTE: After thawing, immediately use the competent cells. If all the cells in the aliquot is not used up, do not refreeze but discard the remaining cells.

- Incubate the competent cells and DNA mixture on ice for 30 min.
- Heat shock the cells by promptly transferring the tube from ice to a 42°C water bath and by storing the tube for **exactly** 90 seconds (Table **20**). To achieve good results, swirl the tube in the water bath during the heat shock step.
- Place the tube back to ice promptly for 5 minutes.
- Add 1 ml of LB medium (pre-warmed to 37 °C) to each tube of heat-shocked cells.
- Place the tube in a 37 °C incubator shaker and incubate by shaking for 1 hour.

- Take different aliquots of 100 µl and 200 µl of the transformation mixture and spread them on LB/ampicillin plates or other appropriate antibiotic-containing plates.

NOTE: If working with ligation mixtures, the remainder of the transformation mixture may either be stored at 4°C for subsequent plating, or the cells can be concentrated in the following way. Centrifuge the suspension briefly, discard most of the supernatant, and leave a small amount to resuspend the cell pellet. These concentrated cells can be directly spread onto the LB/ampicillin plates.

- When plates are dry, incubate 12 to 16 hr at 37°C.

NOTE I: The amount of the DNA to be transformed should be decided by the transformation frequency of the vector. A high transformation frequency of the vector should yield many transformants per nanogram of DNA transformed, and relatively few transformants would be yielded for vectors with low transformation frequency.

NOTE II: The $CaCl_2$ concentration should be maintained at 100 µM or above, and therefore, the volume of the DNA to be added to the competent cells is critical. The ideal volume of the DNA to be transformed should be less than 5% of the volume of the competent cells (e.g., no more than 5 µl of DNA per 100 µl of competent cells is to be added).

NOTE III: DNA from ligation reactions can be used directly, but one should keep in mind that the components of the ligation reaction may interfere with DNA uptake. Thus, the addition of a larger volume of ligation mixture leads to the carryover of more ligation reaction components to the transformation mixture, which would affect the efficiency of transformation. Dilute the ligation mix if there is >1 µg of DNA in the ligation reaction or if the ligation reaction is from low gelling/melting temperature agarose.

NOTE IV: DNA amounts above 2 ng for the volumes of competent cells used do not yield more transformants. To obtain more of the transformants, especially for recombinant clones, parallel transformation reactions should be used instead of using a larger volume of DNA.

- In the following morning, count the number of transformed colonies to estimate the efficiency of transformation.

PROTOCOL 17: HIGH-EFFICIENCY TRANSFORMATION OF ELECTRO-COMPETENT *ESCHERICHIA COLI* BY ELECTRO-PORATION

Principle

Electroporation, or the use of a high electric field to introduce DNA, has become the most efficient method for transforming *E. coli* with plasmid DNA. Electroporation yields 100-1000 times higher competence than chemically competent cells [1, 4, 5]. A specially designed apparatus called electroporator equipped with accessories is widely used for this method, which applies an electric field of approximately 12.5 kV/cm^2 in 5-10 milli second pulses to the *E. coli* cell suspension [4, 5]. The electric pulses are thought to induce the transient formation of small holes in the bacterial cell membrane, through which the DNA enters promptly before the holes become repaired. This method is worth considering when transformants are required for high efficiencies. Transformation efficiency by this method depends on the purity of the plasmid DNA and on the nature of electro-competent cells. Very pure plasmid DNA increases the transformation efficiency up to $10^{10}/\mu g$, which is rarely available otherwise. Cells with even minute traces of growth medium and salts reduce the transformation efficiency drastically. Hence, the bacterial cells need to be washed thoroughly and repeatedly in water to become electro-competent prior to electroporation.

Materials

A single colony of the appropriate strain of *E. coli* cells

LB medium

Ice-cold, sterile, deionized H_2O

Ice cold 10% glycerol,

SOC medium (see ***RECIPE*** at the end of ***CHAPTER 6***)

LB plates containing antibiotics (at the end of the ***CHAPTER 7***)

Recovery medium.

Equipment

Electroporation apparatus with a pulse controller or 200- or 400-ohm resistor, Chilled electroporation cuvettes, 0.2-cm electrode gap; Beckman J-6M centrifuge (or equivalent).

Beckman JS-4.2 rotor (or equivalent) and chilled ice-cold adaptors for 50-ml narrow-bottom tubes.

Glassware/Plasticware

1-liter centrifuge bottle, 50-ml narrow-bottom polypropylene tube, and microcentrifuge tubes, additional reagents and equipment for growth of bacteria in liquid media (***UNIT 2.3.4.1***).

NOTE: All materials and reagents must be sterile.

Procedure

Preparation of Competent Cells

- Grow an overnight culture by inoculating 5 mL of LB medium with a single colony of freshly grown *E. coli*. Grow the cells at 37°C overnight with shaking.
- Inoculate a fresh 500 ml LB medium in a sterile 2-liter flask with 2.5 ml overnight culture. Grow the cells by incubating at 37°C with vigorous shaking at 300 rpm until the OD_{600} reaches 0.5-0.7. Typically, it takes about 2.5 h.

NOTE: Harvesting cells at a*n* OD_{600} *of ~0.5 to 0.6. gives the best results.*

- Place the culture flask in an ice-water bath for 10 to 15 minutes and chill the cells. Transfer the chilled culture to a pre-chilled 1-liter centrifuge bottle.

NOTE: From this point onwards, the cells are not allowed to warm up and should be kept cold for all subsequent steps. The glass pipettes, microtips, microfuge tubes, and centrifuge tubes should all be at a chilled temperature.

- Harvest the cells by centrifugation at 6000Xg for 10 minutes at 4°C in a pre-cooled rotor or 20 min at 4200 rpm in a Beckman J-6M rotor at 2°C.
- Save the pellet and discard the supernatant. Resuspend the cell pellet in 500 ml ice-cold, sterile water.
- Harvest the cells again by centrifuging the cells, as in step 4. Save the pellet and discard the supernatant (water).
- Add 5 ml ice-cold water to resuspend the cell pellet well, leaving no cell clumps. To this dispersed suspension, add 500 ml of ice-cold water and mix well. Centrifuge cells as in step 4.
- Decant the supernatant immediately and redissolve the pellet by swirling in the remaining liquid.

NOTE: At this stage, the pellet is very loose, so the supernatant should be discarded immediately and cautiously. Substituting ice-cold sterile HEPES (1 mM, pH 7.0) for the ice-cold water in step 5 can make the pellet tighter.

Prepare Either Fresh or Frozen Cells for Transforming

- To use the freshly prepared electro-competent cells for transformation, place the cell suspension in a pre-chilled, narrow-bottom, 50-ml polypropylene tube and harvest by centrifugation for 10 min at 4200 rpm in a Beckman J-6M centrifuge with JS-4.2 rotor and adaptors at 2°C.

NOTE: Better results are obtained from fresh cells than frozen cells. The ideal cell density should be ~2×10^{11}/ml. This is accomplished by resuspending the cell pellet in a small volume of water. For example, from a 500-ml culture, the volume of the water to be used for resuspension should be 500 µl. So, the cell concentration would be very high when the pellet is resuspended in such a small volume of ice-cold water (on ice). Then, the cell suspension is dispensed into pre-chilled microcentrifuge tubes in aliquots of 50 µl to 300 µl cells. The cells should be used immediately.

- In order to freeze the cells for later use, add 40 ml ice-cold 10% glycerol to the cells and mix well. Centrifuge cells as described in step 9a.

NOTE: Dissolve the pellet in ice-cold 10% glycerol. The amount of glycerol to be added will depend on the size of the pellet/cells (on ice). Aliquot 50µl to 300µl aliquots of cells into pre-chilled microcentrifuge tubes and freeze on dry ice (not in liquid nitrogen). Store at −80°C. For some strains, such as BW313/P3 and MC1061/P3, prolonged incubation of cells in ice water at all stages can increase transformation by >3-fold.

Uptake of DNA by Electroporation

- Set the electroporation apparatus to 2.5 kV, 25 µF, and the pulse controller to 200 or 400 ohms.

NOTE: The pulse controller is necessary when high-voltage pulses are applied over short gaps in high-resistance samples.

- Add 5 pg to 0.5 µg plasmid DNA (in 1 µl) to an aliquot of 100 µl of electro-competent cells (either fresh or frozen) with the tube kept on ice. Mix the contents gently by tapping or swirling the cells with the pipette.

NOTE: For all practical purposes, the amount of DNA used in electroporation ranges from a minimum of 5 pg to 200 ng DNA, along with 100 µl cells at a

density of 10^{11} cells/ml. To avoid reducing the cell density, DNA (be it 5pg or 200 ng) must be in a small volume of 1µl.

- Take an electroporation cuvette that has been chilled for 5 min on ice. Transfer the contents of the tube (DNA in electro-competent cells) into it. To settle the cells to the bottom of the cuvette, the cuvette is slightly shaken. Wipe the ice and water from the outer surface of the cuvette with a Kim wipe.

NOTE: The volume of DNA added plays a key role in transformation efficiency. The transformation efficiency would decrease 2- to 3-fold when DNA up to one-tenth of the cell volume is added to the cells. Hence, the volume of the DNA added needs to be small. Make sure that the addition of the DNA to the cells does not increase the total salt concentration in the cuvette by >1mM since the resistance of the sample then increases.

- Place the cuvette into the sample chamber of the electroporator apparatus.
- Follow the operating instructions of the equipment and apply the pulse by pushing the button or flipping the switch.
- Immediately after electroporation (after the switch is released), take out the cuvette from the apparatus and transfer the cells with a Pasteur pipet into a sterile culture tube containing 1 ml of recovery medium or 1 ml SOC medium. Outgrow the cells in a 37°C incubator shaker with moderate shaking for 1 hour.

NOTE: If the actual voltage and time constant of the pulse are displayed on the electroporation apparatus, note this information. Verify that the set voltage was actually delivered, and record the time constant of the pulse so that you may vary it later if necessary.

- Take out different aliquots of the transformation culture and plate on LB plates containing antibiotics.

SPECIAL INFORMATION PANEL: ELECTROPORATION

• This method uses a high electric field to introduce DNA into *E.coli* cells. It is thought that transient hydrophobic pores of diameter 2nm to a maximum of several nm are formed as a result of the dimpling of the membrane caused by a sharp pulse of electricity [4, 5].
• Through the open pores, the foreign DNA enters from the medium into the cytoplasm.
• Closure of the pores can be delayed by keeping the cells at low temperatures.
• As the transmembrane voltage is increased, some of the larger hydrophobic pores are converted to hydrophilic pores. The energy needed to create and maintain these hydrophilic pores requires less energy.
• The transmembrane voltage required for the formation of large hydrophobic pores varies in direct proportion to the diameter of the target cell.
• Three important parameters of the pulse affect the efficiency of electroporation:
• The length of the pulse is determined mainly by the value of the capacitor and the conductivity of the medium.
• Field strength is directly proportional to the applied voltage and is inversely proportional to the distance between the electrodes.
• Shape is determined by the design of the electroporation device. The waveform produced by most commercial machines is simply the exponential decay pattern of a discharging capacitor.

6.8. SCREENING OF THE APPROPRIATE DESIRED RECOMBINANT DNA

The next step in the cloning procedure involves screening the desired recombinant clone once the ligated DNA is introduced into the appropriate host bacterial cells. Note that the ligation mixture contains a re-ligated vector (if possible and this is an undesired product), ligated vector and insert DNA (desired product), and ligated insert (if generated and this product is unable to replicate inside the host bacterium). Therefore, when the entire ligation mixture is introduced into the host cells, the former two products of ligation (re-circularized vector and recombinant DNA) propagate within the host using its machinery. The self-circularized vector, therefore, contributes to the background, and typically, the recombinant DNA needs to be screened out from the vector DNA. This last step is called screening. Various strategies exist for the successful and efficient screening of recombinant DNA. These strategies and the detailed methods involved in these screening processes are presented in the next chapter (Chapter 7). The rest of the current

chapter discusses three complete protocols to subclone a piece of DNA using different strategies.

6.9. SUB-CLONING DNA FRAGMENTS

PROTOCOL 18: PROCEDURE FOR CLONING OF DNA FRAGMENTS WITH COHESIVE TERMINI

Materials

Vector (plasmid) DNA

Insert (source) DNA (plasmid or PCR product)

Suitable Restriction Enzyme(s)

10X Restriction Enzyme Reaction Buffer(s)

Shrimp alkaline phosphatase (SAP) or Calf Intestinal Phosphatase (CIP)

10X Phosphatase Buffer

dNTP mix (1 mM stock)

Klenow fragment of *E. coli* DNA polymerase I *or* T4 DNA polymerase (***UNIT 6.5.1.3***)

T4 DNA ligase (measured in cohesive-end units) (***UNIT 6.6***)

5X T4 DNA ligase buffer

Additional reagents and equipment are required for restriction endonuclease digestions (***UNIT 6.4.1***), transformation of *E. coli* cells (***UNIT 6.7***), plasmid DNA preparations in mini scale (***UNIT 3.2.1***), and agarose or polyacrylamide gel electrophoresis (***UNIT 5.2*** or ***5.7***).

Procedure

Preparation of Vector

- Isolate the appropriate plasmid to be used as the vector by standard plasmid isolation procedure by alkaline lysis (***UNIT 3.2.1***). Analyze the DNA by spectrophotometry (for quantification) (***UNIT 5.8***) and agarose gel electrophoresis (***UNIT 5.2***).
- Set up a pilot digestion reaction in 20 µl with the appropriate restriction enzyme(s) (Table **1**).

- Check the digestion in the agarose gel. Stop the reaction by inactivating the enzymes by heating them at the specified temperature for the required time if the digestion is complete.
- Set up large-scale digestion of 50-100 µl with more DNA (µg), reagents, and the appropriate amount of restriction enzyme (Table **2**).

NOTE 1: If the vector needs to be digested with two different enzymes (done either sequentially or together, depending on the buffer compatibility), check the completion of the first digestion on the gel. However, completion of the second digestion should be checked by ligating and transforming the double-digested DNA into an appropriate host. The expectation is that either no or a few colonies will be observed as the two ends of the vector would not be compatible for ligation if the second digestion is complete.

- After the digestion is complete (by checking in the agarose gel), dephosphorylate the 5′-end of the vector using either shrimp alkaline phosphatase or calf intestinal phosphatase. This enzymatic manipulation can be carried out sequentially without further buffer changes (Tables **10** and **11**).

NOTE 2: Check dephosphorylation by ligation and transformation. The expectation is that a dephosphorylated vector will not be ligated due to the absence of 5′-PO_4^- and hence, no phosphodiester bond will be formed. Consequently, the self-ligation of this dephosphorylated vector will not yield any colony after transformation into the appropriate host. If the reaction is incomplete, add more units of the CIP/SAP and again check for completion.

- After the reaction is complete, inactivate the dephosphorylating enzyme.
- Once confirmed, purify the vector band by eluting it from agarose gel.
- Estimate the eluted band in the gel by comparing it with a standard ladder.

Preparation of Insert (Using Restriction Enzyme Digestion)

- Isolate the insert-bearing plasmid by alkaline lysis (***UNIT 3.2.1***). Estimate the DNA concentration by spectrophotometry (***UNIT 5.8***) and assess the quality by agarose gel analysis (***UNIT 5.2***).
- Set up a pilot digestion of the plasmid 20 µl reaction volume with the required enzyme(s) (as mentioned in the ***PROTOCOL 6.1*** and ***6.2***) (Table **1**).
- Check the digested sample in an agarose gel to confirm the band pattern using the standard DNA ladder as a reference.
- If everything looks fine, set up a large-scale digest of the same in a total of 50-100 µl reaction volume using the same enzyme(s) (Table **2**).
- If two different enzymes are used, then check for complete digestion (as mentioned in NOTE 1 above).

- Elute the required band from the agarose gel using a standard procedure (***UNIT 5.6***).
- Estimate the band by comparing it with a standard ladder in agarose gel.

Preparation of Insert (Using Polymerase Chain Reaction)

- The insert can also be generated by a standard PCR reaction using a template DNA (***UNIT 6.4.2***) (Tables **4**, **5**, **6** and **7**).
- Check the product in the gel. If the PCR product contains only the desired band and does not contain any spurious undesired band, then purify the PCR product using a PCR purification kit. Alternatively, if the PCR product contains additional non-specific and undesired bands, then the desired band needs to be purified from the agarose gel by excision, followed by purification of the band from the agarose matrix (***UNIT 5.6***).

NOTE: If the primers used in the PCR contain restriction sites, then the product needs to be purified, followed by digestion. And then again purified.

- Finally, estimate the band of the purified insert by comparing it with a standard ladder in agarose gel.

Setting up Ligation Reactions

- As mentioned in ***UNIT 6.6*** (***PROTOCOL 6.14***), set up a ligation reaction, as shown below. (Tables **17** and **18**)

9 µl component (Vector and Insert) DNAs (0.1 to 5 µg)

4 µl 5X ligase buffer

1 µl 10 mM ATP

20 to 500 U (cohesive end) T4 DNA ligase

Add appropriate dH2O to 20 µl

- Incubate 1 to 24 hr at 15°C.

NOTE: Two fragments having 4-bp 3′ or 5′ overhanging ends require much less ligase than blunt-end ligations. The amount of ligase needed will depend on the quality of the DNA; hence, both the vector and the insert need good purification. Be sure to set up a control reaction, as discussed in ***UNIT 6.6.2***.

Transformation of Ligation Reactions

- Transform 1 to 10 µl of the ligated products into competent *E. coli* cells and check for transformants on appropriate antibiotic plates that are present on the vector (***UNIT 6.7***) (Table **20**).
- From individual *E. coli* transformants, purify plasmid DNAs by mini-scale preparation procedures and screen for recombinants.

PROTOCOL 19: PROCEDURE FOR CLONING OF DNA FRAGMENTS WITH BLUNT ENDS

Materials

Vector (plasmid) DNA

Insert (source) DNA (plasmid or PCR product)

Restriction Enzyme(s) and appropriate enzyme

Shrimp alkaline phosphatase (SAP) or Calf Intestinal Phosphatase (CIP) and buffer

dNTP mix (0.5 mM each; *UNIT 3.4*)

Klenow fragment of *E. coli* DNA polymerase I *or* T4 DNA polymerase (***UNIT 6.5.1.3***)

Oligonucleotide linkers (optional)

0.2 mM dithiothreitol (DTT)

T4 DNA ligase (measured in cohesive-end units; (***UNIT 6.6***)

2X T4 DNA ligase buffer

Additional reagents and equipment are required for restriction endonuclease digestions (***UNIT 6.4.1***), transformation of *E. coli* cells (***UNIT 6.7***), Plasmid DNA Preparations in mini scale (***UNIT 3.2.1.***), and agarose or polyacrylamide gel electrophoresis (***UNIT 5.2*** **or** ***5.7***).

Procedure

Preparation of Vector

- Isolate the plasmid to be used as the vector by standard plasmid isolation

procedure by alkaline lysis (***UNIT 3.2.1***). Analyze the DNA by spectrophotometry (***UNIT 5.8***) and agarose gel electrophoresis (***UNIT 5.2***).

- Set up a pilot digestion reaction in 20 µl with the appropriate restriction enzyme(s) (Table **1**).
- Check the digestion in the agarose gel. Stop the reaction by inactivating the enzymes by heating them at the specified temperature for the required time if the digestion is complete.
- Set up large-scale digestion of 50-100 µl with more DNA (µg), reagents, and the appropriate amount of restriction enzyme (Table **2**).
- To generate blunt ends of the vector, an appropriate amount of the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase is added to carry out the filling-in or trimming reaction, respectively. Along with the enzyme, add 1 µl of a solution containing all 4 dNTPs (1 mM each) and incubate at the required temperature (***UNIT 6.5.1.3***). Inactivate the enzyme by heating 15 min to 75°C (Tables **13** and **14**).

NOTE: The Klenow fragment works in nearly all restriction buffers and, hence, is added to the reaction directly. Therefore, it is unnecessary to inactivate the restriction endonuclease, change buffers, or purify the DNA.

- If oligonucleotide linkers are to be added, then add the following to the reaction containing blunt-ended DNA (***PROTOCOL 6.15***) (Table **19**).

0.1 to 1.0 µg of an appropriate oligonucleotide linker

1 µl of 10 mM ATP

1 µl of 0.2 M DTT

20 to 100 cohesive-end units of T4 DNA ligase

- Incubate overnight at 15°C.
- Inactivate the ligase by heating for 15 min at 75°C.

*NOTE: Prior to ligation, the oligonucleotide linkers can be phosphorylated but not essentially (Table **8**). For ease of ligation, the linkers harbor restriction recognition sites, which, when digested, will yield cohesive ends, following which ligation becomes much simpler.*

- Set up a digest with the restriction endonuclease recognizing the oligonucleotide linker (***PROTOCOL 6.15***). Check the buffer conditions and accordingly adjust the concentration of the salts, *etc.*, if necessary (Table **1**).
- Electrophorese the DNA sample and isolate the required band segments by gel electrophoresis (***UNIT 5.6***). Other methods of purification can also be used.

NOTE: For many cloning experiments, purifying the DNA may not be required; however, a clean DNA preparation is always desired.

- Cut out the desired band(s) under longwave UV light and purify the DNA from the gel using procedures as described in ***UNIT 5.6***).

NOTE: The DNA band isolated from the gel should be prevented from getting damaged. Therefore, the use of longwave UV light should be done for slicing out the band. For isolation of the band, low gelling/melting temperature agarose can be used, and ligation reactions can be set up directly in the gel slice.

- Set up the following ligation reaction (Tables **17** and **18**):

9 μl component DNAs (0.1 to 5 μg)

10 μl 2× ligase buffer

1 μl 10 mM ATP

20 to 500 U (cohesive end) T4 DNA ligase

- Incubate for 1 to 24 hr at 15°C.

NOTE: Blunt-end ligations are difficult, and hence, more ligase enzymes are required for the reaction. The amount of ligase needed will depend on the quality of the DNA. Also, control reactions are set up as mentioned in ***UNIT 6.6.2***. (Table **17**).

- Transform 1 to 10 μl of the ligated products into competent *E. coli* cells and select transformants on proper antibiotic plates present on the vector (***UNIT 6.7***) (Table **20**).
- From individual *E. coli* transformants, screen by preparing plasmid DNAs in a mini-scale for correct recombinant clones.

PROTOCOL 20. PROCEDURE FOR CLONING OF PCR AMPLIFIED FRAGMENTS

Principle

This protocol calls for amplifying a specific DNA fragment harboring a gene of interest from a template genomic DNA using specific sets of primers containing new unique restriction sites followed by enzymatic manipulations and finally sub-cloning into a vector containing compatible restriction sites. Following the transformation of the ligated product, the candidate sub-clones are further analyzed for correct recombinant clones.

Materials

Vector (Plasmid) DNA

Template DNA (1 to 10 ng of plasmid DNA; 20 to 300 ng of genomic or cDNA)

Oligonucleotide primers (0.6 to 1.0 mM)

TE-buffered phenol and chloroform

100% ethanol

TE buffer, pH 8.0

T4 DNA Polymerase

Vector DNA

Calf intestinal phosphatase (CIP) or Shrimp Alkaline Phosphatase (SAP)

Additional reagents and equipment are required for phosphorylating of the PCR products (***UNIT 6.5.1.1.***), agarose and polyacrylamide gel electrophoresis (***UNIT 5.2*** and ***UNIT 5.7***), purification of DNA by electroelution from agarose gels (***UNIT 5.6***), ligation of DNA fragments and transformation of *E. coli* (***UNIT 6.6*** and ***6.7***).

Preparation of the Insert DNA: Amplification of the target DNA

- Either the plasmid or genomic DNA sample may serve as the template DNA. Use the necessary protocol to prepare the appropriate template DNA as described in ***CHAPTERS 3 AND 4***.

NOTE: Either rapid or purified by CsCl equilibrium sedimentation gradient, preparations of plasmid and genomic DNA can be used as the source of target DNA. However, if the preparation contains nucleases, first they should be removed by heating the sample at 100°C for 10 minutes, followed by slow cooling of the DNA sample at room temperature.

- Depending on the type of ligation, the oligonucleotide primers are modified accordingly. For example, a blunt end ligation would need phosphorylation of the 5′ hydroxyl end of the oligonucleotide primers (Table **8**).

NOTE I: A 5′ phosphate and a 3′-OH are needed to form a phosphodiester bond. If the vector ends are modified by the treatment with phosphatases, then the ends of the PCR products, which serve as the insert, certainly need to be

*phosphorylated for the linkage to be formed with the 3'-OH of the vector (**UNIT 6.5.1.1**).*

NOTE II: Because the purity of the oligonucleotides does not seem to affect the PCR reaction, primer purification (as detailed in UNIT 8.5) may not be necessary.

- With the template DNA, set up a standard amplification reaction (***UNIT 6.4.2***). Set up a program in the automated thermal cycler for 25-30 cycles. Calculate the melting temperature of the primers and then decide on the annealing temperature. An additional extension of 10 min at 72°C in the last cycle makes the products as complete as possible (Tables **6** and **7**).

*NOTE: Set up the amplification reactions following **UNIT 6.4.2**. Use a high fidelity thermostable DNA polymerase with 3′→5′ exonuclease to prevent mis-incorporation of nucleotides. Q5®DNA polymerase (New England Biolabs) has this activity (follow manufacturers' instructions). Also, set up appropriate negative control reactions of no template DNA, no polymerase, and each oligonucleotide alone, as well as several different oligonucleotide:template ratios.*

- Check the success of the amplification reaction in agarose or polyacrylamide gel using about an aliquot of 4 to 8 µl of each reaction mix and verify that the amplification has yielded the expected product and did not yield any non-specific spurious product(s).
- Purify PCR products from unincorporated nucleotides, oligonucleotide primers, unwanted PCR products, and template DNA either using electroelution or phenol freeze method (***UNIT 5.6***). Nowadays, PCR purification kits are available and used widely in laboratories.

NOTE: It is wise to clean up the PCR product from unused oligonucleotide primers, which can inhibit downstream reactions of the ability of the restriction enzymes to digest the amplified PCR product.

Preparation of the Amplified Fragment for Ligation

- For a blunt end ligation, repair 3′ ends of the PCR fragment with T4 DNA polymerase (Table **13**).

NOTE: Taq DNA polymerase adds a non-templated nucleotide (usually dA) to the 3′ ends of PCR fragments hence the 3′ needs to be repaired.

- Set up the digestion reaction of the PCR products harboring flanking restriction sites (originally present in the primers). It is wise to use half of the amplified DNA for digestion. With the appropriate restriction enzyme(s), set up the

reaction in a reaction volume of 20 µl. Use an excess of enzymes and digest them for several hours (Table **1**).

NOTE: The other undigested half of the amplified DNA is kept in reserve for future use, if necessary.

Preparation of the Vector

- Prepare the recipient vector for cloning using the alkaline lysis method (***UNIT 3.2.1***). Digest 0.2 to 2 µg or more DNA in 20 µl with compatible restriction enzymes (Table **1**). To prevent unproductive vector end ligation, dephosphorylate the vector DNA with CIP or SAP (***UNIT 6.5.1.2***) (Tables **10** and **11**).
- Purify the linearized vector by separating it from the uncut vector in agarose or low-gelling/melting temperature gel electrophoresis followed by electroelution or phenol freeze method (***UNIT 5.6***).

Ligation of Amplified Fragment and Vector

- Set up a ligation reaction with the prepared PCR fragment and the digested vector following the procedure outlined in ***UNIT 6.6*** (***PROTOCOL 6.14***) (Table **18**).

NOTE: Set up all the control reactions with/without insert, ligase, vector, etc. This will enable us to troubleshoot any problems if necessary (Table **17**).

- Transform an aliquot of each ligation into *E. coli* by following ***UNIT 6.7*** (Table **20**). Prepare plasmid DNAs by mini-scale preparation procedures and screen for recombinants (***UNIT 3.2.1***).

6.10. PROCEDURE FOR GATEWAY CLONING

Introduction

An improved cloning technology dubbed “Gateway Cloning” surfaced in recent years, which has been widely adopted in the research community, promising a much faster method to clone genes [5]. This high throughput technology circumvents the problems faced during traditional cloning, reduces the total time for obtaining a clone, and supports the cloning of multiple DNA fragments in parallel in a highly efficient manner into multiple vector systems for functional analysis and protein expression. For multidisciplinary scientific studies, this cloning technology has become very useful.

The Rationale of Gateway Cloning

The Gateway cloning technology takes advantage of the site-specific recombination process and the switch between the lytic and lysogenic pathways of bacteriophage λ (Fig. **8**). It relies on the highly specific integration and excision of genes into and out of the *Escherichia coli* genome. When bacteriophage λ integrates into the *E. coli* genome, it employs a site-specific recombination process catalyzed by the IHF (encoded by *E. coli*) and integrase (dubbed Int, encoded by bacteriophage λ) (Fig. **8**). In contrast, the reverse reaction, dubbed excision is carried out by IHF, integrase, and excisionase (dubbed Xis, encoded by bacteriophage λ) (Fig. **8**) during the induction of the phage from the bacterial genome during UV treatment or any other stress. The integration into the bacterial genome relies on highly conserved sites called *attP* and *attB* present in the phage and bacterial genomes, respectively (Fig. **8**). Successful recombination of the phage genome into the bacterial genome results in the formation of *attL* and *attR* sites on the left and right termini of the sites of integration, respectively (Fig. **8**).

Gateway technology employs the basic biological principle involved in the site-specific recombination reaction *in vitro* to create a recombinant clone. Gateway vectors harbor modified versions of the *att* sites that allow incorporation of the insert fragment carrying *attB*-like sequences into the vector molecule that harbors *attP*-like sequences. Typically, the *att* sites vary from 25–242 bp long, which are much longer than most of the widely used *cis*-acting elements used in traditional molecular cloning procedures. Consequently, they are less likely to be present even by chance in arbitrarily chosen DNA fragments (inserts) in contrast to traditional *cis*-element such as restriction sites, which is a mark of their site specificity. Thus, this unique feature allows repeated uses of identical sets of recombinases (Int, Xis, IHF) enzymes that catalyze the site-specific recombination reaction to robustly clone multiple fragments of variable size in parallel reactions.

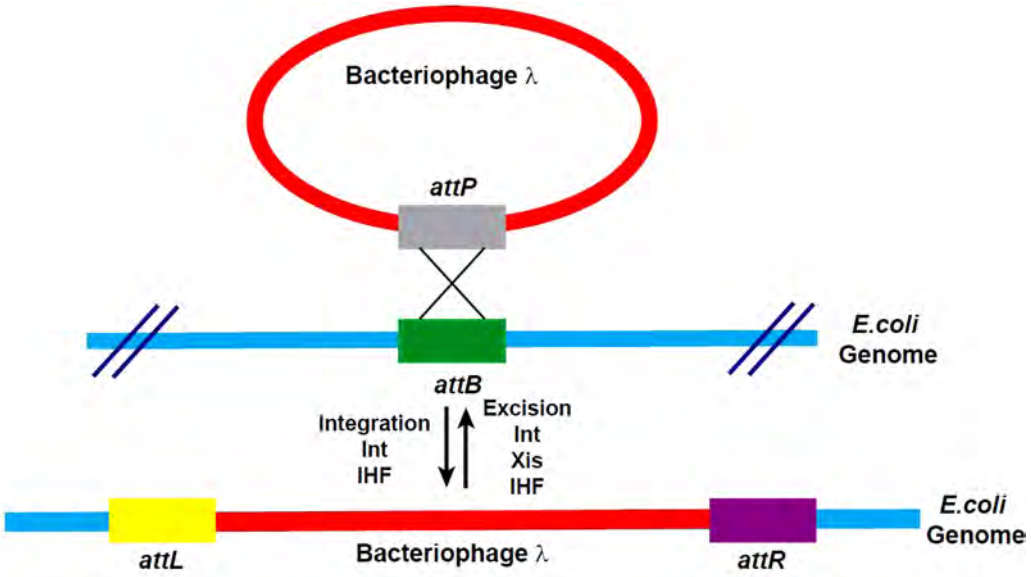

Fig. (8). Schematic diagram showing the integration and excision reactions between bacteriophage λ and E. coli genome. Site-specific recombination between attP and attB sites generates attL and attR sites.

Steps Involved in Gateway Cloning

In principle, there are two major steps in Gateway Technology: the **BP Reaction** and the **LR reaction**. The **BP Reaction** is the first step that occurs between the *attB* (called *attB1* and *attB2*) sites that flank the insert molecule and the *attP* (called *attP1* and *attP2*) sites flanking the *ccdB* gene, which is present in the donor vector. This first reaction is catalyzed by the BP Clonase enzyme mix, which creates the entry clone containing the gene of interest (GOI) flanked by *attL1* and *attL2* sites and the *ccdB* gene (essentially a by-product) that is generated by excision from the donor vector (Fig. **9A**).

In the next step, the **LR Reaction**, catalyzed by the LR Clonase enzyme mix, is carried out between the *attL1* and *attL2* sites of the entry clone generated in the previous BP reaction and the *attR1* and *attR2* sites of the destination vector. This reaction thus creates an expression/screening clone (see below) with the gene of interest (GOI) flanked by *attB1* and *attB2* sites generated in the LR reaction. As usual, a DNA fragment containing the *ccdB* gene is excised from the destination vector and is subsequently incorporated in another circular DNA molecule, which is a toxic by-product (Fig. **9B**).

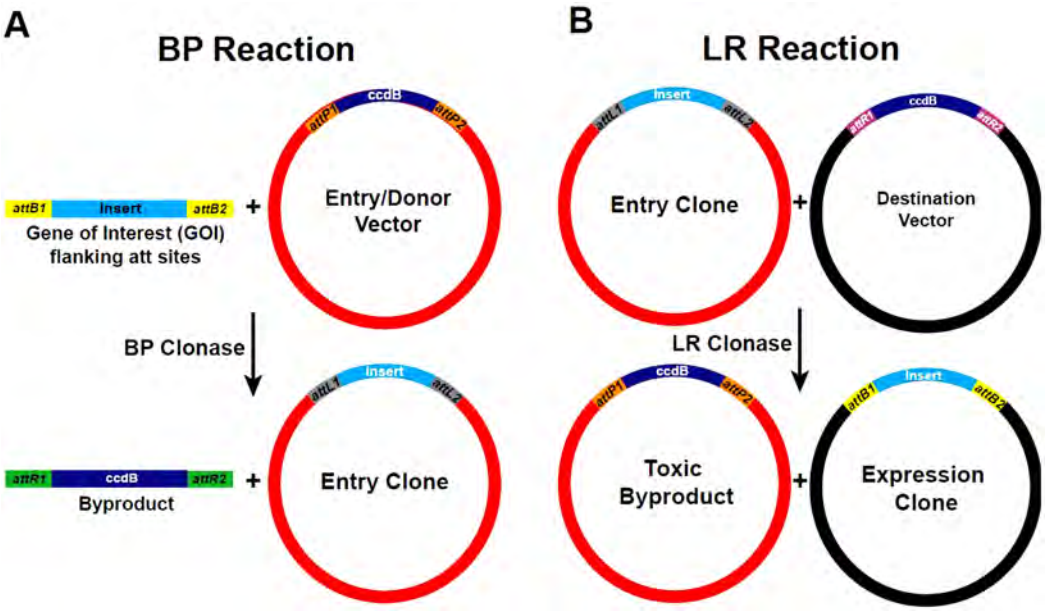

Fig. (9). Schematic diagram showing the thematic views of the BP (**A**) and LR (**B**) Reactions. Note that the BP Reaction produces an entry clone with the insert flanked at the attL site, whereas the LR reaction generates the expression/screening clone suitable for expression in diverse systems and other applications.

Following the BP and LR reactions, the products are finally transformed into competent *E. coli* cells, and the selection of the correct clones is made. Since, typically, the entry and destination vectors carry different antibiotic resistance markers, it allows the user to conveniently select the destination clone. The vector containing the *ccdB* gene does not grow under the selection condition since the *ccdB* gene kills the host strain.

Methodologies Involved in Gateway Cloning Technology

Step 1: Creation of Entry Clones and Vectors

In the first step of the Gateway cloning procedure, an "Entry clone" is first generated by a BP Gateway cloning reaction in which a DNA fragment of interest (*e.g.*, promoter, ORF, 3′-UTR) is incorporated into the "donor vector" (Fig. **9A**). Typically, the DNA fragment of interest (GOI) containing either the ORF or regulatory sequence may be produced on a large scale in any of the following three different methods.

Method 1: PCR Amplification: In this method, *attB1/B2* sites are incorporated at either end of the GOI using long (~50 bp) tailed overhanging primer sets. Note that the 3′-part of the primers is specific for the DNA of interest (indicated in blue

in Fig. **10**), and the 5′-tail of the primers carries the sequence corresponding to the appropriate *att*B sites (indicated in yellow color in Fig. **10**). The product of this PCR reaction is then transferred into an *attP*-donor vector (such as pDONR221, see below) with compatible *att*P (*e.g.*, *attP1* and *attP2*) sites (Fig. **10**).

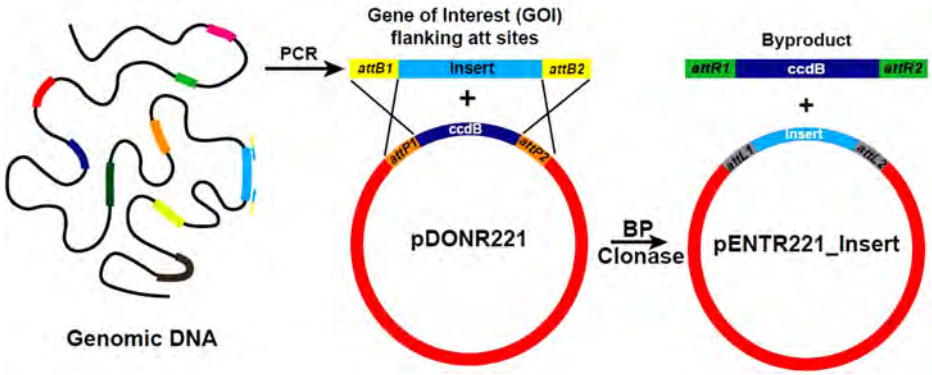

Fig. (10). Schematic diagram showing method 1 to generate entry clone using PCR amplification using overhanging primer sets.

Method 2: Topo-Cloning: In this method, the desired gene of interest (GOI) is introduced into an *attL* containing entry vector with short extensions of GTGG (such as pENTR/D-TOPO, see below) at one end (Fig. **11**). An extension sequence of CACC, which is complementary to GTGG, is added during the PCR amplification of the desired GOI from the genomic DNA using a specially designed forward primer. The addition of a complementary extension of CACC facilitates the cloning of the PCR product in pENTR using the Topo-cloning method (Fig. **11**).

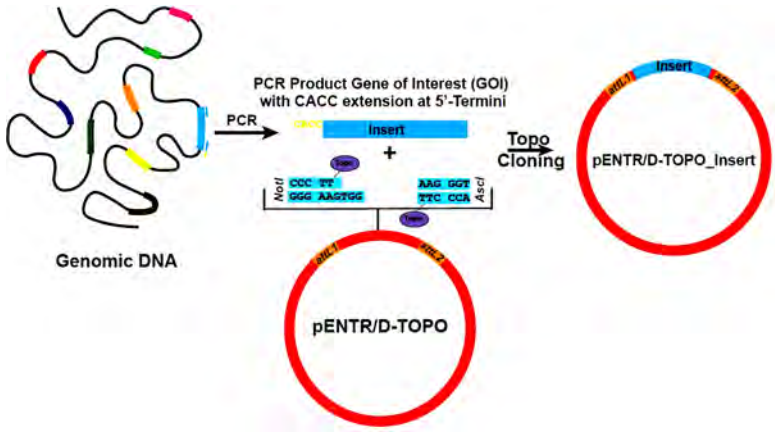

Fig. (11). Schematic diagram showing the Topo cloning method to generate entry clone using PCR amplification followed by Topo cloning of the PCR Product.

Method 3: Restriction-Cloning: This method involves the production of the insert DNA containing the gene of interest by direct restriction digestion with appropriate enzymes (if the restriction sites are naturally present in the GOI). Alternatively, the desired fragment, along with the appropriate restriction sites, can also be generated by PCR amplification in which the appropriate restriction sites are incorporated, followed by the digestion of these enzymes (if the desired restriction sites are absent in the GOI). Finally, the resultant fragment is inserted into the MCS of a suitable *attL* containing an entry vector (such as pENTR11) (Fig. **12**).

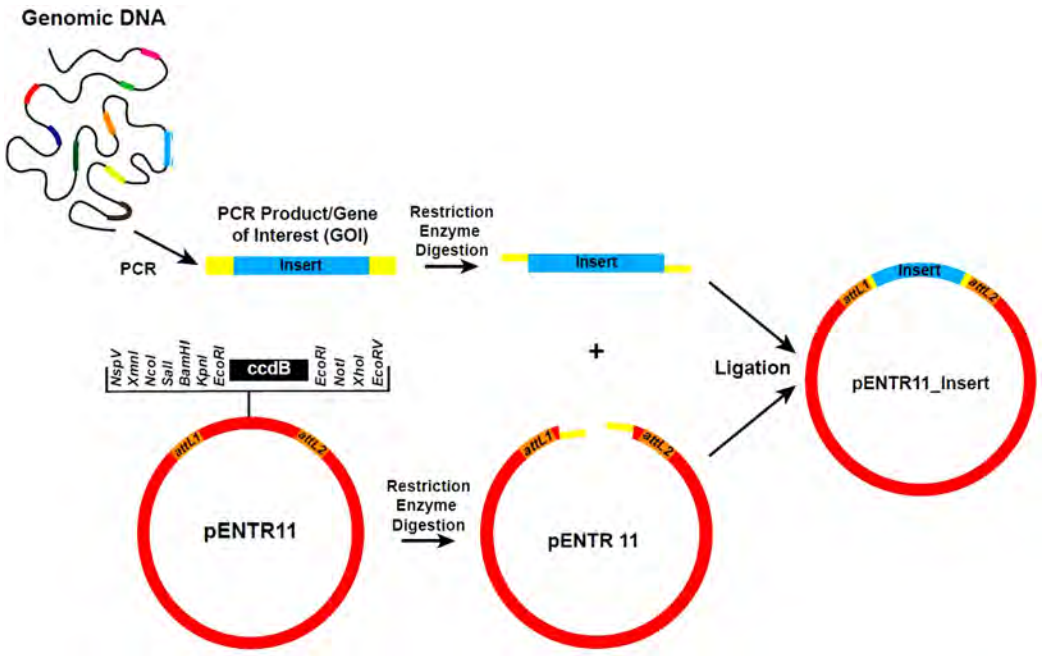

Fig. (12). Schematic diagram showing the restriction digestion method to generate entry clone followed by ligation of the restriction fragment.

Step 2: Creation of Expression/Screening Clones by Transferring the Insert Containing Entry Clone into the Destination Vectors

In the next step, the insert containing GOI is transferred from the entry clone to the destination vector to generate the desired expression/screening clone by LR reaction. Since, a wide range of destination vectors are available, it is vital to select the appropriate destination vector that fits best for a particular experiment/application. This choice relies on a number of parameters, such as the organism of interest, desired expression level, whether genetic screening (such as two-hybrid screening) is the final goal of the experiment, *etc.* (Fig. **13**).

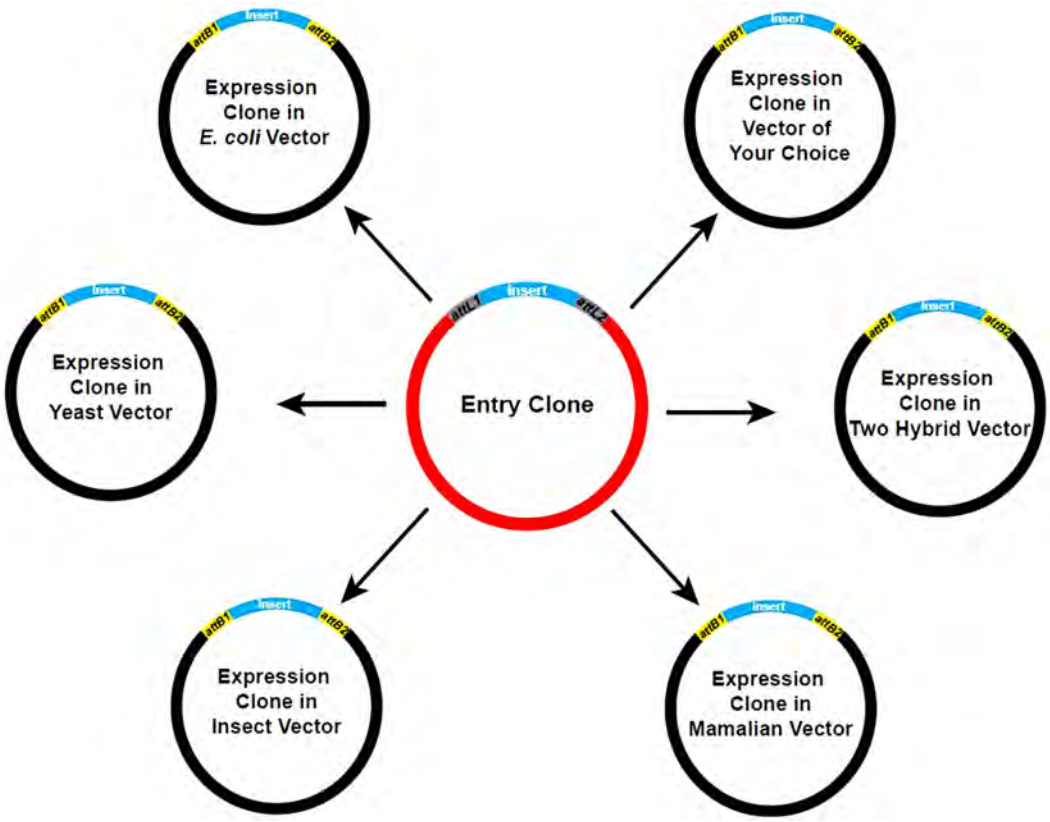

Fig. (13). Gateway technology provides possibilities of cloning genes into and back out of multiple vectors *via* site-specific recombination.

For example, for mammalian lentiviral expression, one may select pLenti CMV Puro DEST (w118-1) or the doxycycline-inducible pLIX_403. The selected *attR* destination vector will recombine with the *attL*-entry clone to create the final expression/screening clone (Fig. **14**).

More advanced "multisite" Gateway LR reactions are now available to incorporate the DNA fragments from more than one entry clone (*e.g.*, promoter and ORF) into a single destination construct (Fig. **15**). This approach is extremely useful for generating complex constructs in which a promoter and ORF are fused together into a GFP-encoding ORF. The strategy involves a combination of two entry clones with a single destination vector, and, just as in the LR reaction, the LR clonase enzymes recombine the matching subtypes of compatible *attL* and *attR* sites, as shown in Fig. (**15**).

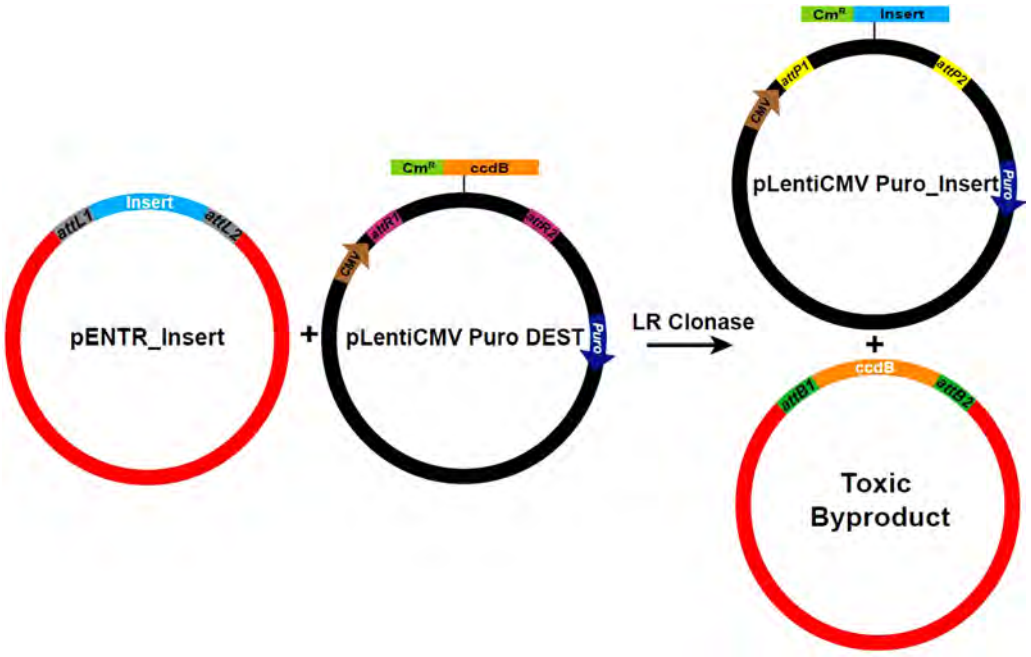

Fig. (14). Schematic diagram showing the strategy of generation of expression clone in the mammalian expression vector pLenti CMV Puro DEST.

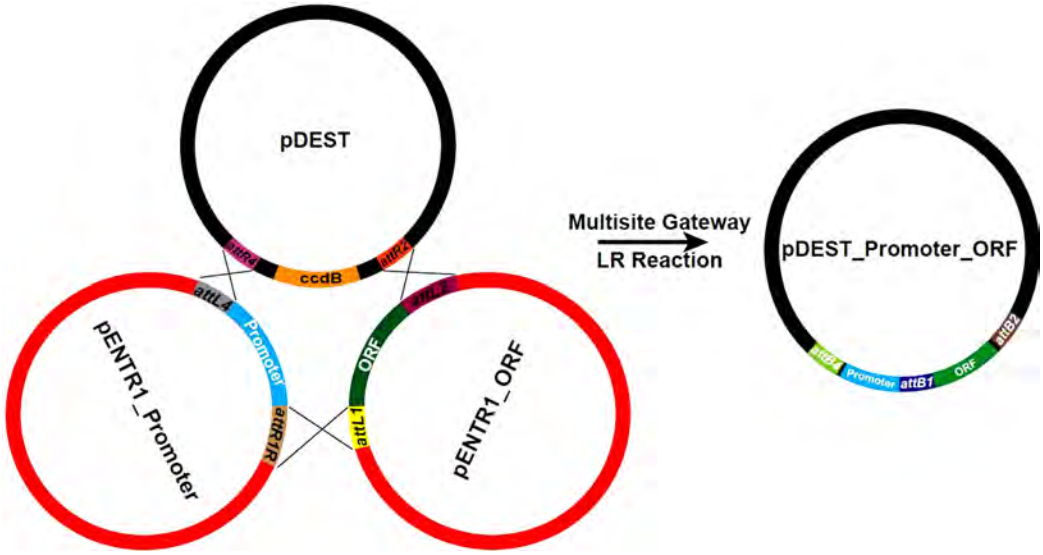

Fig. (15). Schematic diagram showing the strategy of generation of complex expression clone *via* fusing a given promoter and orf in tandem with a destination vector employing multisite gateway cloning strategy.

Step 3: Express the Gene of Interest

Finally, after generating the desired expression/screening clone in the destination vector, the integrity of the final expression/screening clone is ensured *via* sequencing or restriction digest. Then, these clones are transformed or transfected into the appropriate cells that the experiment demands, followed by the verification that the final desired construct is functional.

Since Gateway cloning technology is a proprietary technology of Thermo-Fisher Scientific, varieties of kits, associated products, and the recommended protocols for all the techniques are available from the manufacturer, we prefer not to narrate/describe any specific protocols in this manual. Readers interested in using the Gateway Cloning Platform are advised to consult the website of Thermo Fisher Scientific (https://www.thermofisher.com/in/en/home/life-science/cloning/gateway-cloning.html) to learn more specific information and procedures.

Essential Features of Different Components of Gateway Cloning Technology

Recombination Components

DNA Recombination Sequences (Att Sites)

It is the *att* site where recombination occurs between the interacting DNA molecules. Each *att* sites are characterized by a 25-bp core "recognition region" (where the excision occurs that is followed by the strand exchange), which is flanked by the presence/absence of "arms" on either side (Fig. **16**). The arms contain interaction/binding sites for the recombination enzymes. Within the recognition region, a 7-bp "asymmetric overlap" is present, which represents the site where the DNA is excised and re-joined (Fig. **16**). To improve the efficiency and specificity of the Gateway System, the wild-type λ *att* recombination sites have been modified in the following ways. Mutations have been introduced in the (i) core regions of the *att* sites to eliminate stop codons and to maintain orientation and reading frame and (ii) short (5 bp) regions flanking the 15-bp core regions of the *attB* sites to minimize secondary structure formation in single-stranded forms of *attB* plasmids (*e.g.*, phagemid ssDNA or mRNA). The *attR* has been modified by removing a 43 bp portion to make the *attL* x *attR* reaction irreversible and more efficient. In addition to the above changes, site-specific point mutations have been produced to some *att* sites to increase recombination efficiency, resulting in sequence variations among the *att* sites, which do not affect the specificity of the recombination reactions or the functionality of the vectors. Some specific properties of various modified *att* sites and the specificities of interactions between various attP sites with different attB sites are presented in Tables **21** and **22**, respectively.

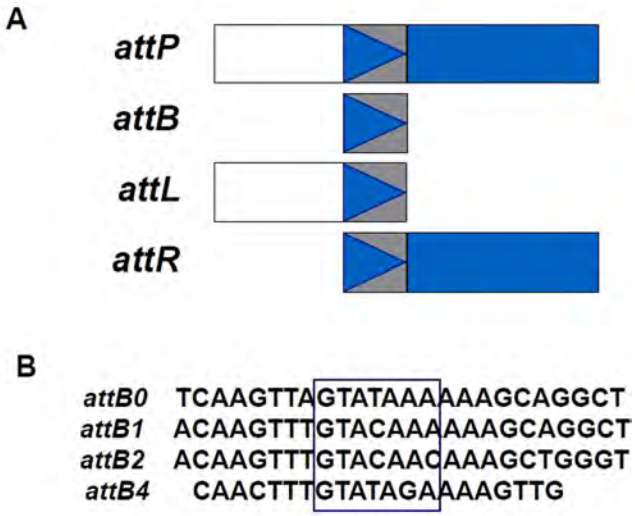

Fig. (16). Features of the sites involved in site-specific recombination. (**A**) Schematic representation of attP, attL, and attR sites with recognition "arms" either on one (for attB or attR) or on both sides (attP) of the "recognition region" (indicated by a grey box with a blue arrowhead within). In contrast, attB sites have no arms at all. These arms harbor binding sites for the recombinases and are dubbed "left" and "right" with respect to the asymmetric overlap. Note that attL sites carry an arm at 5′ (left) side of the overlap (indicated with a white box), whereas attR sites have an arm at 3′ (right) side of the overlap (indicated with a blue box), and attP sites have both arms. (**B**) attB sites are ~25 bp and feature a central 7-bp "asymmetric overlap" (boxed) that determines where DNA is cut and re-joined. attB0 is the naturally occurring site in the *E. coli* genome that is used by bacteriophage λ. Mutagenesis has been used to generate the other varieties of attB sites.

Table 21. Characteristics of the Modified *att* sites.

Site	Length	Found In
*att*B	25 bp	Expression vector, Expression clone
*att*P	200 bp	Donor vector
*att*L	100 bp	Entry vector, Entry clone
*att*R	125 bp	Destination vector

Table 22. Specificity of Interactions with different *att* sites.

Donor Site	Interacting with
*att*B1	*att*P1
*att*B2	*att*P2
*att*L1	*att*R1
*att*L2	*att*R2

Proteins that Mediate the Recombination Reaction

A blend of lambda and *E. coli*-encoded recombination proteins (*i.e.*, Clonase enzyme mix –BP Clonase and LR Clonase) is typically used for integrating Lambda into the *E. coli* chromosome to mediate intermolecular DNA recombination. The enzymes used in these reactions cause two pairs of excision, strand exchange, and ligation of the DNAs in a novel form by binding to specific sequences, thereby bringing the target sites together, cleaving them, and covalently attaching the complementary partner DNA. In this phenomenon, the DNA segments flanking the recombination sites are switched, which results in hybrid *att* sites consisting of sequences donated by each parental vector, and hence, there is no net gain or loss of nucleotides. For example, as mentioned above, *attL* sites are created from the recombination between the sequences from *attB* and *attP* sites during the integration of the bacteriophage genome into the host genome. The site-specific recombination between *attB* with *attP* sites used in the BP reaction is catalyzed by the BP clonase enzyme mix, thereby generating the *attL* and *attR* sites, whereas the LR clonase enzyme mix catalyzes the reverse reaction to regenerate *attP1/P2* and *attB1/B2* (Fig. **9,** Table **23**). It should be noted here that the strategy of preparation of these enzyme mixes relies on the specificity of the integration and excision of the bacteriophage into and out of the *E. coli* genome, respectively, and are extremely specific. The choice of the BP and LR clonase proteins depends on which pathway (either lytic or lysogenic) lambda utilizes (Table 23). BP clonase enzyme mix consisting of bacteriophage λ Integrase (Int) and *E. coli* Integration Host Factor (IHF) proteins catalyzes the lysogenic pathway whereas LR clonase enzyme mix made up of bacteriophage λ, Int, Excisionase (Xis) proteins, and the *E. coli* Integration Host Factor (IHF) of the lytic pathway. Therefore, BP clonase enzymes never use *attL* or *attR* sites and hence generate only one set of hybrid. The same is also true for the LR clonase mix, and the recognition of *att* sites is extremely specific.

Table 23. Specificity of the various site-specific recombination reactions and composition of recombinase enzyme mixes used to catalyze them.

Pathway Reaction	Reaction	Catalyzed by	Composition
Lysogenic	*attB* X *attP* → *attL* x *attR*	BP Clonase	Int, IHF
Lytic	*attL* X *attR* → *attB* x *attP*	LR Clonase	Int, Xis, IHF

BP Reaction: BP Clonase enzyme mix recombines an *attB* substrate (*attB*-PCR product or a linearized *attB* expression clone) with an *attP* substrate (donor vector), creating an *attL*-containing entry clone.

LR Reaction: LR Clonase enzyme mix catalyzes the recombination of an *attL* substrate (entry clone) with an *attR* substrate (destination vector) to create an attB-containing expression clone (see diagram below).

Features of the Gateway Vectors

Three different types of Gateway®-adapted vectors are available from Invitrogen/Thermo Fisher Scientific. Their properties are summarized in Table **24**.

Table 24. Salient features of different vectors used in major applications of gateway technology.

Gateway Vector	Nature of *att* site carried	Used for
Donor vector (pDONR™)	*attP*	Cloning *attB*-flanked PCR products to generate entry clones.
Entry vector (pENTR™)	*attL* sites with single T extension	Cloning PCR products or restriction fragments that do not contain *att* sites to generate entry clones.
Destination vector contains sites	*attR*	Recombination with the entry clone in an LR reaction to generate an expression clone. Contains elements necessary to express the GOI in the appropriate system (*i.e.*, *E. coli*, mammalian, yeast, insect).

Common Features of the Gateway Vectors

- Two *att* sites flank a cassette to enable recombinational cloning and efficient selection of entry or expression clones.
- The cassette contains the *ccdB* for negative selection (present in donor, destination, and supercoiled entry vectors).
- Chloramphenicol resistance gene (Cm^R) for counter-selection (present in donor and destination vectors)
- After a BP or LR recombination reaction, the cassette may be replaced by the gene of interest to generate the entry clone and expression clone, respectively.

Advantages of Gateway Cloning

The Gateway recombination cloning system uses a one-hour reversible recombination reaction without using ligase, sub-cloning steps, or screening of countless colonies, thereby saving a substantial amount of time, money, and effort. The benefits of this procedure include:

- **High Efficiency:** 95% cloning efficiency is delivered.
- **Authenticity:** Maintains the orientation and reading frame of the DNA sequence throughout the cloning process

- **Seamless:** Efficient cloning of single fragments into multiple vectors simultaneously.
- **Flexibility:** Offers an ease to clone multiple gene fragments into a single construct.
- **Simple:** No need for ligase, sub-cloning steps to screen countless colonies as needed in traditional restriction enzyme cloning.
- **Fast reactions**: A one-hour room-temperature cloning reaction causes a rapid and highly efficient transfer of DNA sequences into multiple destination vector systems, maintaining orientation and reading frames.
- **Versatile:** Easily shuttle DNA material/ insert from one vector to another vector. The insert from the entry clone can be transferred into multiple destination vectors conveniently.
- **High Throughput Compatible:** Appropriate for adaptation to high-throughput (HTP) format.

6.11. TROUBLESHOOTING GUIDE FOR CLONING

It is strongly recommended to run the following controls during transformations. These controls may help troubleshoot which step(s) in the cloning workflow has failed.

- Transform 100 pg to 1 ng of uncut vector to check cell viability, calculate transformation efficiency, and verify the antibiotic resistance of the plasmid.
- Transform the equivalent quantity (100 pg to 1 ng) of the digested linear vector to determine the amount of background contributed by the undigested plasmid. The number of colonies in this control should be less than 1% of the number of colonies in the uncut plasmid control transformation (from control #1).
- Transform a vector only in the ligation reaction. The ends of the vector should not be able to re-ligate because either they are incompatible (*e.g.*, digested with two restriction enzymes that do not generate compatible ends) or the 5′ phosphate group has been removed in a dephosphorylating reaction (*e.g.*, blunt ends treated with rSAP). This control transformation should yield the same number of colonies as control #2.
- Digest vector DNA with a single restriction enzyme, re-ligate, and transform. The ends of the vector DNA should be compatible and easily joined during the ligation reaction, resulting in approximately the same number of colonies as control #1.

6.12. Recipes Of Different Buffers And Reagents

10X Buffers for the Restriction Endonuclease from New England Biolab

NE Buffer 1.1: 10 mM Bis-Tris Propane-HCl, 10 mM $MgCl_2$, 100 µg/ml BSA (pH 7.0 @ 25°C).

NE Buffer 2.1 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 100 µg/ml BSA (pH 7.9 @ 25°C).

NE Buffer 3.1 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 100 µg/ml BSA (pH 7.9 @ 25°C).

CutSmart Buffer 20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 100 µg/ml BSA (pH 7.9 @ 25°C).

A wide variety of restriction endonuclease buffers are available. However, the supplier provides the buffer along with the enzyme. Generally, each company has categorized the buffers into four types, which are again color-coded. In this book, the buffer composition from New England Biolabs (NEB) is referred to here, which, in our experience, works best for all the restriction enzymes procured from NEB.

Dephosphorylation Buffers

BAP (Bacterial Alkaline Phosphatase) Buffer

0.5 M Tris-HCl, pH 8.0

10 mM $ZnCl_2$

CIP (Calf Intestine Phosphatase) Buffer

0.2 M Tris-HCl, pH 8.0

10 mM $MgCl_2$

10 mM $ZnCl_2$

0.5 mg/ml BSA or gelatin.

SAP (Shrimp Alkaline Phosphatase) Buffer

20 mM Tris-HCl, pH 8.0,

100 mM $MgCl_2$

E. coli DNA ligase Buffer

400 mM Tris-HCl, pH 8

0.1 M $MgCl_2$

50 mM DTT

0.5 mg/ml BSA

E. coli DNA Polymerase I or Klenow fragment Buffer

0.5 M Tris-HCl, pH 7.5

0.1 M $MgCl_2$

10 mM DTT

0.5 mg/ml BSA or gelatin

Mung Bean Nuclease Buffer

0.3 M sodium acetate, pH 5.0

0.5 M NaCl

10 mM zinc acetate

0.5 mg/ml BSA or gelatin

T4 DNA Polymerase Buffer

0.5 M Tris-HCl, pH 8.0

50 mM $MgCl_2$

50 mM DTT

0.5 mg/ml BSA or gelatin

10X Taq DNA Polymerase Buffer

0.1 M Tris-HCl, pH 8.4

15 mM $MgCl_2$

500 mM KCl

1 mg/ml gelatin

Note:

The concentration (x) of $MgCl_2$ depends upon the sequence and primer set of interest. The optimal concentration is first determined empirically using a 10× amplification buffer minus $MgCl_2$. Based on these results, a 10× amplification buffer containing $MgCl_2$ at the optimal concentration is prepared.

Terminal Transferase Buffer

1 M sodium cacodylate, pH 7.0

10 mM CoCl2

1 mM DTT

0.5 mg/ml BSA or gelatin

T4 Polynucleotide Kinase Buffer

0.5 M Tris-HCl, pH 7.5 (forward reaction) or

500 mM imidazole-HCl, pH 6.6 (exchange reaction)

0.1 M $MgCl_2$

50 mM DTT

0.5 mg/ml BSA or gelatin

dNTP mix, pH 7.0

Deoxyribonucleoside triphosphates (dNTPs) can be purchased as ready-made 100 mM solutions, which is the preferred method of shipping and storage. Alternatively, they can also be purchased in lyophilized form and prepared in deionized water as follows:

- Dissolve in water to an expected concentration of 25 mM; adjust to pH 7 with 1 M NaOH. dNTPs will undergo acid-catalyzed hydrolysis unless they are neutralized.
- Determine the actual concentration of each dNTP spectrophotometrically, using their extinction coefficients.
- Prepare 5mM working solutions for each dNTP from concentrated stocks of nucleoside triphosphates.

- Prepare dNTP solutions (mixes) containing equimolar amounts of all four DNA precursors as follows:

1 mM 4dNTP mix: 1 mM each of dATP, dTTP, dCTP, dGTP

0.5 mM 4dNTP mix: 0.5 mM each of dATP, dTTP, dCTP, dGTP

Store all the aliquots at -20°C.

SOC medium /Recovery Medium for Transformation

Bacto-tryptone 2 g

Bacto-yeast extract 0.5 g

NaCl 0. 05 g

Add dH_2O to make the volume to 100 ml

Check the pH, adjust to 7.0-7.2 with NaOH, and sterilize by autoclaving at 121°C, at 103.5kPa (151b/in2) for 20 min. Immediately before use, add aliquots of autoclaved or filter-sterilized stock solutions to give the following final concentrations: 2.5 mM KC1, 20 mM MgSO4, 20 mM glucose.

CONCLUSION

This chapter describes make various steps involved in the successful creation of recombinant DNA molecules more user-friendly for a newcomer trying to clone a gene of interest. They can easily choose an appropriate strategy from a variety of different types of cloning strategies that have been described in detail throughout the chapter. The criteria for selection can be based on the advantages and disadvantages of each procedure. The know-how of restriction enzymes, modification enzymes, and the protocol for the preparation of insert and vector DNA samples using these enzymes provide important tools for molecular cloning. Last but not least, the ligation of the two DNA molecules concludes this process, and most importantly, this section deals with protocols for joining molecules with different ends. The preparation of competent cells and their transformation with protocols bring an end to the cloning procedure. Finally, a brief description of the newly emerged Gateway Cloning Technology is also included, which will offer the user a choice of adopting this emerging technology. A new student can imbibe all the facts from the vivid description of each section for successful cloning.

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CHAPTER 7

Identification of Recombinant Clones

Abstract: Identification of correct recombinant clones using an appropriate screening strategy following ligation reaction and transformation constitutes one of the most vital methods in molecular cloning. Introducing the commonly used screening strategies used to identify the correct recombinant clone to a newcomer forms the basis of this chapter. We include various methods to verify true recombinants, beginning with a classic way of initial selection of the transformants on antibiotic-containing medium followed by powerful means of blue-white colony screening strategy, a precise screening strategy using restriction enzyme digestion, and a quick screening strategy using polymerase chain reaction (PCR). The reagents and solutions required for each process can be found at the end of the chapter.

Keywords: Antibiotics, Alpha complementation, Blue white screening, PCR primers, Recombinant clone.

7.1. INTRODUCTION

Identifying recombinant clones constitutes the last but vital step in the cloning workflow. Following all the essential steps of cloning and introduction of ligated products into host *E. coli* cells, the desired recombinant clones need to be screened out from the non-recombinant ones [4 - 6]. This crucial step is accomplished in a sequential manner employing multiple screening strategies. The first step toward this goal involves screening the actual transformants from the non-transformants. Note that during transformation, not all of the competent cells present in the transformation mixture take up the ligated products, thereby creating these two classes of cells. Consequently, the cells that have taken up the ligated products (transformants) must be screened and separated from the cells unable to receive any ligated products (non-transformants). This step is usually carried out employing a positive selection strategy by growing the transformation mixture in the presence of an antibiotic (usually ampicillin) that permits the growth of only the transformants (see ***UNIT 7.2.1***) [5].

The second step involves the screening of the recombinant clones (desired product) from the non-recombinants (background and undesired product). As mentioned in ***CHAPTER 6*** (see ***UNIT 6.7***), the transformants that are selected

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and propagated successfully in the presence of ampicillin harbors the ligated products that contain both the vector plasmid (either undigested or self-circularized) and the desired recombinant clones (vector plasmid ligated to a desired insert). This phenomenon necessitates a successful screening strategy of the recombinant clones from the vector using a positive selection strategy. A large number of strategies have so far been developed to identify the correct recombinant clones that finally verify that the gene of interest is successfully cloned. As described below, these strategies include the classic way, the powerful way, the precise way, the quick way, or the most accurate way. This chapter deals with screening strategies of various candidate clones (consisting of both recombinant and non-recombinant) from mixtures of colonies generated after transformation in *Escherichia coli* as the host organism.

7.2. METHODS TO VERIFY IF YOUR GENE OF INTEREST IS SUCCESSFULLY CLONED

7.2.1. Initial Selection of the Transformants on Antibiotic-Containing Medium

Selective growth of the transformed host bacterial cells harboring recombinant/non-recombinant plasmid constitutes the very first step towards the screening procedure of the recombinant clones from non-recombinants. As mentioned in ***CHAPTER 6***, following the ligation reaction, the ligated products are introduced into the host bacterium by transformation (see ***UNIT 6.7***). During this process, an aliquot of the transformation mixture that contains both transformed and non-transformed *E. coli* cells is spread onto a selective growth medium containing a specific antibiotic (usually ampicillin). The rationale for the use of the selective medium involves enabling the growth of the transformed cells harboring the recombinant/non-recombinant plasmids in a selective fashion while preventing the growth of the non-transformed cells. To prevent non-transformants from growing, the selective medium has to be chosen carefully, bearing two criteria in mind:

- Non-transformed cells that lack any plasmid should not grow.
- Transformed cells that harbor the recombinant/non-recombinant plasmids should be able to grow in a selective manner owing to the presence of an antibiotic-resistance gene present in the vector moiety.

Note that the latter feature will distinguish the transformed colonies (harboring the recombinant/non-recombinant plasmids) from the non-transformants lacking any plasmids. The vectors used for cloning processes carry selectable markers based on which the medium for plating the transformants is chosen [4 - 6]. For most of the modern-day plasmid vectors, antibiotic resistance genes like

ampicillin, tetracycline, chloramphenicol, etc., are routinely used in the medium for promoting the growth of the transformants.

PROTOCOL 1: SELECTION OF THE TRANSFORMANTS FROM THE NON-TRANSFORMANTS BY GROWTH ON THE ANTIBIOTIC-CONTAINING SELECTIVE MEDIUM

Principle

Most of the modern-day plasmids that are routinely used in molecular cloning applications, including pUC-series, pBluescript-series, and pET-series of vectors, contain an ampicillin-resistance gene as the selectable marker. As noted above, the transformation of the ligated product containing both the religated vector (if any) and recombinant plasmid (vector and insert together) confer an antibiotic (ampicillin) resistance property to the host *E.coli*. All the transformed bacterial cells consequently display an ampicillin resistance property that permits their growth on the ampicillin-containing growth medium while preventing the growth of the non-transformed cells lacking any plasmid. This selection strategy screens out non-transformed cells from the transformants, which constitutes an essential first step toward the screening of recombinant clones.

Materials

5 mL liquid LB Medium in 25 mL culture tube

Liquid LB medium

LB agar plates supplemented with 50 µg/ml ampicillin

Equipment

Microcentrifuge, microfuge tube, microtips, micropipettes, incubator, spreaders for plating, sterile toothpicks, laminar flow hood.

Procedure

- Prepare four to six LB-ampicillin plates and one LB plate and dry them properly as described in ***CHAPTER 2***.
- Inoculate 5 ml liquid LB medium (in 25 mL culture tube) with 1/100 the volume of an overnight culture of the host *E. coli* strain (such as DH5α or XL1 Blue).
- Have the outgrowth of the transformed cells ready, which has been previously transformed with a ligation mixture (see ***UNIT 6.7***).
- Set up the following plates:
  - Spread 200 µl of transformed cell mixture onto an LB plate.

- Spread 200 µl of transformed cell mixture onto an LB ampicillin plate.
- Spread 200 µl of the overnight culture (untransformed cells) onto an LB ampicillin plate. Use the spread-plate technique (see ***CHAPTER 2, PROTOCOL 2.7***).

- Store all three plates in a 37°C incubator overnight.

Observations

- Count the colonies that appear on the individual plates the following morning.
  - The plate from step 3a should show confluent growth, confirming that the cells are viable. The plate from step 3b shows discrete colonies derived from individual amp^r cells, and the plate from step 3c shows no colonies at all.

NOTE I: The transformed colonies from Step 3b harbor both the non-recombinant (vector only) and recombinant (vector and insert) plasmid in the cells. Therefore, the cells that have taken up any of the vector or recombinant plasmids will only be able to grow on an LB ampicillin plate, producing discrete colonies. These cells are able to disable the antibiotic as a selectable antibiotic resistance marker is present on the plasmid itself. On the other hand, the untransformed cells from Step 3c do not contain this ampicillin marker containing plasmid and, hence, are unable to grow in LB ampicillin agar plates.

Troubleshooting

Occasionally, problems in this experiment may arise, though the procedure appears straightforward and easy.

• No transformed colonies are obtained

- In case no transformants appear on the plate from step 3b, check for the growth on LB plates of the same cells for cell viability.
- If confluent growth is obtained from step 3a, then possibly the DNA uptake has been unsuccessful. This could be due to a number of reasons. While pouring plates, the quantity of stock ampicillin added in the LB medium could have been added in a higher quantity than actually required, or the competent cell preparation might not have been done appropriately.

• Confluent growth on the LB-ampicillin plates

In most cases, this observation indicates that ampicillin has become inactive. While pouring plates, the molten agar needs to be cooled appropriately (below 50°C) before the ampicillin is added. Otherwise, the ampicillin usually becomes degraded and inactivated as it is very heat sensitive.

• Colonies on the LB-ampicillin plate are surrounded by haloes of smaller colonies

Haloes of smaller colonies of non-transformed cells (called satellites) appear when working with high copy number plasmids. Typically, the concentration of the ampicillin used prevents the growth of the untransformed cells but does not kill them. The β-lactamase produced by ampicillin-resistant bacteria is secreted outside the cells (extracellular), which slowly diffuses into the agar medium around the resistant colonies and degrades the ampicillin. As time passes, the ampicillin slowly becomes depleted, and its effective concentration gradually reduces, which in turn allows the non-transformed cells to grow, giving rise to small white colonies [1, 4, 6]. These colonies, when inoculated separately in an LB ampicillin medium, will fail to grow. Since the high copy number vectors direct the synthesis of relatively large amounts of β-lactamase, this problem can be solved by adding more ampicillin in the medium. Try increasing the ampicillin concentration to 60 or 70 μg/ml and make sure that no inactivation is occurring during medium preparation [5]. Do not incubate the plates for too long (incubation should not exceed for more than 16 hours).

7.2.2. Screening Recombinant Clones using Blue-White Colony Screening: A Classic Way

Once the transformants are selected from the non-transformants by growing the transformation mixture on the LB-ampicillin plates, the transformants need to be screened in the next step for selecting the subsets of transformants harboring the correct recombinant clones using the strategy of the blue-white screening method. This selection system employs genetic principles involved in bacterial lactose metabolism as an indicator of successful cloning of foreign insert. As the screen suggests, this classic way makes use of blue/white colors to distinguish the transformants harboring recombinant clones (appear as white colonies) from the non-transformants carrying only empty vectors (appear as blue colonies) when they are grown on the ampicillin containing plates supplemented with a chromogenic substrate X-gal (see the **Information Panel on X-gal** below) [4, 6] (Fig. **1**).

Sounds complicated? Here is how it works. For utilization of this principle, the experimenter needs to select specific vector plasmids for cloning and the host bacterium for transformation and propagating the recombinant clones, which are specially designed for this kind of screening strategy. In these vectors, a DNA fragment harboring the sites of the multiple restriction enzymes in tandem (MCS) is embedded in the regulatory sequence, and the first 146 amino acids of β-galactosidase enzyme (called *lac Z′* allele encoding the α-donor fragment) are

inserted in such a way that the reading frame of this fragment is neither destroyed nor shifted. The host bacterium carries the α-acceptor fragment encoding the carboxyl-terminal fragment of the β-galactosidase gene in their genome (see the **Information Panel on α-complementation** below). The expression of either of these fragments (such as that of α-acceptor fragment in the host cell when no vector is present) does not lead to the production of active β-galactosidase enzyme. However, when the undigested or religated vectors harboring α-donor fragment gets inside an appropriate host, the expression of both the α-donor and α-acceptor fragments leads to their association into a functional β-galactosidase enzyme. This phenomenon is known as α-complementation [4 - 6]. Any interruption in the reading frame of the α-donor fragment (such as insertion of an insert in the MCS of the vector) leads to the failure of the α-complementation. Importantly, the phenomenon of α-complementation is visually detected by growing either the host bacterium alone or the host bacterium carrying the empty vector alone or recombinant DNA in an LB ampicillin plate that is supplemented with a chromogenic substrate called X-gal. X-gal is a lactose analog, which itself is colorless but is converted to a product with an intense blue color when hydrolyzed by β-galactosidase (Fig. **2**). A vector harboring a DNA insert of interest inside its MCS will not express any α-donor peptide, leading to the formation of non-functional β-galactosidase. Consequently, in the transformant colonies carrying recombinant clones, the substrate X-gal will not be hydrolyzed, thereby giving rise to white colonies. Thus, the growth of the host alone or the host carrying recombinant clone on X-gal plates produces white colonies, while the host carrying an empty (religated) plasmid generates blue colonies. Therefore, the thumb rule of this strategy is if a transformant carries a recombinant DNA, it will give rise to white colonies, whereas if a transformant harbors an empty plasmid DNA, it will produce a blue colony [4 - 6].

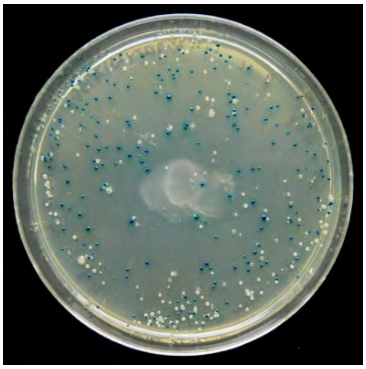

Fig. (1). A sample picture showing the growth of blue (transformants carrying vector DNA only) and white (transformant carrying putative recombinant clones) E. coli host colonies on LB plate containing ampicillin, IPTG, and X-gal.

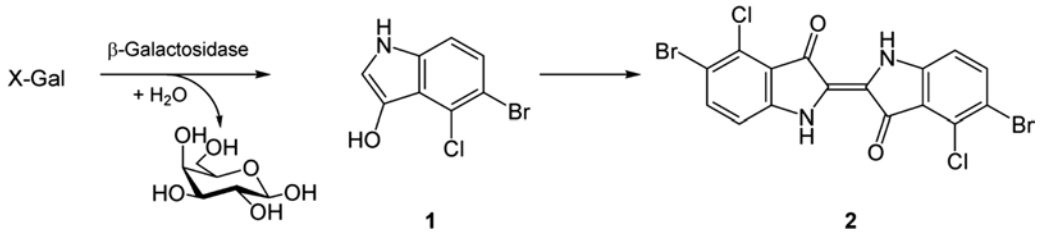

Fig. (2). The X-gal reaction carried out by β-galactosidase.

7.2.2.1. Lac Selection of Plasmids

pUCI8 plasmid is typically used as a vector for blue-white colony screening as it harbors the *lac Z′* sequence within its multiple cloning site. It also carries an ampicillin-resistance gene, so transformants can grow to produce ampicillin-resistant colonies on the LB ampicillin plate. When a piece of foreign DNA is inserted into a restriction site within its *lac Z′* sequence, the transformants carrying such recombinants now become $amp^{r}lacZ^{-}$ and the transformants harboring non-recombinants are $amp^{r}lacZ^{+}$. The two types of colonies are readily distinguishable by their respective color when a lactose inducer IPTG (Fig. **3**), is added with X-Gal in the plates - the recombinants being white and non-recombinants being blue.

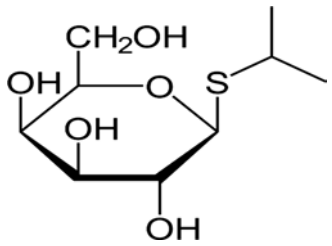

Fig. (3). The chemical structure of IPTG.

PROTOCOL 2: RECOMBINANT SELECTION WITH A *LAC* PLASMID VECTOR PUC18

Materials

Transformed and non-transformed *E. coli* cells

LB agar plates without ampicillin

LB agar plates containing ampicillin

LB medium

Reagents

100 mM IPTG (Stocks can be stored at -20°C) (see ***APPENDIX A.1***)

2% (w/v) X-gal in dimethylformamide (see ***APPENDIX A.1***)

Caution: Avoid direct skin contact with dimethylformamide; follow the supplier's safety guidelines.

Equipment

Microcentrifuge, microfuge tube, microtips, micropipettes, incubator, spreaders for plating, sterile toothpicks, laminar flow hood.

Procedure

- Prepare LB-ampicillin and plain LB plates and dry them as described in ***CHAPTER 2***.
- Inoculate a 5 ml LB medium with the appropriate host *E. coli* strain and grow overnight at 37°C. Also, 1ml of transformed cells should be ready (after ligation followed by transformation).
- Add 50 µl of 2% (w/v) stock solution of X-gal, along with 10 µl of 100 mM stock of IPTG, to the LB-ampicillin plates.
- Immediately spread the contents of each tube onto an LB-ampicillin plate.
- Prepare the following plates:
  - Spread 200 µl of transformed cells onto an LB plate.
  - Spread 200 µl of the overnight culture (untransformed cells) onto an LB ampicillin plate.
  - Spread 200 µl of transformed cells onto an LB ampicillin plate with X-gal and IPTG.
  - Centrifuge the rest (800 µl) of the transformed cells briefly at top speed. Discard most of the supernatant, leaving approximately 100-150 µL, and resuspend the pellet in that leftover supernatant. Spread the entire cell suspension on the LB ampicillin plate with X-gal and IPTG.
- Incubate all the plates overnight at 37°C.

Observations

- Plates from step 4a) should show confluent growth of the transformed cells on the LB plate.
- Plates from 4b) should show no growth of the untransformed cells on LB-ampicillin c & d. The transformed cells (200 µl & 800 µl) from steps 4c) and 4d) should produce discrete colonies on LB-ampicillin plates. Two varieties of

colonies, blue and white, may appear (Fig. **1**). Non-recombinants being blue and recombinants being white.

NOTE I: To switch on the expression of lac Z', IPTG is needed as it is a non-metabolizable inducer (gratuitous inducer) of the lac operon.

NOTE II: An additional control should be added to check whether the color reaction is working with a digested and dephosphorylated vector that is expected to yield no non-recombinants. Since it is possible to obtain false positives, transform an aliquot of cells with 1 ng undigested pUC18 vector and plate on an LB-ampicillin plate, which will produce a blue-colored colony.

NOTE III: The Blue white Colby screening method is very sensitive.

Troubleshooting

The X-gal system is not perfect, thereby frequently yielding false positives. Thus, the screening for recombinant candidates can become tricky. Following problems may arise, which can be troubleshooted as follows.

- **All the colonies are white:** It is possible that X-gal has been degraded, or the IPTG stock may not be functional due to prolonged storage. Make sure to use a fresh X-gal stock in clean microfuge tubes. Check the label on the bottle of IPTG and make sure that the stock is no more than 3 months old.
- **The color reaction is faint:** In such a case, for a better intense color reaction, keep the plates at 4°C for 4-6 hours even after the colonies have grown on them. Storage of the transformation plates at 4°C yields satisfactory results for certain vectors. However, if the color change is still ambiguous, then suspect partial inactivation of the X-gal.
- **The colonies are not uniformly colored:** There are two common variants:
  - The periphery is more densely colored than the center; usually, this is a non-recombinant.
  - The colony is white, but there is a faint blue region in the center; typically, this is a recombinant.
- **Some blue colonies are recombinants:** This condition arises when the foreign DNA insert is inserted into the *lac Z′* gene without inactivating it and still maintaining the reading frame. Generally, this occurs when you are cloning a very small fragment of DNA. This means that some recombinants will still produce active β-galactosidase, and so will appear blue on X-gal plates. Do not disregard the blue colonies in that case! An alternative strategy, *e.g.*, colony hybridization, can be used to screen these blue colonies.
- **White colonies occasionally do not contain insert:** Sequence analysis of isolated plasmid from such white colonies shows that the vector usually contains

a small deletion in the *lac Z′* gene, which results from the excision of the inserted DNA at some stage in colony growth. This can happen if the cloning vector, especially the host strain, is not chosen with care or if the inserted DNA contains a sequence that forms secondary stem-loop structures, which in turn enhances recombination and rearrangement events. Also, the polylinker in *lacZ′* vectors stimulates stem-loop formation under certain circumstances.

SPECIAL INFORMATION PANEL: X-gal

X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactoside) is an analog of lactose. It is actually a chromogenic substrate that is used to carry out *in vitro* assay of the activity of the *E. coli* β-galactosidase enzyme that catalyzes the conversion of disaccharide lactose into monosaccharide glucose. X-gal is a colorless compound by itself. However, when the β-galactoside linkage present in the molecule is cleaved by the enzyme, the 5-bromo-4-chloro-3-indolyl group is released from the parent substrate 5-bromo-4-chloro-3-indolyl- β-D-galactoside. The latter then spontaneously dimerizes and is oxidized into 5, 5'-dibromo-4,4'-dichloro-indigo-2, an intensely blue product that is insoluble. (Refer to Fig. **7.2**) Therefore, the presence of a blue-colored product can be used as an indicator for the presence of active β-galactosidase. This also allows for bacterial β-galactosidase (so-called *lacZ*) to be used as a reporter in various applications, as mentioned earlier in this chapter. Furthermore, it is also used to detect the activity of β-galactosidase in histochemistry [4, 5].

SPECIAL INFORMATION PANEL: α-COMPLEMENTATION

α-Complementation results when two catalytically inactive fragments of *E. coli* β-galactosidase associate to form a functional enzyme. This method was developed by independently producing the two genetically inactive fragments of *lacZ* by engineering this gene. These fragments are known as α-receptor and α-donor fragments, respectively. The α-receptor fragment is created by deleting the 5' termini of the *lacZ* gene encoding the initiating methionine residues that result in the C-terminal fragment of the enzyme in which translation initiates at a downstream methionine residue. The α-donor fragment was constructed by

inserting deletion or chain-terminating mutations towards the 3′-end of the β-galactosidase gene, thereby generating an amino-terminal fragment that contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene. Note that neither the α-donor nor the α-acceptor fragment is enzymatically active by themselves. However, if these two inactive fragments of the enzyme are present at stoichiometric amounts, they can associate to form an active β-galactosidase both *in vivo* and *in vitro* [4 - 6].

Many of the new generation plasmid vectors harbor the α-donor fragment. Within this fragment, the multicloning site (MCS) is embedded in such a way that it still maintains the reading frame of the α-donor fragment, which leads to the harmless interpolation of a small number of amino acids into the N-terminal fragment of β-galactosidase gene. These vectors are used in conjunction with the host cells that express the C-terminal α-receptor fragment of β-galactosidase. As mentioned above, neither the host-encoded nor the plasmid-encoded fragments of β-galactosidase are functional by themselves. They can associate to form an enzymatically active protein. This type of complementation is called α-complementation [4 - 6].

The *lac*$^+$ *E. coli* cells that result from α-complementation are readily detectable because they form blue colonies in the presence of the chromogenic substrate X-gal (see the information panel on X-GAL). Notably, the insertion of a piece of exogenous DNA into the MCS of the plasmid during the cloning procedure customarily results in the production of an N-terminal fragment that fails to carry out α-complementation. Bacteria carrying recombinant plasmids, therefore, form white colonies. The development of this simple development-based assay system has greatly eased the identification of recombinants constructed in plasmid vectors.

SPECIAL INFORMATION PANEL: IPTG

Isopropyl β-d-1-thiogalactopyranoside (IPTG) is a molecular mimic of allolactose, a lactose metabolite that withdraws the repression of the lac operon. IPTG is widely used to induce the expression of a test protein whose gene is experimentally placed under the control of the *lac* operator P_{lac} [4, 5].

Just like allolactose, IPTG binds to the *lac* repressor bound to the operator/promoter and triggers the dissociation of the tetrameric repressor from the *lac* operator in an allosteric manner, thereby stimulating the activation of transcription of *lac* genes, including β-galactosidase. However, unlike allolactose, the sulfur (S) atom present in the molecule creates a chemical bond that is non-hydrolyzable by the enzyme β-galactosidase, thereby preventing its metabolism. (Refer to Fig. **3**) Therefore, its concentration remains constant during an experiment. In the blue-white screening assay for recombinant clone selection, IPTG is used along with X-gal.

7.2.3. Screening Recombinant Clones with Restriction Enzyme: A Powerful and Precise Way

In a systematic screening procedure, the candidate recombinant clones that are screened out from the transformant colonies harboring the empty vector using blue-white colony screening require further confirmation. Screening candidate recombinant clones using a thorough analysis by restriction enzyme digestion constitutes a more precise and confirmatory method to identify correct recombinant clones [4 - 6]. Moreover, for cloning methods that do not employ the vector/host system amenable to the blue/white assay system, this method appears to be the only reliable method to screen out the appropriate recombinant clones. In this method, if the sequence information of the insert is known, the location of the restriction sites can be easily deduced. However, if the sequence information of the insert is not available, a thorough analysis of the restriction enzyme digestion needs to be performed to establish its restriction map. This information is later used to develop a strategy in which appropriate restriction enzymes can be used to easily identify the presence of the insert within the vector. The initial analysis should be carried out with the same set(s) of restriction enzymes that were originally used to prepare both the vector and the insert. Digestion, in this case, should yield the free insert band when checked on an agarose gel. For further confirmation of the existence of an appropriate insert, one needs to carry out digestion with additional restriction enzymes to verify the recombinant clone. The sizes of the restriction fragments from different combinations of restriction enzymes would establish the identity of the correct insert in the plasmid and reveal the orientation of the insert relative to a specific landmark of the vector molecule. Hence, this method is unequivocally precise.

PROTOCOL 3: SELECTION OF RECOMBINANT CLONES WITH RESTRICTION ENZYME DIGESTION ANALYSIS

Materials

Plasmid DNA samples prepared from selected candidate transformed colonies

Combinations of restriction enzymes(s) and their buffers

Components and reagents required for plasmid DNA preparation from mini scale (see ***PROTOCOL 3.1***)

Components and reagents required for agarose gel electrophoresis (see ***PROTOCOL 5.1***)

Equipment

Microcentrifuge, microfuge tube, microtips, micropipettes, incubator, shaker-incubator, water bath, apparatus for agarose gel electrophoresis.

Procedure

- Choose twelve well-isolated bacterial colonies from the transformation plates. If the vector host system involves the *Lac Z′* allele and α-complementation system, choose and select twelve white colonies.
- Circle these colonies with a black marker and number them on the base of the plate.
- Take an agar plate (called a master plate) containing solid LB medium containing 50 μg/mL ampicillin and divide the plate into thirteen one-square grids (5 mm x 5 mm each) (see Fig. **4**). Label the first square as the vector control and label the rest twelve squares with numbers 1 to 12.

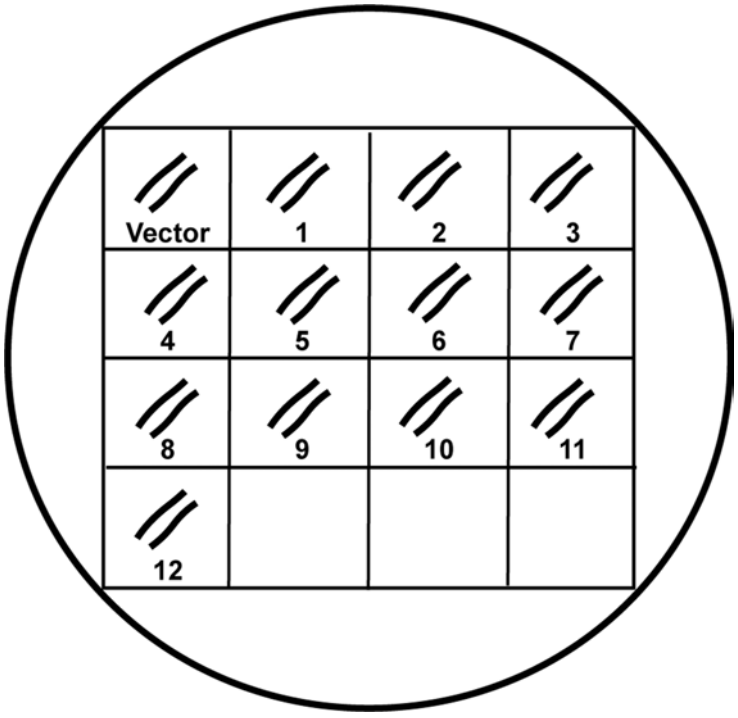

Fig. (4). chematic diagram showing the design of the grid on the master plate and the shape of the cell patch/streak within each grid.

- Label thirteen 25 mL culture tubes containing 5 mL liquid LB medium containing ampicillin (50 μg/ml) as the ‘vector control’ and 1-12 as before.

- Pick up a colony of the host bacterium that has been previously transformed with the empty vector or blue colony (if applicable) with a flat sterile toothpick/microtip. Streak into three small parallel patches on the grid labeled 'vector control' on the agar plate, as shown in Fig. (**4**).

- Use the same toothpick/tip to inoculate liquid LB medium labeled 'vector control'. Discard the toothpick/tip.
- Repeat steps 5-6 to patch on the LB plate containing 50 μg/mL ampicillin grids and to inoculate with twelve white colonies from the transformation plate that underwent the blue/white screening method as described in ***PROTOCOL 7.2.*** In case the blue/white screening strategy is not employed, pick up twelve randomly identified transformed colonies and inoculate twelve separate 5 mL liquid LB medium containing 50 μg/ml ampicillin.
  - Grow the LB+ampicillin plates as well as the thirteen cultures overnight in an incubator shaker with vigorous shaking (at 250 rpm) at 37°C.
  - Isolate the plasmid DNA samples on a small scale from each candidate overnight culture.
  - Use a small aliquot of 2-5μl of each DNA sample and analyze them in an agarose gel using the vector DNA sample as the negative control.
  - Look for candidates that are migrate slowly than the vector plasmid. True recombinants will be those that are larger than the vector molecule owing to the presence of the insert and hence will be migrated at a slower speed relative to the vector (see Fig. **5**). Identify all the slow-migrating plasmid DNA candidates. They should represent candidate recombinant clones.

- Purify these candidate plasmid DNA samples using extraction with phenol-chloroform-isoamyl alcohol followed by recovery by standard ethanol precipitation.
- Set up digestion reactions for all the candidate DNA samples (keeping vector DNA as the negative control) with the appropriate choice of one or with a combination of two restriction enzyme(s). Note that, for a correct candidate recombinant clone, the pattern of restriction fragments yielding from a given set of restriction enzymes would be predictable.

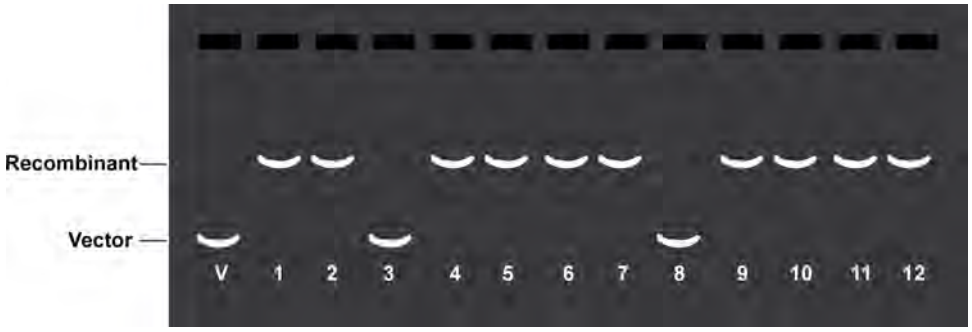

Fig. (5). Schematic diagram of a sample and hypothetical agarose gel showing the relative migration of the vector plasmid DNA (V) and candidate plasmid DNA sample 1-12. Note that clones 3 and 8 are not recombinants, whereas all other candidates are recombinant clones displaying much slower migration in the gel owing to the presence of the insert DNA.

NOTE: While screening for the correct recombinant clones by restriction enzyme digestion analysis, one needs to set up a master mix, which includes all the reagents except the DNA. The master mix is then dispensed in the tubes, each containing a plasmid DNA clone that needs to be screened (see ***PROTOCOL 6.3****).*

- Analyse the digestion products from each candidate plasmid on an agarose gel to verify that the sizes of the vector backbone and insert (or the observed pattern of restriction fragments if the given enzyme(s) does not yield vector and insert directly) confirm the expected sizes using a specific set of restriction enzymes (Fig. **6**).

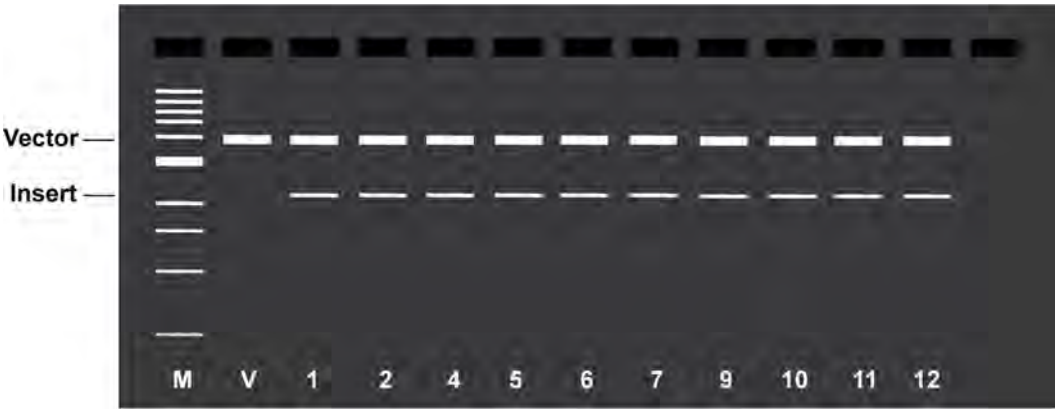

Fig. (6). Schematic diagram of a sample agarose gel image showing the profile of vector and insert bands arising from restriction digestion of the vector and slow migrating clones from the experiment described in Fig. (**2**) with restriction enzymes BamHI and EcoRI with which the insert was originally cloned. M: Standard 1 Kb plus Ladder DNA, V: vector. Note that a 2.2 kB BamHI-EcoRI insert was cloned in the 3.9 kb vector DNA.

7.2.4. Selecting Recombinant Clones by Direct Screening of the Transformant Colonies using Polymerase Chain Reaction (PCR): A Quick Way

This method is the most rapid method that involves screening the colonies at the initial stage to determine the presence of the insert DNA. It bypasses both the isolation of the candidate plasmid DNA samples and their restriction digestion reaction. Since the latter two steps consist of a relatively longer procedure requiring at least three to four days, this method provides the experimenter with an alternative option that is a rapid, fast, easy, and inexpensive solution for screening cloned recombinant constructs [4, 5]. Screening the candidate transformant colonies with polymerase chain reaction (PCR) involves setting up parallel PCR reactions on a small scale by designing appropriate sets of forward and reverse primers that either encompass the flanking sequences of the insert DNA or can be vector-specific. These primer sets will amplify the foreign gene of interest present only in the recombinant clones in a PCR reaction, thereby identifying them faithfully. This process involves the lysis of the bacteria rapidly followed by the amplification of a portion of the plasmid with either insert-specific or vector-specific primers. To determine the orientation of your insert, it is recommended to use orientation-specific primer sets for PCR analysis (see ***UNIT 7.2.4.1***). One drawback of this method is that it works when the insert DNA is typically shorter than 3 kb. The key steps in this process are:

- Designing primers to detect the presence of insert.
- Setting up a standard PCR reaction (primers, dNTPs, polymerase) using the supernatant of lysed bacteria as the template.
- Analyze the PCR products on an agarose gel for their sizes. In some cases, a portion of the individual candidate colony needs to be added directly to a PCR master mix, with the remaining portions being used to inoculate a culture plate or liquid media with appropriate antibiotics for downstream applications.

7.2.4.1. Designing Colony PCR Primers

Designing PCR primers is the first and most important step in the colony PCR procedure [4, 5]. Three strategies (Fig. **7**) are generally adopted for primer designing that include:

- Insert-specific primers.
- Vector backbone-specific primers.
- Orientation-specific primers.

- ***Insert-specific primers*:** Insert-specific primers encompass the entire length of the insert and anneal to insert-specific flanking sequence (Fig. **7**). If the desired

insert is present in the clone, an amplified product will be obtained following a PCR reaction, whereas no specific product will be observed for the empty vector molecules. Thus, this is a direct "yes or no" kind of verification. Note that, despite this test confirming the presence of the insert almost unequivocally, it is impossible to determine its orientation and directionality.

- ***Vector backbone-specific primers*:** Designing vector backbone-specific primers is another option where the primers anneal to specific sequences in the plasmid backbone flanking the insert (Fig. **7**). A positive clone will produce a much larger sized product than a negative clone without the insert [4, 6]. Additionally, this method confirms the correct size of the insert and ensures whether the insert resides within the plasmid backbone. Since these primers anneal to the backbone, they can be used to amplify any insert cloned in the same plasmid and hence serve as universal primer sets. This is one of the major advantages of using this kind of primer. However, the main drawback of using this type of primer is that it does not provide information about the orientation of the insert.

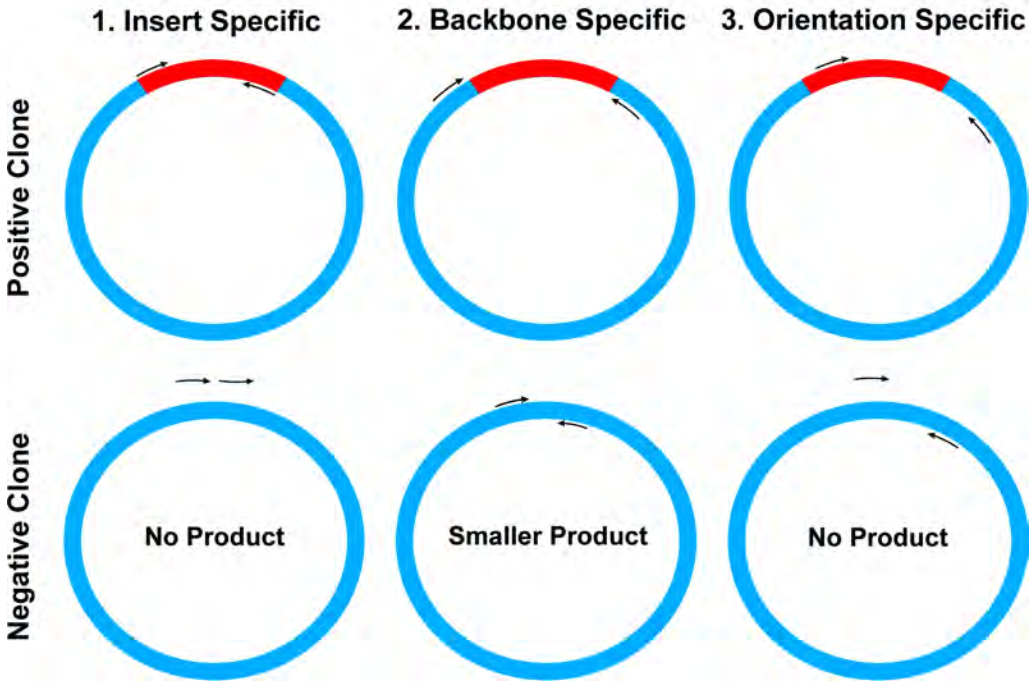

Fig. (7). Schematic diagram showing various strategies of using colony PCR reaction with insert-specific, vector-backbone specific, and orientation-specific primer sets to distinguish between recombinant and non-recombinant clones.

- ***Orientation-specific primers*:** These primers are useful in screening the orientations of the inserts that can be ligated in both orientations [4, 6]. Recall

that the inserts that are either prepared by a single restriction enzyme or with those restriction enzymes that generate a blunt end can be ligated in both orientations. One of the two primer pairs anneals to a specific sequence within the insert (thereby acting as the sense or forward primer), while the second primer binds to the vector backbone at the flanking region of the MCS (thereby acting as the antisense or reverse primers) (Fig. **7**). One of the ways to create this kind of primer pair is to mix-and-match insert-specific and backbone specific primers as mentioned above.

PROTOCOL 4: SELECTING RECOMBINANT CLONES BY DIRECT SCREENING OF THE TRANSFORMANT COLONIES USING POLYMERASE CHAIN REACTION (PCR)

Materials

LB Plates containing 50 μg/mL ampicillin

Transformed colonies grown on LB plates containing ampicillin

Standard Reagents for PCR Reactions (*Taq* DNA polymerase, buffers, dNTPs) (see ***UNIT 6.4.2***)

Specific Sets of Primers (Insert-Specific, Backbone-Specific or Orientation-Specific).

Components and reagents required for agarose gel electrophoresis (see ***PROTOCOL 5.1***)

Equipment

Microcentrifuge, microfuge tube, microtips, micropipettes, PCR thermocycler, apparatus for agarose gel electrophoresis.

Procedure

- Choose twenty well-isolated bacterial colonies from the transformation plates.
- Circle these colonies with a black marker and number them on the base of the plate.
- Label twenty-two 0.5 mL PCR tubes from 1-20 and designate the last two as the empty vector and no template negative controls, respectively.
- Take an agar plate containing solid LB medium containing 50 μg/mL ampicillin and divide the plate into twenty one square grids (5 mm x 5 mm each) (see Fig. **8**). Label each of the twenty squares with numbers 1 to 20.
- In parallel, prepare a PCR master mix with the insert-specific PCR primers, Taq

DNA polymerase, that is enough for ~25 PCR reactions.

- Aliquot the appropriate volume of this master mix into twenty-one previously labeled PCR tubes from step 3.
- Using a pipette tip, pick up a small cell clump from each colony of interest. Dip the tip in and out of the mix in the first PCR tube five times, followed by swirling the tip thoroughly.

NOTE: Make sure that some cell clump/patch is visible on the tip; approximately 1 mm3 = 1 µl volume of the cell is enough. It is important to realize that transferring too many bacterial cells to the PCR reaction may actually inhibit the PCR reaction or even cause non-specific products to show up on your gel.

- With the same tip, transfer the remaining cells into a patch on grid number 1 on your agar plate by scratching the tip across the surface of the agar a few times (make a set of three closely spaced parallel lines) (see Fig. **8**). Discard the tip.

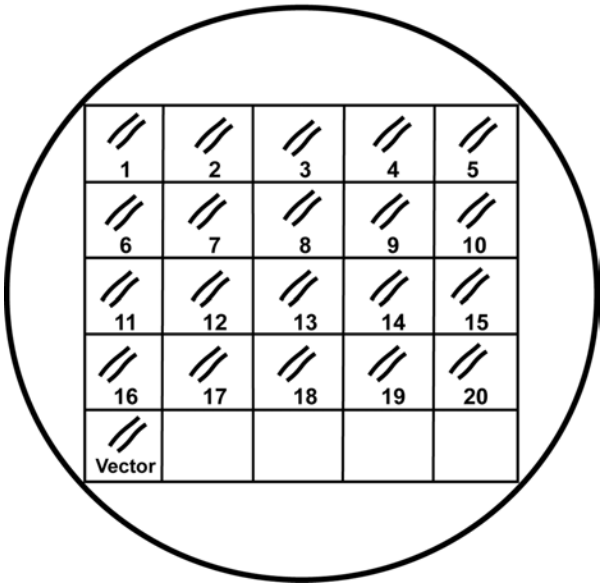

Fig. (8). Schematic diagram showing the design of the grid on the master plate and the shape of the cell patch/streak within each grid.

NOTE: It is important to stay away from the borders of each square to prevent the clones from touching each other when they grow up.

- Repeat steps 7 and 8 with the remaining 19 candidate clones. A small amount of cells from the bacterial host transformed with the empty vector should be added to the empty vector tube. No cells/colonies should be added to the tube, which is

marked as no template control.

- Run the PCR reactions by transferring the tubes to the thermocycler using an appropriate PCR program, incubate the agar plate at 37°C overnight, and save the plate for further downstream applications in case a positive clone is identified from screening clones 1-20.

NOTE: The bacteria-water suspension from step 7 will serve as the template for the PCR reaction. The bacterial cells needed to be lysed in order to release the resident plasmid DNA. The bacteria will be lysed during the initial heating step of the PCR reaction.

- Analyze the PCR products on a 1% agarose gel and note which clones give a positive PCR product with the predicted size. Those will be the putative recombinant clones (Fig. **9**).

NOTE I: If none of the tested clones is positive, some more clones (e.g., another 20) should be screened in the same way. However, in case no clones are identified as the desired clone of interest after screening ~40 clones, something is wrong. In that case, all your ligation and transformation controls, primer sequences, and thermocycling conditions should be checked. In the worst case, the cloning process needs to be repeated. It is also possible that the gene that is to be cloned is toxic to the host cells – you may need to try a different vector or different host or different incubation conditions.

NOTE II: This method works best when the insert is shorter than 3 kb.

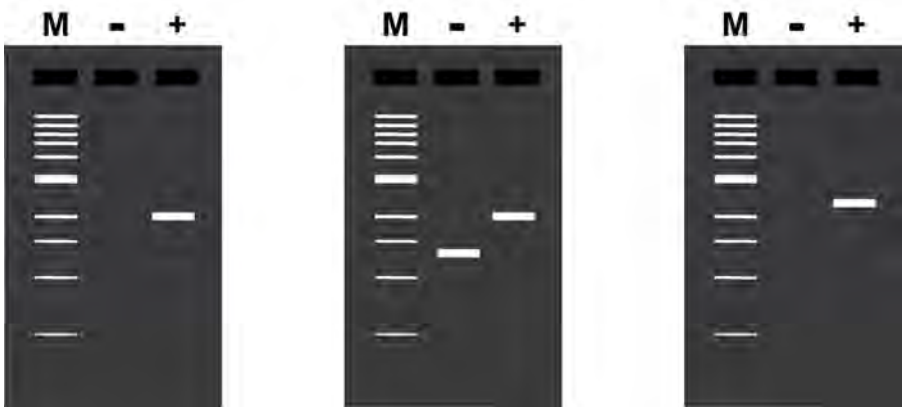

Fig. (9). Hypothetical gel image showing expected results from colony PCR screening procedure with one non-recombinant (empty vector only) and one recombinant (vector with insert) clone using the three different sets of primers as described in **UNIT 7.2.5.1**. When using insert-specific primers (1), positive clones (+) will produce a positive band, while a negative clone (-) will not yield any product. Backbone-specific primers (2) generate larger-sized PCR products for positive clones (+) compared to negative clones. Finally, orientation-specific primers (3) yield the same band (+) or no band (-) result as insert-specific primers but also reveal the

orientation of the insert.

- If a fraction of the tested clones yield the desired band using an insert-specific primer-set as described above, it is important to confirm the observation by repeating the screening procedure with the selected clones using both the vector backbone and orientation-specific primer-sets by colony PCR (Fig. **9**).
- It is also advisable to validate the identity of the positive clones identified from the colony PCR screening procedure by restriction digestion procedure (see ***PROTOCOL 7.3***).

NOTE: Regardless of the method of selection to screen the recombinant clones, identification of the correct recombinant will bring your cloning workflow to a successful end!

7.3. Recipes Of Different Buffers And Reagents

Liquid LB Medium (Luria-Bertani Medium)

Per liter:

To 950 ml of deionized H2O, add:

Tryptone 10 g

Yeast extract 5 g

NaCl 10 g

Shake or mix using a magnetic stirrer thoroughly until all the ingredients are dissolved. Adjust the pH to 7.0 with 5M NaOH (usually requires ≈ 2 mL). Adjust the volume of the medium to 1000 mL using deionized water. Sterilize by autoclaving for 20 minutes for 15 psi on a liquid cycle.

Solid LB medium with 50 µg/ml of ampicillin

Per liter:

To 950 ml of deionized H_2O, add:

Tryptone 10 g

Yeast extract 5 g

NaCl 10 g

Bacto Agar 15 g

Add all the ingredients except for Bacto Agar. Shake or mix using a magnetic stirrer thoroughly until all the ingredients are dissolved. Adjust the pH to 7.0 with 5M NaOH (usually requires ≈ 2 mL). Add the bacto agar and mix for another 3-5 minutes (note the agar at room temperature would not dissolve). Adjust the volume of the medium to 1000 mL using deionized water. Sterilize by autoclaving for 20 minutes for 15 psi on a liquid cycle. After sterilization, store the hot medium at room temperature for 40 minutes until the temperature of the medium reaches 45°C-50°C. Add 1 µl sterile solution of 50 µg/ml ampicillin. Mix the suspension for another 5 minutes and pour it into the plates, as described in **Chapter 2.**

IPTG (20% w/v, 0.8 M)

Prepare a 20% solution of IPTG by dissolving 2 g of IPTG in 8 ml of deionized water. Adjust the volume of the solution to 10 ml with deionized water and sterilize by passing it through a 0.22-µm disposable filter. Dispense the solution into 1-ml aliquots and store them at -20°C.

X-gal Solution (2% w/v)

Prepare a stock solution by dissolving X-gal in dimethylformamide at a concentration of 20 mg/ml solution in a glass or polypropylene tube. Wrap the tube containing the solution in aluminum foil to prevent damage by light and store at -20°C. It is not necessary to sterilize X-gal solutions by filtration.

CONCLUSION

The most important step in molecular cloning involves the identification of the correct recombinant clones that appear on the transformed plate, as discussed in the previous chapter. Throughout this chapter, the rationale for screening strategies, the concept of 'false positives', and several methods of screening are discussed in a highly illustrative manner that would benefit beginners. We have included the screening methods in a stepwise manner, starting with antibiotic-containing plates, followed by blue-white colony screening, a precise way using restriction enzymes, and finally, a quick way of setting up a PCR reaction. Necessary information on panels and critical factors, as provided, will enlighten a new student regarding cloning and troubleshooting failures. Lastly, we believe that this book will be useful to the students in designing experiments and working effortlessly and independently in the laboratory.

FURTHER READING

[1] Green MR, Sambrook J. Cloning and transformation with plasmid vectors Cold spring harb protoc. New York: Cold Springer Harbor Laboratory Press 2021; p. 11.

[2] Green MR, Sambrook J. Cloning in plasmid vectors: Directional cloning Cold spring harb protoc. New York: Cold Springer Harbor Laboratory Press 2020; p. 11.

[3] Green MR, Sambrook J. Cloning in Plasmid Vectors: Blunt-End Cloning Cold spring harb protoc. New York: Cold Springer Harbor Laboratory Press 2020; p. 11.

[4] Sambrook J, Green RM. Molecular cloning a laboratory Manual. 4th ed., New York: Cold Springer Harbor Laboratory Press 2012.

[5] Brown TA. Gene cloning and DNA analysis: An introduction. 6th ed., Chichester: Wiley Blackwell, John Wiley & Sons, Ltd. Publication 2010.

[6] Ausubel FM, Brent R, Kingston RE, *et al.* Current Protocols in Molecular Biology. Hoboken, NJ: John Wiley & Sons Inc. 1988.

APPENDICES

Preparation of Reagents, Buffers, and Stock Solutions

29:1 (w/w) Acrylamide/bis-acrylamide

Acrylamide 29 g

N, N'-methylene-bis- acrylamide 1.0 g

Distilled deionized water (dH_2O) to 100 mL

Place the resultant solution on a hot plate maintained at 37°C-40°C and keep stirring the solution using a magnetic stir bar until the acrylamide and bis-acrylamide are completely dissolved. Store at ≤1 month at 4°C.

CAUTION: Acrylamide is a potent neurotoxin. Do not forget to use gloves and a mask while handling the solutions or powder of un-polymerized acrylamide monomer.

10M Ammonium acetate (M.W. 77.08)

Ammonium acetate 385.4 g

dH_2O to 500 mL

Add 385.4 g of sold ammonium acetate to 150 mL dH_2O and stir the solution until the solid completely dissolves. Adjust the volume to 500 mL with H_2O. Sterilize the solution by filtration. Store in bottles at 4°C.

10%Ammonium Persulfate (APS)

APS 1 g

H_2O 10 mL

Dissolve 1g of ammonium persulfate in 10 mL of dH_2O. Store the solution at 4°C. This solution is good for 2-3 weeks as APS decays over time and hence should be replaced.

Bromophenol Blue (0.04% W/V aqueous)

Bromophenol Blue 0.04 g

H_2O to 100 mL

Dissolve 0.04 g of bromophenol blue in 50 mL of water, then dilute to 100 mL. Store the solution at room temperature (pH indicator).

1M $CaCl_2$ (M.W. 147.01)

$CaCl_2.2H_2O$ 147 g

H_2O to 1 L

Dissolve 147g of $CaCl_2.2H_2O$ in 800 mL of H_2O (Milli-Q or equivalent). Increase the volume to 1000 mL with dH_2O. Filter sterilize the solution by passing through 0.22µm membrane and store in 1-mL aliquots at –20°C.

NOTE: During the preparation of the competent cells while carrying out the transformation, slowly melt one aliquot of 1 M $CaCl_2$ by keeping it on ice and make a 10X dilution of the stock solution (final concentration would be 100 mM) to 100 mL with sterile dH_2O. Filter sterilize the solution through a 0.45-µm filter and then chill the solution to 0°C by keeping the tube on ice.

1M Cobalt (II) chloride (M.W.237. 95)

$CoCl_2 \cdot 6H_2O$ 23.8 g

H_2O to 100 mL

1M Dithiothreitol (DTT) (anhydrous M.W. 154.25)

DTT 15.45 g

H_2O to 100 mL

Dissolve 15.45 g of DTT in 80 mL of dH_2O. Make up the volume to 100 mL with dH_2O. Distribute into 1 mL aliquots. Make sure the bottle is stored in the dark (wrapped in aluminum foil) at –20°C (indefinitely).

NOTE: Never autoclave either DTT or any solutions that contain DTT.

5-7% DMSO

Dimethyl Sulfoxide (V/V) 7%

H_2O to 100 mL

Get a high grade of DMSO, dilute it as shown above, and aliquot 1 mL of the diluted solution in multiple sterile tubes. Save the tubes at -20°C. Note that every aliquot is designated for single use. Reject after single use.

25mM dNTP mix, pH 7.0

Deoxyribonucleoside triphosphates (dNTPs) are commercially available and can be procured as ready-to-use solutions of 100 mM concentration. Otherwise, they are also available in lyophilized/powdered form and can be reconstituted as solution of desired strength using dH_2O as solvent as follows:

- Suspend the powdered stock in dH_2O to the desired concentration (typically 25 mM is used) and adjust pH to 7.0 with 1 M NaOH.

NOTE: dNTPs suffer hydrolysis in acidic pH unless they are neutralized to pH 7.0.

- Once their solution is prepared, find out the real strength of each dNTP from their extinction coefficients using a spectrophotometer.
- Make working solutions of every dNTP with 5 mM strength from their concentrated stocks.
- Make a mixture of all four dNTPs containing equimolar amounts of all four of them as follows:

1 mM 4dNTP mix: 1 mM each of dATP, dTTP, dCTP, dGTP

0.5 mM 4dNTP mix: 0.5 mM each of dATP, dTTP, dCTP, dGTP

Store at −20°C.

Ethidium bromide (EtBr), 10 mg/mL

Ethidium bromide 1g

H_2O to 100 mL

Add 1g EtBr powder to 100 mL dH_2O and mix well using a magnetic stirrer (usually, it is necessary to stir the solution of EtBr for a few hours). Transfer the resultant solution to a dark bottle and save it at 4°C.

CAUTION: *EtBr is a powerful mutagen, and appropriate caution should be exercised when handling a solution of EtBr carefully.*

0.5 M EDTA (Disodium Ethylene Diamine Tetra-Acetate, pH 8.0)(M.W. 372.3)
$Na_2EDTA•2H_2O$ 181.6 g

NaOH pellets ~20 g

dH_2O to 1 L

Preparation

Note that $Na_2EDTA•2H_2O$ is only soluble at basic pH=8.0. Combine 800 mL of dH_2O in a beaker containing 181.6 g di-sodium salt EDTA. Stir the solution using a magnetic stir bar along with the addition of NaOH pellets (~20 g of NaOH is required) until the pH comes to 8.0. Bring the volume of the resulting solution to 1000 mL after transferring the solution to a graduated cylinder with dH_2O. After autoclaving, store at room temperature.

OR

Dissolve 186.1 g $Na_2EDTA.2H_2O$ in 700 mL dH_2O

Adjust pH to 8.0 with 10N NaOH (~50 mL)

Add dH_2O to 1 L

NOTE: The EDTA powder will not completely dissolve until the pH of the solution reaches 8.0 by the addition of NaOH.

6X Gel Loading Buffer: Glycerol-based

30% (v/v) glycerol 4°C

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol

10X Gel Loading Buffer: Ficoll-based

Ficoll 400 20%

Disodium EDTA, pH 8 0.1 M

sodium dodecyl sulphate 1.0%

bromophenol blue 0.25%

1M Glucose $C_6H_{12}O_6$, (MW 180.16)

Anhydrous dextrose 180.16 g

dH_2O 1 L

Sterilize by filtration

50X Glucose (150-mL Stock Solution)

D-Glucose (2 M) 54 g

H_2O to 150 mL

Add 54 g glucose powder to 100 mL dH_2O, followed by solubilization of the powder by stirring in a magnetic stirrer. Sterilize *via* filtration and save at 25°C to 27°C.

80% Glycerol (75 mL)

dH_2O 15 mL

glycerol 60 mL

Add 15 mL dH_2O to 60 mL of glycerol and stir thoroughly using a magnetic stir bar with slight heating on a hot plate until the solution becomes homogeneous. Note that glycerol is very viscous, and special care must be exercised when pipetting out 100% glycerol from the stock bottle/container and transferring it to another container. Sterilize by autoclaving.

0.8M IPTG (isopropyl thio-β-D galacto pyranoside) 20%w/v (M.W. 238.3)

IPTG 2.0 g

H_2O to 10 mL

Add 2 g of IPTG to 8 mL of dH_2O and vortex gently to allow complete dissolution. Bring the volume of the solution to 10 mL with dH_2O. Filter sterilize with a 0.22 μm filter. Store at -20 °C in small (typically one mL) aliquots.

1M Magnesium Acetate (M.W.214.46)

Magnesium acetate •$4H_2O$ 214.46 g

H_2O to 1 L

Add 214.46 g of solid magnesium acetate•4H_2O in 800 mL of dH_2O and stir the solution in a magnetic stirrer. Make up the final volume to 1000 mL with dH_2O. Filter sterilize the solution by passing through a 0.22 μm filter. Store the solution at 25°C to 27°C.

1M Magnesium Sulphate [$MgSO_4$ (M.W. 120.37)]

$MgSO_4$ (anhydrous) 120g

OR

$MgSO_4$•7H2O 246g

H_2O to 1 L

Either add 120 g of solid anhydrous $MgSO_4$ or add 246 g of $MgSO_4$•7H_2O to 1 L of distilled H_2O. Sterilize by autoclaving. Store the solution at 25°C to 27°C.

2M Magnesium Chloride [$MgCl_2$ (M.W. 95.21)]

$MgCl_2$ (anhydrous) 95 g

OR

$MgCl_2$•6H2O 203g

H_2O to 1 L

Either add 190 g of solid anhydrous $MgCl_2$ or add 406 g of $MgCl_2$•6H_2O to 1 L of distilled H_2O. Sterilize by autoclaving. Store the solution at 25°C to 27°C.

β-Mercaptoethanol (β –ME)

Typically, *β* –ME is commercially available as a 14.4 M solution. Store in dark bottles. Always open the vial/bottle containing *β* –ME within the fume hood and never expose the chemical to open air. Do not autoclave *β* –ME or solutions containing it.

NOTE: β –ME is highly volatile and a potential neurotoxin. Avoid inhalation of vapors of the solutions containing β –ME.

50% PEG 8000

Working solution ranges from concentrations of 13% to 40% (w/v). Dissolve the desired amount (depending on the concentration in percentage) of PEG 8000 in sterile water. Warm the solution if necessary for dissolving. Filter sterilize by

filtration through a 0.22 μm filter. Store for up to 1 to 2 years at -20°C for long-term storage or at room temperature.

Phenol/chloroform/isoamyl alcohol (25:24:1)

Mix 25 parts v/v phenol (equilibrated in 150 mM NaCl/50 mM Tris-HCl (pH 7.5)/1 mM EDTA) with 24 parts v/v chloroform and 1 part v/v isoamyl alcohol. Add 8-hydroxyquinoline to 0.1%. Store in aliquots at 4°C wrapped in aluminum foil or in a dark-colored glass bottle. Discard after 6 months.

NOTE: The phenol should look like a yellow-colored liquid due to the addition of 8-hydroxyquinoline, which is added as an antioxidant that prevents the oxidation of phenol.

Phosphate-buffered saline (PBS) 10X stock solution, 1 L

NaCl 80 g

KCl 2 g

$Na_2HPO_4.7H_2O$ 11.5 g

KH_2PO_4 2 g

H_2O to 1 L

Concentrations of different components in the working solution, pH ~7.3:

NaCl 137 mM

KCl 2.7 mM

$Na_2HPO_4.7H_2O$ 4.3 mM

KH_2PO_4 1.4 mM

3 M Potassium acetate solution pH ~5.5 (M.W. 98.14)

potassium acetate (3 M final) 294 g

90% formic acid (1.18 M final) 50 mL

H_2O to 1 L

Store at 25°C to 27°C indefinitely.

3M Potassium acetate (M.W. 98.14)

5M Potassium acetate 60 mL

Glacial acetic acid 11.5 mL

H_2O 28.5 mL

The resulting solution becomes 5M with respect to acetate and 3M with respect to potassium. Store at 25°C to 27°C.

0.1 M Potassium acetate buffer

Solution A: 11.55 mL glacial acetic acid/L (0.2 M).

Solution B: 19.6 g potassium acetate ($KC_2H_3O_2$)/L (0.2 M).

Consult the table depicting the volumes of glacial 0.2M acetic acid and 0.2M potassium acetate to arrive at a specific pH. Combine the indicated volumes of solutions A and B, as given in the Table **1**, to get the desired pH, followed by making up the final volume to 100 mL with dH_2O.

NOTE: It is a good idea to prepare either a 5X or a 10X concentrated stock by dissolving 5X or 10X the amount of potassium acetate in the same volume of the solvent. In this regard, it should be kept in mind that acetate buffers exhibit concentration-dependent pH alterations. Thus, the user should verify the pH of the diluted solution after diluting an aliquot to the concentrated stock solution. Also, if you plan to make buffers with an intermediate pH between the points listed in the same pH table, make a solution with the closest higher pH, then titrate with 0.2M glacial acetic acid.

1M Potassium Chloride (KCl) (M.W. 74.55)

KCl 74.55 g

H_2O to 1 L

Add 74.55 g of KCl to 900 mL of dH_2O, and stir the solution until the KCl crystals are completely dissolved. Make the final volume of the solution up to 1000 mL with dH_2O. Autoclave the solution for complete sterilization.

1M Potassium Glutamate (M.W. 203.24)

Potassium Glutamate 20.3 g

H_2O to 100 mL

0.1 M Potassium phosphate buffer

Solution A: 27.2 g KH_2PO_4 per L (0.2 M).

Solution B: 34.8 g K_2HPO_4 per L (0.2 M).

In order to obtain 0.1M potassium phosphate buffer with pH=7.0, add 39 mL of solution A and 61 mL of solution B, followed by dilution of the resulting solution to 200 mL with dH_2O. Notably, phosphate buffers display concentration-dependent pH alterations. Thus, it is always advisable to verify the final pH of the diluted solution.

3 M Sodium acetate

Sodium Acetate•$3H_2O$ 408 g

H_2O to 1 L

Add 408 g of sodium acetate•$3H_2O$ to 800 mL dH_2O, followed by complete dissolution of the solid sodium acetate by stirring the solution in a magnetic stirrer. Adjust the pH of the resulting solution to 4.8 or 5.3 (as desired) with 3M acetic acid. Make up the volume of the solution with dH_2O to 1000 mL. Sterilize by autoclaving. Stand at room temperature.

0.1 M Sodium acetate buffer

Solution A: 11.55 mL glacial acetic acid/L (0.2 M).

Solution B: 27.2 g sodium acetate ($NaC_2H_3O_2.3H_2O$)/L (0.2 M).

For pH = 4.8, mix 20 mL solution A and 30 mL solution B, then dilute with H_2O to 100 mL.

1M Sodium Bicarbonate (M.W. 84.007)

$NaHCO_3$ 12.6 g

H_2O to 150 mL

Add 12.6 g of $NaHCO_3$ to 100 mL of dH_2O. Stir the resultant solution in a magnetic stirrer until the solute is completely dissolved. Make the volume up to 150 mL. Sterilize *via* filtration through a 0.22 μm filter and let the stock solution stand at 25°C to 27°C.

1M Sodium Cacodylate, pH 7.0 (M.W. 214.03)

Sodium Cacodylate 21.4 g

H_2O to 100 mL

Dissolve 21.4 g Sodium cacodylate in 80 mL of dH_2O. Adjust the pH of the solution to 7.0 with HCl. Make the volume up to 100 mL with additional dH_2O.

NOTE: The cacodylate buffer contains toxic metalloid arsenic and hence should be handled very carefully, avoiding direct contact with skin and eyes and inhalation.

5M Sodium Chloride (NaCl)

NaCl 292 g

H_2O to 1 L

Add 292.2 g of crystalline NaCl in 800 mL of dH_2O, followed by thorough mixing of the resulting solution in a magnetic stirrer. Make the volume up to 1000 mL with dH_2O. Sterilize by autoclaving and store stock solution stand at 25°C to 27°C.

0.1M sodium citrate, (M.W. 294.10)

Sodium citrate 24.269 g

Citric Acid (M.W. 192.12) 3.358 g

dH_2O to 1 L

Immerse 24.269 g of solid sodium citrate dihydrate to 800 mL of dH_2O, followed by thorough mixing in a magnetic stirrer until the solute sodium citrate is completely dissolved. Then, keep adding 3.358 g of solid citric acid crystal slowly and gently to the solution to allow gradual solubilization of the citric acid crystal. Adjust the pH of the resulting using 0.1N HCl (typically maintained at pH ≈ 6.0). Add dH_2O to bring the volume to 1000 mL.

10% or 20% SDS (Sodium Dodecyl Sulphate)

SDS: 10/20 g

H_2O to 100 mL

Dissolve 10/20 g of SDS in 100 mL dH_2O. Store the stock solution at 25°C to 27°C.

NOTE I: *It is advisable that instead of adding the entire 10/20 g of the SDS powder to the 100 mL of dH_2O, a small amount of SDS powder should be added to the solvent, followed by stirring the solution very slowly in a magnetic stirrer, avoiding frothing until the entire powder becomes solubilized. When the entire amount of SDS powder is dissolved completely, more powder may be added to the solution to facilitate its complete solubilization. This process should be continued to allow solubilization of the entire solute.*

Note II: *Avoid inhalation of the SDS dust during handling of the SDS powder, as SDS dust is frequently produced. Use a face mask mandatorily during the handling of SDS powder.*

Note III: *Slight heating of the solution during the solubilization of SDS facilitates easy and faster dissolution of SDS powder. SDS is also called sodium lauryl sulfate.*

6M Sodium Iodide (NaI) (M.W. 149.89)

NaI 89.9 g

H_2O up to 100 mL

0.1M Sodium Phosphate Buffer

Solution A: 13.8 g/L NaH_2PO_4 0.1 M

Solution B: 26.8 g/L Na_2HPO_4 0.1 M

To obtain 0.1M Sodium phosphate buffer, pH=7.0, combine 39 mL solution A and 61 mL solution B, followed by the addition of 100 mL of dH_2O to make the final volume 200 mL. Notably, phosphate buffers exhibit alterations in pH as a function of concentration. Determine the final pH using a sensitive pH meter.

8% (W/V) Sucrose

Sucrose 8 g

H_2O to 100 mL

Dissolve 8g of sucrose in 100 mL of dH_2O. Sterilize *via* filtration through a 0.22 μm filter and store the stock solution at 4^oC

NOTE: The sucrose solution will become charred, producing a brown color if autoclaved.

TAE (Tris/acetate/EDTA) electrophoresis buffer: 50× stock solution

Tris base 242 g

Glacial acetic acid 57.1 mL

$Na_2EDTA.2H_2O$ 37.2 g

H_2O to 1 L

TBE (Tris/borate/EDTA) electrophoresis buffer: 10× stock solution

Tris base (890 mM) 108 g

Boric acid (890 mM) 55 g

0.5 M EDTA, pH 8.0 40 mL

DH_2O to 1 L

1M Tris, pH 7.4 to 8.0 (M.W. 121.1)

Tris base 121.1 g

HCL (concentrated)

DH_2O to 1 L

Preparation

Add 121.1 g of Tris base in 800 mL of dH_2O, stirring in a magnetic field until the Tris powder is completely solubilized. Now, determine the pH using a pH meter, which will be between 11.80 and 12.20. Now, start adding 12N HCl and note the resultant pH after each addition. Stop adding HCl as soon as the desired pH is reached. The usual volume of 12N HCl required to arrive at a specific pH from a starting pH of 12.00 is shown below.

Desired pH Volume of 12 HCl to be added

7.4 70 mL

7.6 60 mL

8.0 42 mL

Finally, adjust the volume of the solution to 1000 mL with dH_2O. Autoclave the solution in a liquid cycle to sterilize the solution. Store at 25°C to 27°C.

Note I: The addition of 12N HCl will generate heat and make the solution warm. Before making final pH adjustments, cool the solution to 25°C to 27°C.

Note II: If the color of the 1M solution appears to be slightly yellow in color, the quality of the Tris reagent is not good. Get rid of the solution and use higher-quality Tris reagent to make your solution.

Note III: Make sure the electrode you are using is suitable for determining the pH of Tris, as many types of electrodes are not able to determine the pH of Tris solutions accurately.

Note IV: The pH of Tris solutions varies inversely with the alterations in temperature, which reduces approximately 0.03 pH units for each 1°C rise in the temperature. Therefore, the pH of the Tris-buffered solutions should be determined at the temperature at which they will be used. Please note that it is advisable that Tris buffer must not be used below the pH of 7.2 or above the pH of 9.0 since the pKa of Tris is 8.08.

TE (Tris/EDTA) buffer

10 mM Tris-HCl, pH 7.4, 7.5, or 8.0 10 mL

1 mM EDTA, pH 8.0 2 mL

H_2O to 1 L

Combine 10 mL of 1M Tris-HCl buffer of desired pH with 2 mL of 0.5 M EDTA solution, followed by thorough mixing of the solutions in a magnetic stirrer to make up the final volume to 1000 mL with dH_2O. Usually, the pH of the Tris-HCl used to make the buffer dictates the pH of the TE buffer.

1M Tris.Acetate, pH 7.5

Trizma Base 121 g (0.02 M)

Glacial Acetic Acid 50 mL (0.017M)

H_2O to 500 mL

pH adjusted with glacial acetic acid

5% (W/V) Triton X-100

Triton X-100 5 mL

H_2O 95 mL

Mix 5 mL of Triton X-100 in 95 mL water. Store at 25°C to 27°C.

X-gal Solution 2% (W/V) (5-bromo 4-chloro 3-indolyl β-D galacto pyranoside)

Prepare a 10X stock solution of X-gal by dissolving 2 mg of X-gal powder in 10 mL of dimethylformamide. Store either in a glass or in a polypropylene tube. To protect its light-dependent lysis, protect the solution by wrapping the tube with aluminum foil. Dispense into smaller aliquots and store at -20°C. X-gal solution does not need to be sterilized.

1M Zinc Acetate dihydrate (M.W. 219.49)

Zinc Acetate dihydrate 21.9g

H_2O to 100 mL

1M $ZnCl_2$ (M.W.136.29)

$ZnCl_2$ 13.6 g

12M HCl 1 mL

H_2O to 100 mL

Dissolve 13.6 g of crystalline $ZnCl_2$ in 50mL of water followed by the addition of 1mL of 12M HCl to the resultant solution. Allow slow cooling and dissolution by standing the tube for 10-15 minutes at 25°C. Once the $ZnCl_2$ crystal has completely dissolved, make up the volume to 100 mL with dH_2O. Remember to always add concentrated acids to water and not otherwise.

70% (V/V) Ethanol (EtOH)

Absolute ethanol 70 mL

DH_2O 30 mL

Combine 70 mL of absolute EtOH with 30 mL of sterile dH_2O. It is not necessary to sterilize the EtOH solution. Store the solution at –20°C.

ACIDS AND BASES

10N Sodium Hydroxide (NaOH) (M.W. 40)

NaOH pellets 400 g

H_2O to 1 L

Preparation

In a heavy plastic beaker, add approximately 0.9 L of dH_2O. Weigh out 400 g of NaOH pellets and place them in the beaker, stirring the contents in a magnetic stirrer. After the complete dissolution of the NaOH pellets, bring the final volume to 1000 mL with dH_2O. Sterilization is not necessary. Store at room temperature.

NOTE: The addition of NaOH in water results in an exergonic reaction. Appropriate care should be exercised during the solubilization of NaOH to prevent the breakage of glass containers and avoid personal injury involving chemical burns. Therefore, it is wise to use a heavy plastic beaker. Alternatively, the beaker may be placed in an ice bath. Do not add H_2O to the NaOH pellets.

1N Hydrochloric Acid (HCl) (M.W. 36.5)

Mix in the following order:

DH_2O 913.8 mL

Concentrated HCl 86.2 mL

NOTE: Never add water to acid!

RECIPES FOR *ESCHERICHIA COLI* CULTURE MEDIA AND ANTIBIOTICS

Liquid media

Note that the pH for all nutrient media should be maintained within 7.0-7.2. Thus, after preparing any medium, if the resultant pH lies outside the above range, it must be adjusted with NaOH. Media should be sterilized by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

M9 Minimal Medium

Add the following components to 750 mL sterile autoclaved dH_2O, the

temperature of which is maintained within 45°C-50°C.

5x M9 Salts 200 mL

1M $MgSO_4$ 2 mL

20% Glucose 20 mL

1M $CaCl_2$ 0.1 mL

Sterile dH_2O to 980 mL

M9 Medium is sometimes supplemented with the stock solutions of appropriate vitamins and/or amino acids.

Composition of 5X M9 Salt Solution

$Na_2HPO_4 \bullet 7H_2O$ 64 g

KH_2PO_4 15 g

NaCl 2.5 g

NH_4Cl 5.0 g

Deionized water up to 1000 mL

Dispense into aliquots of 200 mL and sterilize by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

NOTE: Stock solutions of 1M $MgSO_4$, 1M $CaCl_2$, and 20% Glucose should be prepared separately and sterilized either by autoclaving in a liquid cycle for 20 minutes (for $MgSO_4$ and $CaCl_2$) or by filtration through 0.22 μm filter unit. These stock solutions, after preparation, are stored at 4°C and added to the 5x M9 slat solution right before use.

Luria-Bertani Medium (LB)

bacto-tryptone 10 g

bacto-yeast extract 5 g

NaCl 10 g

Deionized water Up to 1000 mL

Add the above components to 900 mL of dH_2O, followed by stirring in a magnetic stirrer to allow their complete dissolution. Adjust the pH of the suspension to 7.0 using 5N NaOH. Bring the volume of the solution to 1000 mL with dH_2O. Sterilize by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

2X YT

Tryptone 16 gm

Yeast extract 10 gm

NaCl 5 gm

Distilled water Up to 1000 mL

Add the above components to 900 mL of dH_2O, followed by stirring in a magnetic stirrer to allow their complete dissolution. Adjust the pH of the suspension to 7.0 using 5N NaOH. Bring the volume of the solution to 1000 mL with dH_2O. Sterilize by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

Terrific broth (TB)

Bacto-tryptone, 12 g

Bacto-yeast extract 24 g

Glycerol 4 mL

Distilled water Up to 900 mL

Add indicated amounts of bacto-tryptone, yeast extract powders, and a specified volume of glycerol to 900 mL of dH_2O and mix well. Sterilize by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min), allow it to cool down the temperature below 60°C, and then supplement with 100 mL of a sterile solution of 0.17M KH_2PO_4, 0.72 M K_2HPO_4.

The 0.17M KH_2PO_4, 0.72 M K_2HPO_4 solution can be prepared by combining 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 in 90 mL of dH_2O. Shake well to allow complete dissolution of the salts, followed by adjusting the volume of the resultant solution to 100 mL and sterilization by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

SOC Broth

Bacto-tryptone, 20 g

Bacto-yeast extract 5 g

NaCl 0.5 g

KCl 0.18g

Distilled water Up to 1000 mL

Add the above reagents to a glass beaker and add 900 mL of de-ionized water, followed by thorough mixing using a magnetic stirrer for their complete dissolution. Adjust the pH to 7.0 with 5N NaOH. Adjust the volume of the broth to 975 mL with de-ionized water. Autoclave for 20 min at 15 psi (1.05 kg/cm^2) on a liquid cycle. After Autoclaving, cool down the medium to room temperature under a laminar airflow chamber. Add 5 mL of 2M $MgCl_2$ and 20 mL of 1M Glucose stock solutions right before use and mix thoroughly.

Prepare stock solutions of 1M Glucose and 2M $MgCl_2$ separately, as stated in the previous section, and filter sterilize them by using a 0.22-micron membrane filter under a laminar airflow chamber. Store them at 4°C for long-term use.

SOLID MEDIA

For making solid media of the above compositions, the required amount of bacto-agar is usually added before autoclaving. All agar plates use a typical concentration of 15g per L.

ANTIBIOTICS

Since antibiotics are thermolabile, they cannot be sterilized by autoclaving. The stock solutions of all antibiotics, therefore, must be sterilized by filtration by passing their stock solution through a 0.22 μm filter and stored at -20°C. These reagents are then supplemented to the autoclaved media recipes right before use. Solid media containing agar, therefore, should be cooled to 45°C to 50°C before the antibiotic can be supplemented.

Ampicillin

Stock: 50 mg/mL in water

Working concentration: 50 μg/mL (plates); 25-50|μg/mL (broth)

For a stock solution of 50 mg/mL:

- 1 g Ampicillin
- 20 mL dH_2O
- Filter sterilize by passing through a 0.22 μm filter.

NOTE: Store the stock solutions of ampicillin at -20°C

Kanamycin

Stock: 10 mg/mL in water

Working concentration: 50 μg/ml (plates); 25-70 μg/mL (broth)

Streptomycin

Stock: 10 mg/mL in water

Working concentration: 50 μg/ml (plates); 25 μg/mL (broth)

Tetracycline

Stock: 5 mg/mL in ethanol

Working concentration: 50 μg/mL (plates); 25 (μg/mL (broth)

Tetracycline is photo-reactive, and therefore, solutions of tetracycline broths and plates containing this antibiotic should be protected from light either by wrapping them in aluminum foil or by storing them in the dark. Tetracycline is inhibited by Mg^{2+} ions.

This antibiotic does not need to be sterilized.

All the antibiotics dissolved in water should be filter sterilized by passing through 0.22 micron filter and stored at -20 degree C.

RECIPES FOR BUFFERS FOR RESTRICTION AND MODIFICATION ENZYMES

DNase-free RNase A

Reagents

Ribonuclease A (RNase A)

Solvent: 10 mM Tris-HCl, pH 7.5, 15 mM NaCl

Suspend 1 mg of lyophilized pancreatic RNase (RNase A) enzyme in 100 µL of 10 mM Tris-HCl, pH 7.5, and 15 mM NaCl to prepare a solution with a concentration of 10 mg/mL. Place the tube into a boiling water bath (100°C) for 15 min. This step will inactivate the pancreatic DNase I, which frequently contaminates the RNase A purified from the same source. Store the tube at room temperature to cool down the enzyme preparation slowly. Dispense into smaller aliquots and store them at -20°C.

Proteinase K (20 mg/mL)

Reagents

Proteinase K

50 mM Tris-HCl pH-8.0, 1.5 mM Calcium acetate

Add 20 mg of lyophilized proteinase K in 1.0 mL 50 mM Tris-HCl pH-8.0, 1.5 mM Calcium acetate. Dispense into 200-100 µl aliquots and store them at -20°C. 3. Use sterile pipet tips when removing the solution. Notably, Proteinase K is resistant to 1% SDS, 4 M urea, and a temperature up to 65°C.

Lysozyme (5 mg/mL)

Reagents

Hen Egg white Lysozyme

25 mM Tris-HCl, pH 8.0

Dissolve 5 mg hen egg white lysozyme in 1.0 mL of 25 mM Tris-HCl, pH 8.0, right before use. Note that lysozyme solution should always be freshly prepared before use.

GENERAL PROCEDURES

PROTOCOL A.4.1: PRECIPITATION OF DNA WITH ETHANOL

Reagents

3 M sodium acetate solution pH 5.3

Absolute ethanol (EtOH) (ice cold)

70% (v/v) ethanol (ice cold)

TE buffer pH 8.0 (***APPENDIX A.1.***)

DNA to be purified (≤1 mg/mL) in 0.1 to 0.4 mL volume

Method

- Measure the volume of the dilute DNA solution to be concentrated.
- Add 1/10th volume (relative to the original volume of dilute DNA) of 3 M sodium acetate pH 5.3 to a microfuge tube containing dilute DNA solution and mix thoroughly.
- Add 2.5 volumes (relative to the original volume of dilute DNA) of ice-cold EtOH (kept at -20°C) and mix well. Store the tube either at -70°C for 30 min or at -20°C for overnight.
- Centrifuge the tube at 15,000 g for 20 min at 4°C. Take the pellet and decant the supernatant.
- Add cold 70% (v/v) EtOH to the pellet and vortex vigorously to break the pellet.
- Centrifuge the microfuge tube at 15,000 g for 10 min at 4°C.
- Discard the supernatant carefully without disturbing the pellet. Note that the DNA pellet at this stage may attach very loosely to the wall of the tube. Care should be exercised while draining the supernatant of the tube. It is wise to pipette out the supernatant gently using a micropipette.
- Let the tube stand at 37°C incubator or on the bench top to allow the ethanol to dry the pellet. The pellet will turn transparent when all the ethanol evaporates.
- Resuspend the DNA pellet in sterile dH_2O or in sterile TE pH 8.0.

NOTE: Ethanol used for this procedure should be stored in the freezer. Ethanol stored at room temperature may cause high-molecular-weight genomic DNA to shear.

PROTOCOL A.4.2.: EXTRACTION OF DNA WITH PHENOL, CHLOROFORM, AND ISOAMYL ALCOHOL

Reagents

Phenol-chloroform-isoamyl alcohol mix (25:24:1, V/V) See ***APPENDIX A.1***

Chloroform-isoamyl alcohol (24:1 v/v)

Combine 24 parts of chloroform and 1 part of isoamyl alcohol and store in a capped bottle.

DNA in aqueous solution

Method

- Measure the volume of the DNA to be purified/deproteinized.
- Add an equal volume (relative to the original volume of the DNA solution) of phenol-chloroform- isoamyl alcohol (25:24:1v/v) to the DNA solution.
- Mix thoroughly the content of the tube by vortexing.

NOTE: The extent of mixing should depend on the nature of the DNA to be handled. While vigorous vortexing is recommended for low molecular weight smaller plasmids, care should be exercised when larger plasmid or high-molecular-weight genomic DNA is handled since vigorous vortexing would lead to physical shearing to those DNAs.

- Centrifuge the tube for 5 min at top speed at room temperature, leading to the formation of two separate phases along with the interface. The DNA would partition in the upper aqueous layer while the denatured proteins dissolved in phenol would stay in the lower organic phase.
- Carefully withdraw the upper layer to a clean tube without disturbing the interface.
- Repeat the steps 2 to 5.
- Add an equal volume of chloroform-isoamyl alcohol (24:1) to the sample.

Note that extraction with chloroform-Isoamyl alcohol removes any residual phenol that might have been present in the aqueous layer containing the DNA sample. This step is crucial, as the presence of phenol in the sample will hinder the downstream reactions.

- Mix thoroughly either by inverting the tube or by vortexing.
- Spin the tube at top speed for 5 min in a tabletop centrifuge at room temperature. Two separate phases will be visible along with the interface.
- Take out the upper aqueous phase and transfer it to a clean tube. Care should be taken to avoid disturbing the interface.
- To purify DNA, perform ethanol Precipitation as stated above (Protocol I).

NOTES 1: When working with samples of genomic DNA, vortexing should be avoided during phenol extraction. Instead, the DNA sample should be mixed with Phenol very gently to avoid shearing.

Preparation of Dialysis Membranes

Dialysis is a very widely used technique in biochemistry and molecular biology that involves the separation of molecules *via* diffusion through a semi-permeable membrane based on their size differences. As discussed in ***CHAPTER 5***, it is used

to purify DNA from agarose gel by a process called electroelution. One of the key elements in this process is the semi-permeable membrane (often dubbed dialysis membrane) that allows the passage of molecules of a certain size in a selective fashion. A wide variety of dialysis membranes with variable thicknesses and pore sizes are available nowadays, either as preformed ready-to-go tubing or dialysis sheets that require pre-treatment prior to use. Notably, pore size is the most important parameter that dictates "molecular weight cut off" (MWCO). It determines the size of the smallest particle that cannot penetrate the membrane of the dialysis bag and thus remains inside the bag. Appropriate knowledge of MWCO information helps the user select the appropriate dialysis bags for the separation of a particular macromolecule from others. For applications like electroelution (***CHAPTER 5 UNIT 5.6***), the dialysis bags are used directly after wetting and rinsing in dH_2O. However, during its manufacturing, the membranes might contain residual sulfides and traces of heavy metals, which need to be removed as they may interfere with the downstream process. The method typically used to pre-treat dialysis membranes is described below.

PROTOCOL A.4.3: PREPARATION OF DIALYSIS MEMBRANES

Reagents

10 mM sodium bicarbonate

10 mM Na_2EDTA, pH 8.0

20% to 50% (v/v) ethanol

Dialysis Membrane

Method

- The dialysis membranes are available as sheets or tubing, which is cut into smaller pieces of usable lengths (usually 8 to 12 inches in length) required for electroelution (***CHAPTER 5 UNIT 5.6***).

NOTE: Wear gloves while handling dialysis membrane as the direct contact of the membrane with skin/hand renders them susceptible to contamination with a number of cellulolytic microorganisms.

- Place the cut membranes in a glass beaker. Add an adequate amount of dH_2O to wet the membrane.
- Add 10 mM sodium bicarbonate in a large excess and boil it for several minutes.
- Pour off the 10 mM sodium bicarbonate solution and add 10 mM Na_2EDTA. Boil for several minutes (about 10 mins).

- Repeat the above step by adding fresh 10 mM Na_2EDTA.

The membranes can also be soaked in the above solution for 30 mins with some agitation. However, boiling improves the overall pre-treatment process.

Add distilled water to the beaker after removing 10 mM Na_2EDTA and thoroughly rinse the membrane with an ample amount of dH_2O.
Store at 4°C in 20% to 50% ethanol to prevent the growth of cellulolytic microorganisms. Ensure that the dialysis membranes always remain submerged and never become dried.

NOTE: Although ethanol is preferred as a storage medium for treated dialysis bags for ease and convenience, some experimenters prefer to use sodium azide and sodium cacodylate for their storage.

PROTOCOL A.4.4: QUANTITATION OF DOUBLE-STRANDED DNA USING ETHIDIUM BROMIDE

Apart from spectrophotometric determination of DNA, other methods have also been developed for quantification of DNA for those samples where either the concentration of the DNA is insufficient (<250 ng/mL) or the sample is contaminated with other molecules that may absorb UV radiation. Consequently, spectrophotometric determination will lead to inaccuracy associated with the quantification of these kinds of DNA samples. In such a case, the property of ethidium bromide to intercalate between the stacked DNA bases and its ability to fluoresce thereafter upon UV exposure is utilized. The amount of DNA present in the sample (typically about 1-5 ng) is assessed by comparing the fluorescent intensity of the individual sample with that of a series of standard DNA samples of known concentrations, assuming that the extent of fluorescence observed is directly proportional to the total mass of DNA.

Minigel Method: This method of determination is employed when the DNA preparation contains significant quantities of RNA, and it is a rapid and convenient procedure.

- Add 0.4 µl of bromophenol blue sucrose solution (see ***APPENDIX A.1***) to 2 µl of the unknown DNA sample and load the mix in the well of a 0.8% small gel prepared with ethidium bromide (0.5 µg/mL).
- Add 0.4 µl of bromophenol blue sucrose solution to each of the 2 µl of a series of DNA solutions of known concentrations (0, 2.5, 5, 10, 20, 30, 40, and 50 µg/mL) followed by their loading into the consecutive wells of the same gel.

NOTE: Ideally, the standard DNA sample should consist of a single species of

DNA having a closely similar molecular weight to that of the DNA sample of unknown concentration. Size comparison of an unknown DNA should always be carried out with a standard DNA whose size matches closely with an unknown sample.

- Electrophorese until the dye front migrated a few centimeters from the well.
- Destain the gel by immersing it in dH_2O containing 0.01 M $MgC1_2$ for 5 minutes.
- Place the gel on the trans-illuminator or gel documentation system, use short-wavelength UV irradiation, and take a photo of the gel. Determine the amount of DNA present in the sample via a comparison of the fluorescence intensity of the unknown DNA with that of the known DNA (standard DNA).

Saran wrap method using ethidium bromide: This is an alternative and fast method to determine the quantity of DNA in a sample, and it is carried out on a saran wrap.

- Place a Saran wrap neatly by stretching on the UV trans-illuminator.
- Spot 1-5 µl of the unknown DNA sample onto the surface of the wrap.
- Spot the same volumes of a series of standard DNA samples (0.1, 2.5, 5, 10, and 20 µg/mL) in an ordered array on the same wrap.

NOTE: Ideally, the standard DNA sample should consist of a single species of DNA having a closely similar molecular weight to that of the DNA sample of unknown concentration. Size comparison of an unknown DNA should always be carried out with a standard DNA whose size matches closely with an unknown sample.

- Add an equal volume of TE (pH 7.6) supplemented with 2 µg/mL ethidium bromide to each spot of unknown and known DNA samples, followed by their thorough mixing by pipetting up and down.
- Shine a short-wavelength UV onto these spots and subsequently determine the concentration of unknown DNA by comparing its fluorescence intensity with that of the standard solutions.

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Satarupa Das

Dr. Satarupa Das is a research scientist in Jadavpur University, Kolkata, India. She spent most of her scientific career in research laboratories both in the USA as well as in India. Her contribution to science are well portrayed in the scientific publication in peer reviewed journals. She graduated at the Bachelor's level with Zoology (major), Masters' level with Biophysics, Molecular Biology and Genetics from the University of Calcutta, and completed her Ph.D in Life Science from Jadavpur University India. Her passion for science evolved from her love for travel, exploring the secrets of life in nature. She is actively engaged in teaching at the post graduate level and training the Ph.D students in their research. Her life in science revolved mostly around the molecular biology laboratories for nearly thirty-two years that prompted her the need to write this book especially for beginners in this field.

Biswadip Das

Dr. Biswadip Das, born in 1965 in Kolkata, earned his B.Sc., M.Sc., and Ph.D. from the University of Calcutta in Biophysics and Molecular Biology. He did postdoc research in mRNA degradation at the University of Rochester and the University of Florida, USA. In 2010, he joined Jadavpur University, becoming a full Professor in 2015 and serving thrice as Department Chair.

He teaches advanced courses in Molecular Biology and Genetics and has published 45+ research papers and a book chapter with Springer. His work, funded by CSIR, DST, and DBT, focuses on nuclear mRNA degradation and gene regulation in disease.

Prof. Das reviews grant proposals for global agencies like the Wellcome Trust and has received the INSA Young Scientist Award. He's a Fellow of the Royal Society of Biology (UK), Indian Academy of Science, and West Bengal Academy of Science and Technology.