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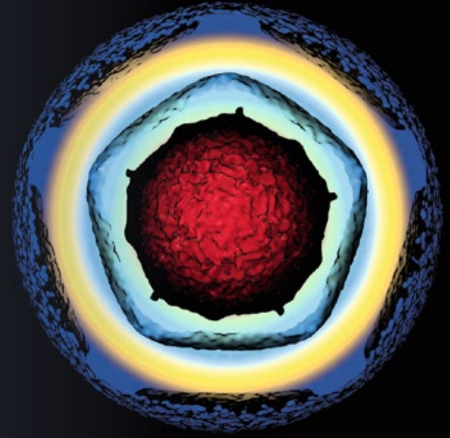

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Editor

African Swine Fever Virus

Methods and Protocols

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Methods in Molecular Biology

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African Swine Fever Virus

Methods and Protocols

Edited by

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Preface

African swine fever virus maintains a unique evolutionary and ecological position, infecting both mammalian and arthropod hosts. Since its emergence in the early twentieth century, the disease has presented challenges to pig farmers, policy makers, and scientists alike. Here, we present a compilation of methods, some refined over many decades, others developed more recently, that will hopefully aid researchers studying the virology, immunology, and vaccinology of this devastating disease of swine.

Woking, UK *Christopher L. Netherton*

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Chapter 1

Working Safely with African Swine Fever Virus

Sarah Gold, Anna Lawton, and Graeme Harkess

Abstract

Risk assessment is the cornerstone of working safely with biological agents. The World Health Organization (WHO) Laboratory Biosafety Manual Fourth Edition Monograph on Risk Assessment provides stepwise guidance for carrying out a risk assessment, from gathering information and identifying hazards to evaluating the risks and developing and implementing controls and review.

To support the development of a mature safety culture within laboratories, it is important that all staff who handle biological agents understand the process of risk assessment and receive training in identifying hazards and mitigating risk. All personnel can partake in risk assessments, and the guidance is written in such a way that it is applicable to all—not just to biosafety professionals, laboratory scientists, or facility managers.

Here we take the guidance from WHO and apply the principles of risk assessment to working with ASFV, illustrating the process using an example activity—the passage of low titer ASFV in cell culture. We discuss other techniques and protocols that you may need to consider when working with ASFV.

Key words Risk assessment, Safety, Risk, Tolerability, Hazard, Risk matrix, Biosafety

1 Introduction

African swine fever virus (ASFV) is a transboundary disease that threatens to spread to countries which have previously never had infections [1]. An outbreak which began in Georgia in 2007 has continued to spread to other countries in Europe such as Poland, Hungary, Romania, and Bulgaria [2]. The virus has now spread into Asia with the first outbreak in China in 2018 [2, 3]. Many countries have concerns that the virus could be imported along with contaminated feed or meat products, in which the virus can survive for long periods [4]. Public awareness campaigns have been implemented to make travelers aware of the risks of bringing in meat products to countries which are disease free [5]. International organizations such as the Food and Agriculture Organization of the United Nations (FAO) are providing recommendations to governments in affected regions on how to apply strict biosecurity

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measures including advice to farmers to improve cleaning on farms, prohibit the feeding of swill to livestock where this is still common practice, and ensure proper disposal of food waste which may contain uncooked pork products [6, 7].

ASFV is consistently ranked worldwide as a high-hazard pathogen. Countries have traditionally classified pathogens from 1 to 4 (usually with 1 being lowest risk to 4 being highest risk) as described in the World Health Organization (WHO), Laboratory Biosafety Manual (LBM), 3rd Edition [8].

In the UK, under the Specified Animal Pathogens Order 2008 (SAPO) Regulations [9], ASFV is classified as a SAPO4 pathogen (the highest classification of risk) due to it being exotic to the UK; the severity of disease in naïve pigs; the lack of vaccine; the threat of incursion from Europe into wild boar populations; and the possibility of severe economic damage an outbreak may cause [1, 2, 10, 11]. SAPO4 pathogens require handling in secure containment level 4 (CL4) facilities [12] under specific license of the national competent authority and government.

The OIE Terrestrial Manual states in Chap. 3.9.1 African swine fever (infection with African swine fever virus) that ASFV should be handled with an appropriate level of biocontainment, determined by risk analysis in accordance with Chap. 1.1.4 Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities [13]. Due to the transmissibility of ASFV, and its potential for spread across national borders, it is one of the 117 OIE-Listed diseases with cases of the virus being notifiable to the OIE [14]. Worldwide, facilities are generally classified by containment level (CL), biosafety level (BSL), or physical containment level (PC). These containment levels are generally (but not always specifically) comparable, i.e., a CL4 facility in the UK may have largely equivalent containment measures to a BSL4 facility in the USA, when the same pathogens are being handled. However, this can lead to confusion; for example, in the UK, the virus is listed as SAPO4 (highest level of animal pathogen) and handled within a CL4 facility [9]. In other countries, where the virus may be endemic, risk analysis may allow for the handling at lower levels of containment. Some countries handle ASFV virus at BSL3 such as Spain at the Center for Animal Health Research (CISA) which is the European Union Reference Laboratory [15], while the USA recommends handling the virus at BSL3 with additional controls of up to ABSL3-Ag for in vivo work with some strains [16]. In Germany at Friedrich-Loeffler-Institut, the virus is handled in BSL4 facilities and on an island away from the mainland [17]. Some countries, such as Australia at the Australian Centre for Disease Preparedness (ACDP), do not handle the virus but maintain surveillance to test imported pork products in PC3 laboratories [18, 19].

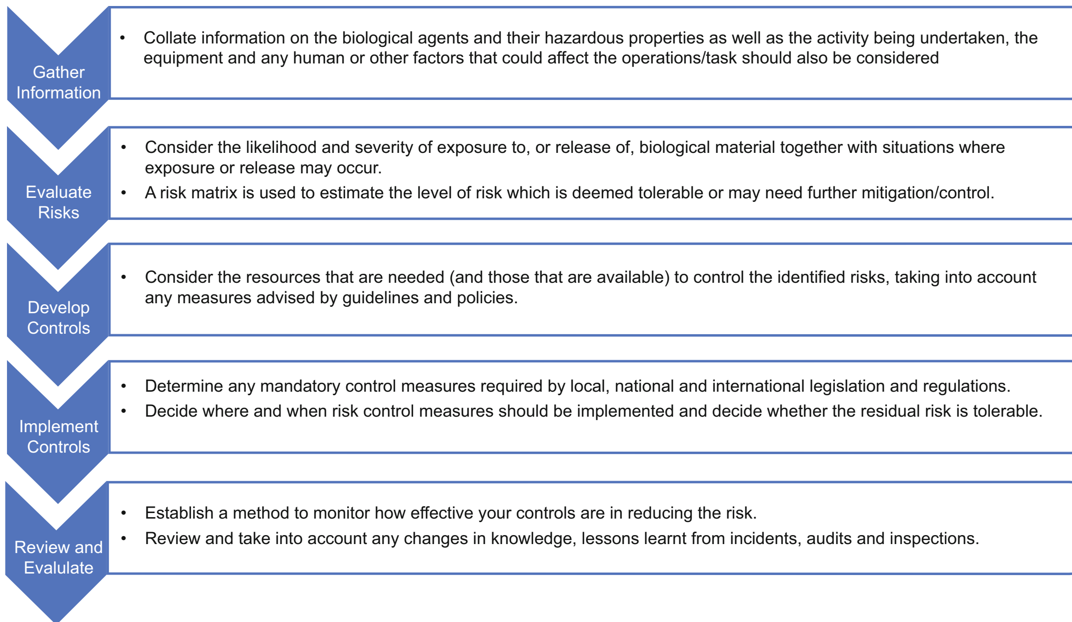

Fig. 1 Flow diagram to show the five steps in the World Health Organization laboratory biosafety manual risk assessment process

To harmonize an approach to working with biological agents worldwide, the WHO released its fourth edition of the Laboratory Biosafety Manual (LBM4) in 2020, in which it no longer classifies pathogens from 1 to 4 but instead promotes a risk-based approach for each pathogen and the related task being undertaken with that pathogen [20]. The manual provides stepwise guidance in carrying out thorough risk assessment for laboratory work with biological agents. The guidance provided is intended for use not only by biosafety professionals, laboratory scientists, or facility managers but also for all personnel who handle biological agents, thereby enabling all personnel in an organization to contribute to assessing the risk of the work and to improve safety culture.

The risk assessment process has five steps, shown in Fig. 1, which are cyclical. This process is based upon Plan, Do, Check, Act methodology [21].

2 Materials

1. WHO Laboratory Biosafety Manual (LBM), 4th Edition.
2. WHO Laboratory Biosafety Manual (LBM), 4th Edition, Risk Assessment Monograph.
3. Risk assessment template.

4. Knowledge of the pathogen properties.
5. Knowledge of or written method for the task to be undertaken.

3 Methods

3.1 Example Risk Assessment for Infecting Cell Culture with ASFV

The process of producing a risk assessment for working with ASFV is demonstrated in this chapter. For ease, a simple, repetitive task which is applicable to many pathogens handled within containment laboratories has been selected. The considerations detailed here may be used for ASFV as well as other pathogens with similar hazards.

A longer template is provided in Annex 2 of WHO LBM4 [20], which has more detailed instructions that assist in breaking down each step and considers each property of the pathogen which may be a hazard (it may be helpful to use the annex in conjunction with this chapter to provide more detail on the process).

This worked example begins with a short protocol of infecting a cell culture within a 175 cm^2 tissue culture flask with ASFV.

1. Prepare a Class 2 Biological Safety Cabinet (BSC) with absorbent material and validated disinfectant.
2. Collect a flask of cells with cell density of 70–80%.
3. Remove a stock virus samples from locked cold storage.
4. Transfer sample vial, to virus laboratory across an open corridor.
5. Defrost unopened sample in the BSC.
6. Briefly centrifuge the virus sample in a benchtop microfuge to ensure no droplets are in the lid.
7. Remove growth medium and add 20-mL reduced serum medium.
8. Add virus inoculum and mix gently.
9. Transfer the labeled culture flask a 37 °C incubator on a bunded tray or contained within a plastic box.
10. Incubate at 37 °C until level of cytopathic effect required is reached.
11. Disinfect liquid waste produced by adding to 2× concentration validated disinfectant (for the required contact time) before discarding to drain via an effluent treatment plant (ETP) (for heat inactivation following a validated process).
12. Disinfect plastic waste produced and dispose via autoclave.

3.2 Hazard Identification

A key part to any risk assessment is gathering information on the hazards posed by the activity being planned. A hazard is defined as “anything that may cause harm,” which may be harm to an

individual; damage to property; or harm to the environment. Almost anything could be seen as a hazard in one way or another, so part of the formal hazard identification process for a risk assessment requires that discretion and professional expertise are used to limit the number of hazards to those capable of causing the most harm and those that will need controlling.

In the above activity, one of the first hazards identified is the handling of ASFV. Information gathering is focused on the things about the virus that may cause harm and information that could be used to mitigate that harm, for example, key information we want to record may include [2, 10, 22].

- ASF causes severe diseases in domestic pigs and persistently infects its natural hosts, warthogs, bushpigs, and soft ticks of the genus *Ornithodoros*, but does not cause disease in humans.
- Virulence of isolates varies greatly.
- Virus is heat inactivated at 60 °C/20 min.
- Virus is inactivated at pH <3.9 or >11.5 in serum-free medium.
- Virus is inactivated by 8/1000 sodium hydroxide (30 min contact time), hypochlorites – between 0.03% and 0.5% chlorine (30 min), 3/1000 formalin (30 min), 3% ortho-phenylphenol (30 min), and iodine compounds.
- Virus can remain viable in blood, feces, and tissues for extended periods of time.
- Can multiply in the tick vector (*see* **Note 1**).
- No vaccines are currently available.

Next, consider the laboratory procedures that will be used for the activity outlined, again focusing on those that have the potential to cause harm (to people, property, or the environment) and any mitigations.

- Transferring of virus from cold storage to laboratory.
- Disinfecting via waste collection vessels.
- Inoculation of cell cultures in standard tissue culture flasks (*see* **Note 2**).
- Using a centrifuge – low speed in microfuge (*see* **Note 3**).
- Transferring cultures to incubator.
- Inactivation of liquid waste and disinfection of plastic waste (*see* **Note 4**).
- Whether the work involves in vivo studies (*see* **Notes 1** and **5**).

At this stage take into account how frequently the activity is performed and consider competency of staff.

These procedures can be broken down into types of equipment that might be needed, including:

- Personal protective equipment (PPE) suitable for the activity.
- Centrifuges.
- Autoclaves.
- Biological Safety Cabinets (BSC).
- Secure cold storage.
- Drop-tested transport containers.
- Microfuge with biosafe rotor lid.
- Standard pipette with filter tips.
- Laboratory plasticware.
- CO_2 incubators with bunded trays.
- Waste sterilized via autoclaving to leave containment area/ building.

Finally, look at the type and condition of the facility the work will be conducted in. This may vary greatly depending on where in the world the work is being carried out, what the national regulatory requirements are for handling and working with the pathogen, and if the disease is already endemic in that country. However, wherever the facility is based, the same basic questions can be asked. These will revolve around the barrier areas of the facility, i.e., how you move things (including people, air, waste, and equipment) into and out of the facility safely (*see* **Note 6**). For example:

- Facility: Is the facility already licensed by the country's competent authority to work with the level of pathogen? Is it a new facility or an established one?
- Air: Does the facility operate at pressure negative to the surrounding area? Does the facility have air handling units to provide directional airflow? Is there an air pressure cascade through the facility? Are there airlocks between spaces?
- Barrier systems: Does the facility have established barrier procedures, including barrier showers, fumigation lobbies, and barrier autoclaves, to allow the safe movement of people, waste, and equipment out of the facility?
- Waste: Are there validated processes in place for the safe removal of waste? How many different types of waste are being generated? Liquid, solid, high-risk, clinical? Is an ETP in place? (*see* **Note 7**)
- Security controls: Is there a system to prevent unauthorized access to the pathogens handled? Is it based on a layered security system (access control, CCTV, secure storage, personnel)?

3.3 Evaluate the Risks

Once the hazards have been considered, the next stage is to evaluate the risk each hazard poses and decide whether this risk is something that can be tolerated by those carrying out the activity.

Table 1
A simple risk matrix. To find the overall risk, make an assessment on the likelihood of the hazard causing harm and the severity of that harm (the consequence) and multiply to get the overall “risk.” Usually, a green risk will be considered acceptable and red unacceptable, and yellow would require further thought before the work could begin

<table>
<tr><td colspan="2" rowspan="2"></td><td colspan="5">Likelihood of harm</td></tr>
<tr><td colspan="2">Unlikely</td><td>Possible</td><td colspan="2">Likely</td></tr>
<tr><td rowspan="3">Consequences of exposure/release</td><td>Severe</td><td colspan="2">Medium</td><td>High</td><td colspan="2">Very High</td></tr>
<tr><td>Moderate</td><td colspan="2">Low</td><td>Medium</td><td colspan="2">High</td></tr>
<tr><td>Negligible</td><td colspan="2">Very low</td><td>Low</td><td colspan="2">Medium</td></tr>
<tr><td colspan="2">Select the overall initial risk.</td><td>Very low</td><td>Low</td><td>Medium</td><td>High</td><td>Very high</td></tr>
</table>

Risk is defined as being a function of the likelihood that a hazard may be realized (how likely is it that the hazard will cause harm?) and the subsequent severity of that harm. This function is usually displayed as a “risk matrix,” a simple example of which is shown in Table 1. Each facility will have its own criteria for how to assess risk, and national competent authorities and regulatory bodies in a country will also have their own expectations.

3.3.1 Evaluating the Risk of Individual Steps of an Activity

In order to assess the risk posed by each hazard, start by looking at the information gathered on the hazards including the process, the equipment, and the facility where the work is being carried out.

Step 4 of the simple activity above (*Transfer sample vial to virus laboratory across an open corridor*) will be used as an example but the same process applies to all steps.

A good starting point would be to assess the risk of handling the virus as this is likely to be the most significant hazard. Using the information gathered above, the major harm that may be caused would be an outbreak of the virus within a susceptible species. Other information relevant at this point includes the lack of a vaccine, and the virus remains viable in blood, feces, and tissues; however, a vector is needed to transmit the virus. Also consider other aspects, i.e.: How big is the sample? Are there multiple samples? Are the samples high-titer virus?

Considering the procedure for moving materials across open corridors: Is there an established process already in place? Is the corridor even and uncluttered? How far do you need to go to move the material? (*see* **Note 8**).

Questions to consider when assessing the risk from the equipment: Is the vial made of glass? Or is it plastic? Has the vial been drop-tested to show it can withstand being dropped onto the floor surface?

Looking at the facility: Is it within a containment facility? Is there air handling with filters on supply and extract? Are the walls and floors sealed? Is the facility secure from non-approved staff/visitor access?

Using the answers about the hazards obtained from the information gathered at the start of the process, it is now possible to evaluate how likely it might be that the activity could cause harm. Using certain assumptions for this example, the work is being carried out in a secure containment building with air handing providing negative air pressure to the external environment, samples are being transferred in 20 glass vials of a 5 mL of pig serum from animals infected with ASFV, and the samples need to be moved between two labs on different floors, so it will need to be transported up a flight of stairs.

The likelihood that one or more glass vials could be dropped onto the floor over such a distance is "possible" (it's a long distance, and moving up a flight of stairs), and the likelihood of the vials smashing on the hard floor would be "likely." The risk of harm to the environment (i.e., the likelihood the virus could get out of the containment space) would depend on many factors, including barrier procedures and PPE (could the person carrying the vials become contaminated and inadvertently take the virus out of the building on their person?). In our case, if it is assumed for this example that there is PPE being worn, there is a barrier shower, and the staff are under quarantine restrictions preventing them having close contact with pigs after leaving the lab area, then it could be argued that the likelihood of harm to the environment is low.

Using all this information, a decision can be made on the likelihood of harm being done (remembering that this part is subjective and requires a level of competence in the process to make an informed judgment). Part of this decision requires a decision to be made on what "harm" would look like. Harm could be defined as finding an infection in the local pig population or virus containing material being outside of containment, or "out of positive control." This is a local decision, but for this example, the harm will be identified as virus "escaping" the containment area.

The severity (or consequence) of this event may also be measured in a number of ways by asking what would be the worst-case scenario? It could be a local outbreak of ASFV. In this

case the severity (or consequence) could be severe, making the overall risk *high* (using the matrix in Table 1). However, it could be argued that if this event occurred there might not be an outbreak, this would reduce the severity to negligible (overall risk therefore being *low*).

However, it is not difficult to imagine that if reports of this event were made public, local residents may start feeling uncomfortable having a facility of this type close to them, and this could affect the reputation of the facility. Even without an outbreak directly attributed to this event, it is foreseeable that there would still be consequences; therefore, it may be appropriate to class the severity as *moderate*.

3.4 Select and Implement Risk Control Measures

Once the risk control strategy has been developed, control measures must be selected and implemented to adequately control the risks associated with each activity.

Firstly, it is important to consider any control measures that are predetermined by local, national, or international regulations or guidelines and ensure that these are implemented.

Next, describe the control measures to be used for each activity. In this example, control measures may include:

- Containing the virus in multiple layers of packaging when transferring from cold storage to the laboratory.
- Using a centrifuge with a sealed rotor lid and using the lowest speed possible to minimize the formation of aerosols during the centrifugation process. This step may include further mitigations by requiring that post centrifugation, buckets are taken to the BSC to be opened.
- Inactivation of liquid waste using validated chemical disinfection followed by heat treatment.

It must be noted that where chemical disinfectants (or other inactivation procedures such as heat treatment) are to be used, they must be validated for the pathogen and the conditions of use. In the UK, iodophor compounds are used for ASFV due to their broad-spectrum activity, which is useful when ASFV is being handled in the same space as other pathogens. Iodophors are also generally less corrosive than other disinfectants (such as hypochlorites) and so are more compatible with the BSCs and drainage systems in the facilities (something to consider when choosing a disinfectant).

After selecting control measures to reduce the risk, it is important to review the activity and evaluate the residual risk that remains (using the matrix in Table 1) to determine whether the risk has been reduced to a tolerable level and work can proceed. If the residual risk is still unacceptable (according to the risk matrix), further action may be necessary to reduce the risk to a tolerable

level. This may include redefining the scope of the work so that it can be done with existing control measures or using an alternative laboratory that has control measures already in place. In this example, it may be decided that the work will take place in a laboratory containing all the equipment required for the entire process, to avoid the need to move infectious material between different laboratories to access different pieces of equipment. It is important to note that it is impossible to remove all risk from an activity, and that, dependent on resources and risks specific to a facility and country, different levels of residual risk may be tolerated. It is essential that each facility sets its own tolerance level to risk taking its specific circumstances into account.

To ensure consistency and provide assurance, it is good practice (and in some countries a legal requirement) that the risk assessment is approved and signed by a competent person or committee, such as the Biological Safety Officer or a Biological Safety Committee. There must then be a mechanism for communicating the hazards, risks, and risk control measures to relevant personnel and for recording that this has been done. Providing personnel with paper copies of the risk assessment is acceptable; however, many organizations use a quality management system (QMS) for this purpose to ensure consistency of approach (particularly important when a facility has a large number of risk assessments across a range of activities).

All control measures identified by the risk assessment must be in place before work commences; therefore, at this stage there must be a defined process and timeline for implementing any control measures that are not already in place.

Finally, written operational and maintenance procedures should be prepared for any equipment to ensure it is kept functional and working as intended and allows users to be properly trained in using the equipment before starting work in the laboratory. Such training should be recorded, either via a paper form (training record) or electronically (e.g., via the QMS).

3.5 Review Risks and Risk Control Measures

A risk assessment must be regularly reviewed and revised where necessary, taking into account new information about the pathogen, changes in laboratory activities or the equipment in use, and any new control measures that need to be implemented.

The frequency of the review will be based on the level of risk. An annual review is typical; however some situations may prompt a review sooner than this. In the example presented here, the review would take place sooner if the following situations arose:

- An accident or incident involving ASFV that happened during one of the risk assessed activities above.
- Changes to personnel or equipment used in the activities, or changes to the laboratory where ASFV is handled.

- Introduction of new technology or processes such as automated liquid handling.
- Changes to local, national, or international regulations or guidelines that apply to work with ASFV.
- Feedback from users or findings from audits and inspections.

The review can be carried out by one designated person, or via a committee or other group. Any review should involve a subject matter expert, as well as the personnel who are doing the work as they will be most familiar with the nuances of each activity.

Following the review, a process, timeline, and owner must be defined for the implementation of any changes or additional control measures. These changes should be reflected in the risk assessment, which may undergo many versions and updates during its life span. It is important therefore to have a system to allow for these changes to be communicated to users as they are made.

4 Notes: Other Considerations for Working with Pathogens

The worked example risk assessment above detailed a simple process of infection of cell culture with a low titer sample. However, many other laboratory procedures with pathogens need to be considered and risk assessments amended, for example, high-speed centrifugation or working with higher titer virus. Another example is working with a larger volume of virus culture; although the hazard may be the same, the risk may be different depending on the laboratory in which it is handled or the disease status of the location. Therefore, different mitigations may be required. The following list presents a selection of possible hazards and the controls that may be suitable in a particular situation:

1. Vector studies: Ensure laboratories are insect proofed and monitoring procedures for escaped insects are in place.
2. Large volume cultures: Use a laboratory with a lobby, employ additional trained personnel to assist with waste management, and ensure robust spill response procedures are in place.
3. Hi-speed or ultracentrifugation: Ensure equipment is maintained (including rotors and buckets, O-rings on lids) and serviced and the procedure is carried out by trained personnel; consider the procedure to reduce spills by using sealed centrifuge vessels; and consider how to clean up any spills within centrifuge buckets, i.e., locate a benchtop autoclave within the same laboratory.
4. High titer cultures: Use a laboratory with a lobby, consider the procedure to reduce movement of samples, and reduce access to laboratory to minimum required to carry out task.

5. Large animal studies: Carry out in specialist high containment animal units by staff trained to the standards set by the national competent authority; document procedures for traceability of samples from collection to the designated testing laboratory.
6. Fomites: All items leaving the laboratory to be disinfected with validated disinfectant; items leaving the containment area must be sterilized via validated procedures such as dunk tank, fumigation, autoclave, or heat treatment of liquid waste.
7. Inactivation of samples for use in lower containment laboratories: In facilities with virus and non-virus handling laboratories, validated and approved methods of inactivation should be in place for removal of samples to "lower containment." Example methods include heat treatment; generation of protein gel samples via heat and the addition of SDS or fixation by formaldehyde.
8. Transfer of samples to other facilities within the country or international: A documented approvals procedure with letter of authority from the receiving laboratory confirming permission/ability to handle the pathogen. Trained staff to package the samples to the standards set out by national or international competent authorities. Vetting of approved commercial couriers dedicated to transporting dangerous goods on behalf of your facility.

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Chapter 2

Necropsy Procedures and Evaluation of Macroscopic Lesions of Pigs Infected with African Swine Fever Virus

Pedro J. Sánchez-Cordón, Fabian Lean, Matthieu Bernard, and Alejandro Núñez

Abstract

Pathology complements and provides a fundamental link to other disciplines for disease investigations supporting molecular biology, genetics, immunology, or virology as core basis of scientific research. Necropsies are an essential tool in veterinary pathology for disease investigation and should be conducted in a routine, systematic, and standard approach. An orderly necropsy procedure will allow the prosector (veterinary clinicians or veterinary pathologists) to determine macroscopically normal or altered structures and allow, through experience, to acquire dexterity, speed, and confidence in the technique. In conjunction with standardized macroscopic scoring protocols, necropsy is a powerful tool especially when using experimental animal models in research. Here, we describe a systematic necropsy protocol to be conducted on pigs infected with African swine fever virus (ASFV). The methodology described only requires rudimentary instruments, and it is not time-consuming. In addition to performing accurate tissue and organ assessment, the technique intends the prosector to carry out sampling of organs and tissues of interest in ASFV-infected pigs.

Key words Necropsy, Pathology, Macroscopic lesions, Pig, African swine fever virus

1 Introduction

Pathology evaluation is a vital first step to enable veterinarians and scientists to understand the mechanisms of disease, interconnecting and complementing other disciplines applied to disease investigation that constitute the fundamental scientific research such as hypothesizing for specific molecular biology, genetic, immunological, or virological questions [1]. The field of pathology offers a plethora of tools that range from basic macroscopic, histopathological, or ultrastructural studies to detailed in situ structural, immunological, or molecular processes, with the advantage of correlating changes that can be appreciated visually and molecular damage, a virtue that other disciplines lack.

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Pathology has contributed enormously to understanding the pathogenic mechanisms and the characterization of the clinico-pathological presentation of African swine fever (ASF) since the disease was first described in Kenya one century ago [2, 3]. ASF is caused by a complex DNA arbovirus, the ASF virus (ASFV), only member of the *Asfarviridae* family, that affects domestic pigs and wild boar (all *Sus scrofa* species) and against which there are not commercial vaccines or treatments available [4, 5]. The acute and subacute forms of ASF, caused by highly and moderately virulent ASFV isolates respectively, result in severe and extensive hemorrhages and edema. On the other hand, subclinical and chronic forms of ASF caused by low virulent ASFV isolates are characterized by the absence of fluid extravasation into body cavities and macroscopic hemorrhagic lesions [3, 6]. The use of pathology has been indispensable in the field especially to establish a preliminary diagnosis given the unambiguity of lesions between acute/subacute versus chronic forms.

Since the incursion of ASFV genotype II into Georgia in 2007, the disease has spread without control first throughout Europe and then, since 2018, throughout Asia [7]. The devastating socioeconomic effects have placed great impact on both poor households, in which pigs constitute the major source of protein, and big pork commercial corporations. Despite the research efforts carried out over the last years and the new information generated, wide gaps in knowledge remain on the molecular mechanisms of ASFV transcription, gene expression patterns, or immunological mechanisms involved in protection, in which these information are critical for the design of suitable antiviral drugs and vaccines [8].

A necropsy is the examination of the carcass of an animal after its death, with the aim of providing evaluation of the degree of anatomic structural alterations or lesions caused by damaging agents, including pathogens such as ASFV [9]. Necropsy is an essential tool in veterinary pathology that should be conducted in a systematic manner to enable differentiation of normal and damaged structures. During necropsy, experienced prosectors may set up macroscopic scoring protocols, converting observations into semi-quantitative values on the extent or severity of injuries. Currently different scoring systems to perform macroscopic assessments of ASFV-inoculated pigs have been proposed [10–12]. Such combination of routine necropsy techniques with standardized macroscopic scoring protocols is particularly useful in research with experimental animals, especially to measure the success of preventative of therapeutic interventions. Along with standard protocols to evaluate virological parameters and immunological responses, scientists involved in ASFV studies, such as new vaccines or antiviral treatments development, characterization of new isolates, or pathogenesis studies, would benefit

from applying such metrics during pathological evaluations to allow meaningful individual, intergroup, and inter-experiment comparison.

Here, we describe a systematic necropsy protocol on pig infected with African swine fever virus (ASFV). The methodology described only requires rudimentary instruments, and it is not time-consuming. In addition to performing accurate tissue assessment, the technique intends the prosector to carry out sampling of organs and tissues of interest in ASFV-infected pigs.

2 Materials

The most important tool required to perform a necropsy is a sharp knife or a scalpel. However, there are other basic surgical instruments and equipment that will facilitate the procedures required to dissect a carcass. In addition, other materials and devices are required to enable sampling of bodily fluids and tissues as well as to examine and record the macroscopic findings. During the necropsy, wearing basic personal protection equipment is also recommended. Disinfectants are also important for decontamination of the necropsy station and to avoid the release and persistence of ASF in the environment given its stability.

2.1 Personal Protection Equipment

1. Wellington boots.
2. Overalls.
3. Boiler suits.
4. Gloves.
5. Cut-proof gloves.
6. Masks.
7. Goggles or face shields for eye protection.

2.2 Equipment and Tools to Carry Out the Necropsy

1. Height-adjustable stainless steel necropsy table.
2. Auxiliary stainless-steel tables.
3. Hoist to move the carcasses.
4. Knives to remove skin and bones.
5. Disposable scalpels.
6. Forceps.
7. Round tip scissors.
8. Bone cutter to cut through ribs and bones.
9. Head bench vice to secure the head during its dissection and exposure of nasal and cranium.
10. Saw, small axe or cleaver to split the skull.

Fig. 1 Tools, materials, and devices to carry out the necropsy, collect bodily fluids and tissues, and record the macroscopic lesions

11. Hammer, hammer mason's chisel or large screwdriver to facilitate removing the cap of the cranial vault and brain removal (see Fig. 1).

2.3 Material to Collect Bodily Fluids and Tissues

Sterile material and containers for sample fluids and fresh tissue for virological, bacteriological, molecular, or immunological studies as required. The following are examples.

1. Specimen jars or pots.
2. Petri dishes.
3. Dry swabs.
4. Swabs in media.
5. Blood collection tubes with and without anticoagulants.
6. Needles and syringes.
7. Containers with specific fluids or tissue storage reagents.
8. Small and large containers with fixatives solutions to fix samples for histopathological and immunohistochemical studies. Among the most extensively used are 4% formalin solutions, Bouin's solution, or zinc fixative (see Fig. 1).
9. Portable refrigerator to keep samples at 4 °C.

2.4 Material and Devices to Examine and Record the Macroscopic Lesions

1. Macroscopic lesions scoring sheets, permanent markers, pens, and pencils (*see* **Note 1**).
2. Craft cutting non-slip mat for placement of organs upon removal from carcass.
3. Digital camera (see Fig. 1).

3 Methods

There are different necropsy procedures, equally useful and practical, used routinely by veterinary practitioners and veterinary pathologists. This protocol describes the procedure for conducting a necropsy on an ASFV-infected pig in an easy and orderly way.

During experimental infections with ASFV, animals may be euthanized at pre-established time points or once they have reached a humane endpoint. After euthanasia, the animals should be weighted and then exsanguinated. Exsanguination will allow a cleaner necropsy and enable an easier and accurate evaluation of the size and weight of the organs as well as for the identification and assessment of macroscopic lesions. In those cases in which animals are found dead, necropsy should be carried out as soon as possible in order to minimize postmortem changes and autolysis which may hinder the macroscopic identification and evaluation of lesions.

3.1 External Examination

Before opening the carcass, perform a complete external examination paying special attention to:

1. Body condition (Fig. 2a, b): prominence of vertebrae, ribs, and bony protuberances (*see* **Note 2**).
2. Eyes, eyelids, and ocular mucosa (Fig. 2c, f): assess the degree of reddening of the ocular mucosa (congestion), the presence of hemorrhages, the swelling of eyelids, and the characteristics and the volume of eye discharge (from clear to cloudy) (*see* **Note 3**).
3. Nostrils and oral cavity (Fig. 2d, f, g): evaluate the nature (seromucous, mucous, or mucopurulent) and severity of nasal discharges as well as the presence and severity of nasal hemorrhages (epistaxis). Also, assess the presence of foamy material in the nostril and mouth (*see* **Note 4**).
4. Anus, perianal area, and hindlimbs (Fig. 2e, h, j, l): examine for soiling and fecal encrusting around the rears, hocks, and tail, which could be indicative of poor consistency of stools or diarrhea as well as the presence of trace of blood in feces (melena). The severity and nature of diarrhea can be influenced by hydration and body condition (*see* **Note 5**).

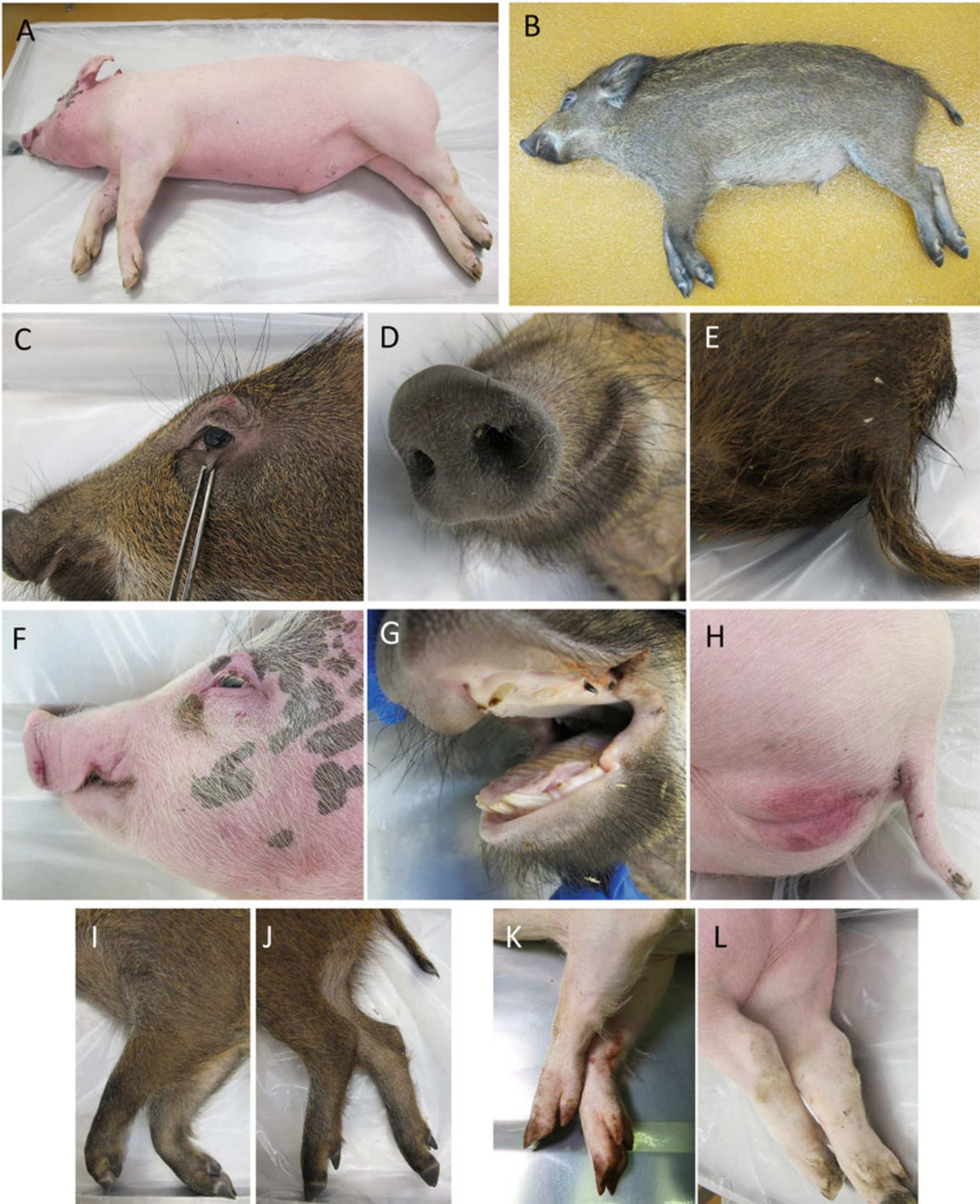

Fig. 2 Before dissecting the carcass (domestic pig or wild boar), perform a complete external examination paying special attention to the body condition and skin (**a**, **b**); eyes, eyelids, and ocular mucosa (**c**, **f**); nostril and oral cavity (**d**, **g**); anus, perianal area, and hindlimbs (**h**, **j**, **l**); joints in forelimbs (**i**, **k**) and hindlimbs (**j**, **l**)

5. Skin (Figs. 2a, b and 3a–d): Evaluate the presence of skin lesions considering the shape, size, color, and distribution (focal, multifocal, diffuse, etc.). Assess hydration by checking the suppleness of the skin and subcutaneous tissues. Special attention should be paid to erythematous, cyanotic, or hemorrhagic lesions, as well as possible presence of foci of necrosis (*see* **Note 6**).
6. Once external evaluation is completed, samples from skin lesions may be collected into sterile containers for bacteriological or virological investigations (virus detection by PCR or virus isolation) or in 4% formalin for histopathological and immunohistochemical studies. Exudates/fluids from the eyes, nostril, mouth, or rectum can also be collected into sterile containers following swabbing for bacteriological or virological studies.
7. During the external examination, joints must be also evaluated to determine the presence of joint swelling (Fig. 2i–l). At this point, joint fluid can be collected using sterile syringes and needles by puncturing through the capsule and inserting the needle between the joint surfaces (arthrocentesis). Alternatively, cut the major joints in the forelimbs (shoulder, elbow, carpometacarpal, and metacarpophalangeal joints) and hindlimbs (hip, stifle, hock, and metatarsophalangeal joints) to collect fluid and visualize the synovial membranes and articular surfaces. To do this first bend the joint and incise the skin with a knife (Fig. 3e). Then, using a sterile scalpel, cut the joint capsule and ligaments and examine the joint surface, the quality and quantity of the synovial fluid, the capsule, as well as the skin and subcutaneous tissue around the joint (Fig. 3f–i). Tissue samples (articular surface, bones, joint capsule, ligaments, skin, and subcutaneous tissue around the joint) can be collected for virological, bacteriological, or histopathological studies (*see* **Note 7**).
8. To evaluate and sample bone marrow, large bones like the femur are the best option. Remove the musculature and soft tissues around the femur (Fig. 3j), release the bone from the hip and knee joint by cutting the articular ligaments, and, using a bone cutter or a saw, fracture the bone obliquely (Fig. 3k) and sample the bone marrow (Fig. 3l, m).

3.2 Carcass Positioning and Initial Incision for the Evaluation of Subcutaneous Areas and Musculature

1. Before dissecting the carcass, position the animal for initial incision. Monogastrics, including pigs, are usually placed with their right side down, which eases the evaluation of more visceral organs in their natural positions. The left forelimb is lifted at right angles to enable incision of axillary space underneath the scapula (left forelimb) and similarly for the left hind limb to facilitate sectioning of the ligament of the head of the

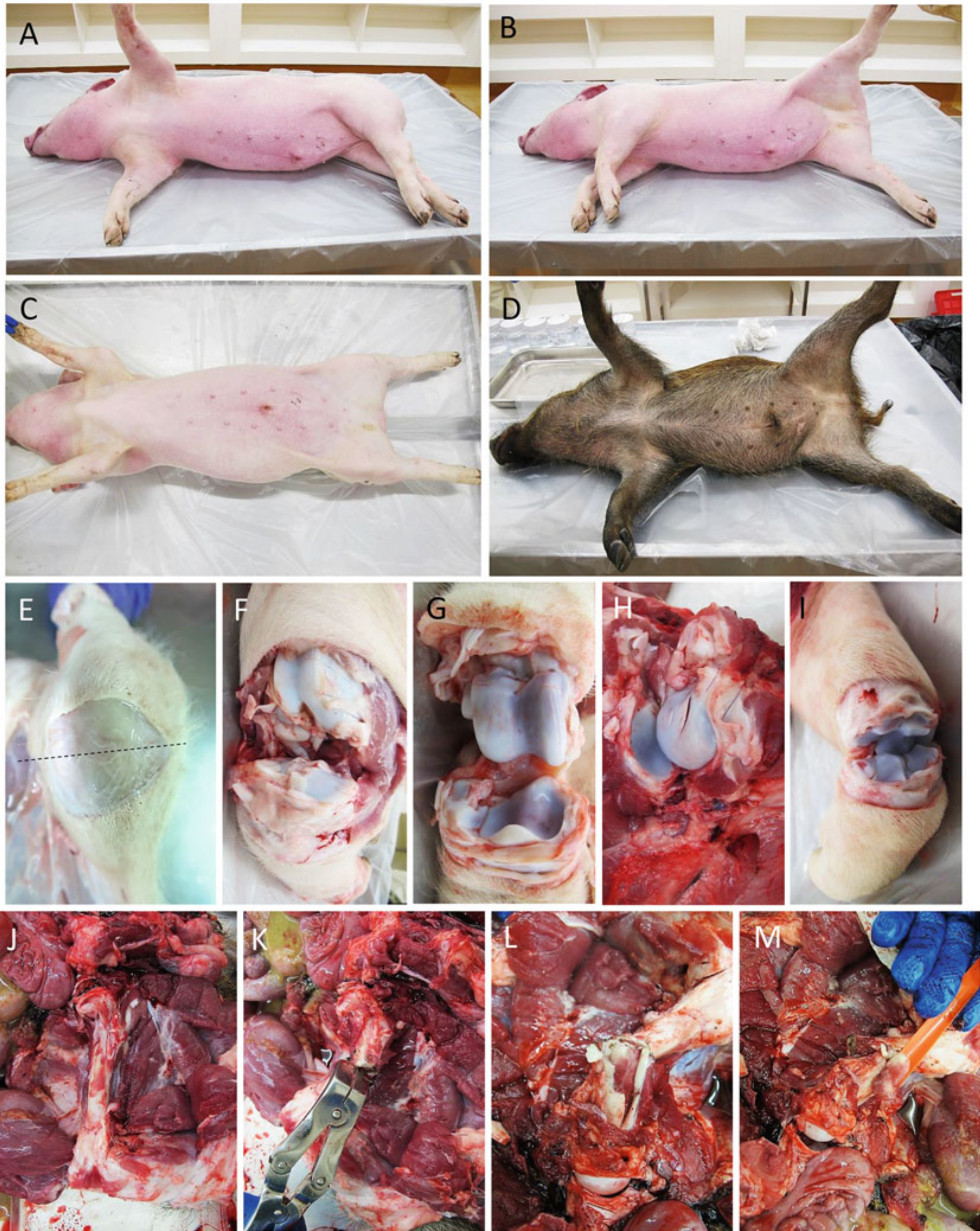

Fig. 3 Evaluation of the skin (**a**–**d**); dissection to open the joints (**e**) and to evaluate the joint surfaces (**f**, stifle; **g**, hock; **h**, shoulder; **i**, metacarpophalangeal) with special attention to the quality and quantity of the synovial fluid, the capsule, as well as the skin and subcutaneous tissue around the joints. Procedure to remove, evaluate, and sample the bone marrow from long bones (**j**–**m**)

femur to reflect the limb from the hip joint (Fig. 4a; black dashes). Then, cut along the ventral midline from the sternum to the pubis symphysis (Fig. 4a; red dashes) and reflect skin dorsally from the left flank to evaluate subcutaneous areas and skeletal muscles. If required, the skin of the limbs can be also removed with a dorsoventral cut (Fig. 4a; blue dashes).

Alternatively, the carcass can be positioned in dorsal decubitus. In this case, proceed in the same way described above by reflecting all four limbs laterally at right angles to the carcass (Fig. 4b) and by reflecting the skin (fully or partially) ventrally in the flanks. If it has not been carried out in a previous step, the joints could be opened and examined at this point of the necropsy (Fig. 4c–e).

2. Check the amount of fat and subcutaneous musculature to complete and complement the evaluation of the body condition (Fig. 4f–g). Evaluate the extension (small, moderate, or large size areas), amount, distribution, and location of subcutaneous edema, hemorrhages (petechiae or ecchymoses), and hematomas. Also, incise into the major muscle masses of the limbs (Fig. 4h) and evaluate the presence and severity of the same lesions among the fascial planes of skeletal muscles (*see* **Note 8**).

   At this point, the mammary glands, scrotum, testicles, and penis as well as superficial inguinal lymph nodes can be evaluated (Fig. 4i, j). Remove and slice the testicles and penis. Open teats through the nipple by incising through the teat canal into the glandular tissue. Examine externally the inguinal lymph nodes (size, consistency, presence of peripheral hemorrhages) and then slice them longitudinally from pole to pole to evaluate the lymphoid parenchyma. This procedure will be applicable to all lymph nodes that will be mentioned throughout the present necropsy protocol. Remember that pigs have an anatomical reversal of the classical lymph node compartments. The medullary sinusoids and cords are located peripherally, while the lymphoid follicles and the diffuse lymphoid tissue are located centrally within the lymph node (*see* **Note 9**).

3. While the pig is side-lying, delimitate a square area in the skin of the neck between the angle of the mandible and the shoulder joint. Carry out two dorsoventral incisions, one caudally to the ears and the angle of the mandible and another one cranially to the scapula (Fig. 5a). Carefully, reflect the skin and subcutis dorsally (Fig. 5b). At this point, parotid (Fig. 5b; black arrowhead), lateral retropharyngeal (Fig. 5b; white arrowhead), and dorsal superficial cervical lymph nodes (Fig. 5b; black arrow) will be easily located. To access the medial retropharyngeal lymph node (Fig. 5c; red arrowhead), cut and reflect the

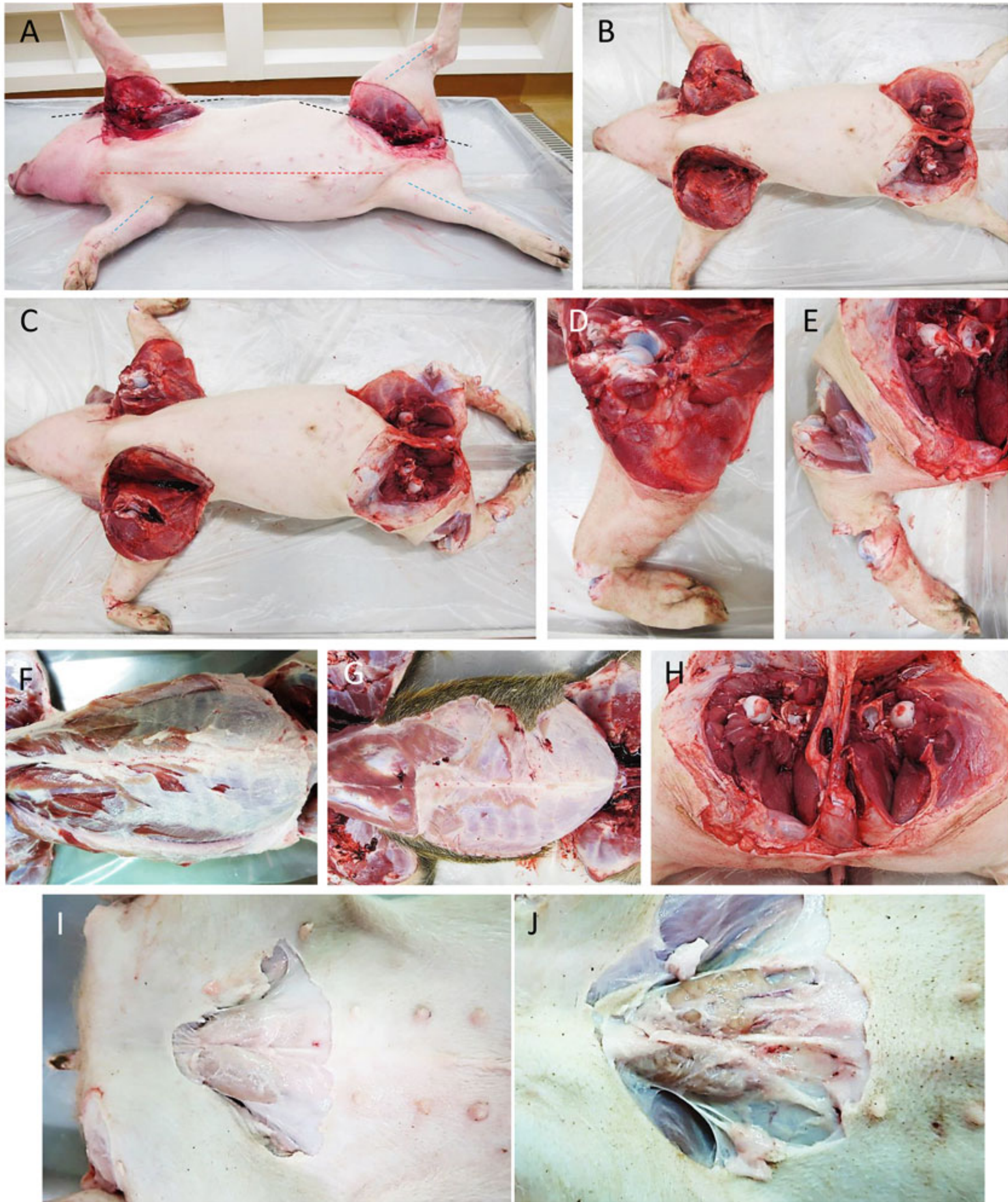

Fig. 4 Carcass positioning and initial incisions to lift the limbs (black dashes), to reflect the skin in abdominal area (red dashes) and limbs (blue dashes) (**a**, **b**), and to dissect and examine the joints (**c**–**e**); evaluation of subcutaneous areas (**f**–**g**) and skeletal muscles (**h**); evaluation of the mammary glands, subcutaneous tissue, and superficial inguinal lymph nodes (**i**, **j**)

cleidomastoideus muscle that is found obliquely in that area (Fig. 5b; red arrow). Repeat the same procedure to evaluate the structures and lymph nodes of the opposite side.

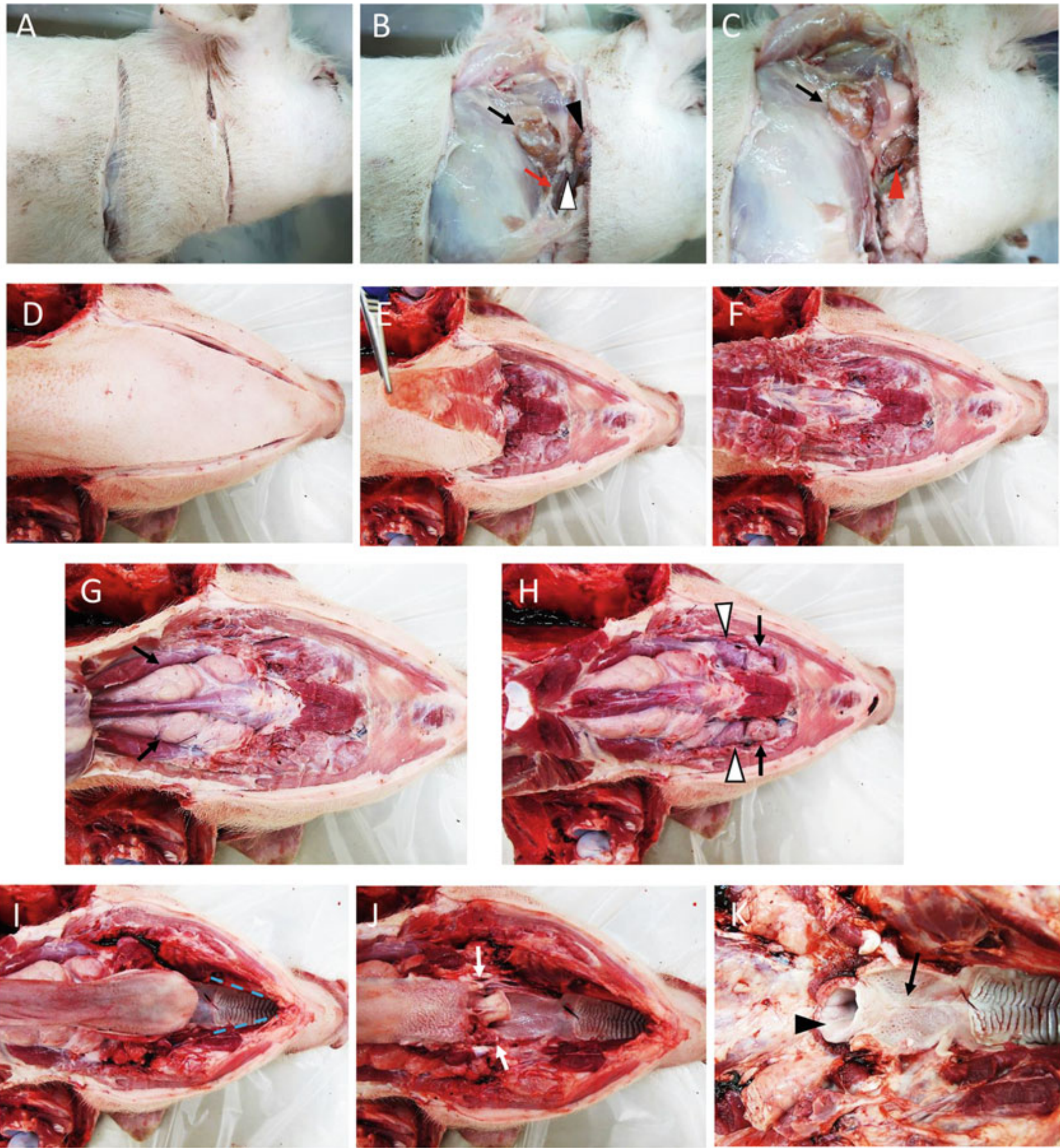

Fig. 5 (**a**–**c**) Dissection of the neck to evaluate the parotid (black arrowhead), lateral retropharyngeal (white arrowhead), medial retropharyngeal (red arrowhead), and dorsal superficial cervical lymph nodes (black arrow) and cleidomastoideus muscle (red arrow); (**d**–**f**) dissection of the submandibular area and evaluation of subcutaneous tissue and muscles; (**g**) evaluation of the thymus (arrow); (**h**) evaluation of the submandibular lymph nodes (arrows) and salivary glands (arrowheads); (**i**–**k**) incision performed to release the tongue (blue dashes) and evaluate the palatine (arrow) and pharyngeal tonsils (arrowhead); hyoid bones (white arrows)

3.3 Evaluation of the Submandibular Area and Opening of the Thoracic and Abdominal Cavity

1. At the submandibular level, carry out a skin incision along the limits on the mandible (Fig. 5d). Expose the intermandibular space and the neck area, reflecting the skin and muscles caudally by keeping the knife blade in parallel to the table and by extending the cut from the mandibular symphysis up to the

level of the thoracic inlet (Fig. 5e, f). At this point, assess subcutaneous area and musculature at submandibular level. In young animals also evaluate the thymus which lies along both sides of the trachea caudal to the larynx (Fig. 5g; arrows). Then, locate and incise the submandibular lymph nodes (Fig. 5h; arrows) and salivary glands (Fig. 5h; arrowheads) (*see* **Note 10**).

2. Remove the tongue to evaluate the oral cavity and tonsils (palatine and pharyngeal). A deep incision should be performed along the medial aspect and close to the mandibular bone bilaterally (Fig. 5i; blue dashes). Push the scalpel or knife through the musculature and soft tissues until reaching the oral cavity and make an incision caudally. Replicate the incision on the opposite side. Release the tongue and pull the tongue ventrally through the incision, cut the hyoid bones (Fig. 5j; arrows), and with steady force use the tongue to guide the removal of the larynx, the trachea, the thyroid, and the esophagus to the thoracic inlet. Examine the thyroid located midline near the thoracic inlet. Finally, evaluate and remove the palatine (Fig. 5k; arrow) and pharyngeal tonsils (Fig. 5k; arrowhead) located in the soft palate and the roof of the nasopharynx, respectively (*see* **Note 11**).
3. To expose the thoracic cavity, keep the blade of the knife in parallel to the table and carcass and pointing caudally. Cut along the costochondral junction but with care to avoid puncturing the pericardial sac (Fig. 6a). Examine the pericardial sac and its contents "in situ" (Fig. 6b) and sample pericardial fluid using sterile syringes or swabs. If the prosector decides to access the abdominal cavity just immediately after opening the thoracic cavity, extend the cut caudally following the ventral midline to the pubic symphysis (Fig. 6c). Avoid perforating the intestine by holding up firmly the package constituted by the skin, muscles, and the sternum and cut with the sharp edge pointing upwards. In this way, the prosector will have a complete view of both major body cavities that will allow examining the thoracic and abdominal organs "in situ". In case a wider window to explore the thoracic cavity is required, use a rib cutter to remove totally or partially the rib cage. In the same way, remove the abdominal wall with a knife to facilitate the inspection. After evaluating the diaphragm for lesions, primarily hemorrhages in subacute forms of ASF, cut into it along the costal arch and remove it (*see* **Note 12**). Collect any thoracic or abdominal fluid in a sterile way using syringes or swabs.

3.4 Evaluation of the Organs of the Thoracic Cavity

1. Up to this point of the necropsy, no organs from thoracic or abdominal cavity have been removed. The tongue will provide a useful handhold for removal the cardiorespiratory system

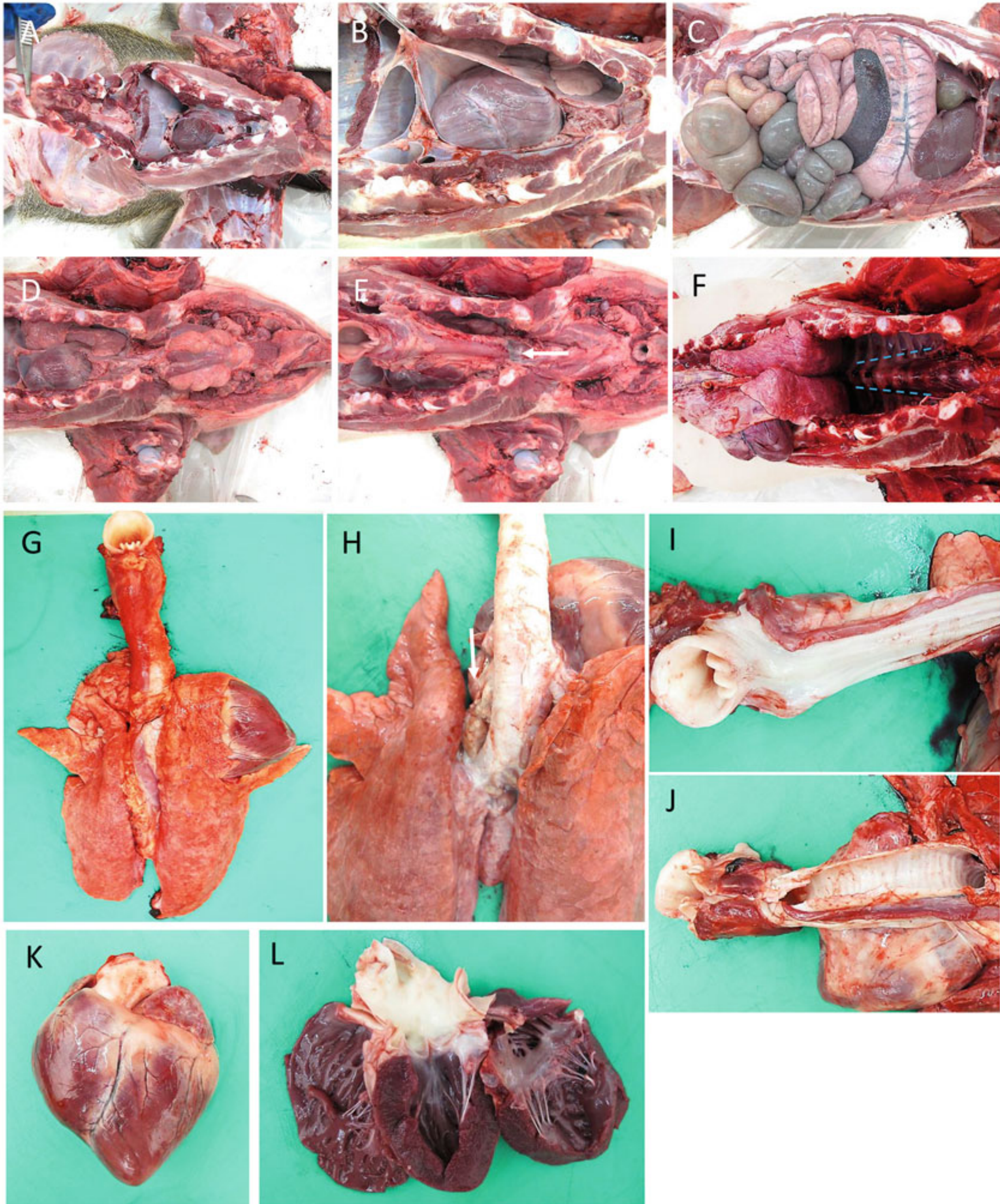

Fig. 6 Thoracic cavity dissection (**a**) and evaluation "in situ" of the pericardial sac, heart, and lungs (**b**); abdominal cavity dissection to examine "in situ" the abdominal organs (**c**); removal of organs from the thoracic cavity by dissecting along the dorsal roof of the thorax cavity adjacent to the trachea (arrow and dashes) (**d**–**f**); evaluation of the esophagus cardiorespiratory system and pulmonary lymph nodes (arrow) (**g**–**h**); dissection and evaluation of the esophagus (I) and trachea (J); external evaluation of the heart (**k**); opening of the heart and evaluation of the great vessels cardiac musculature and valves (**l**)

(Fig. 6d). From the thoracic inlet (Fig. 6e; arrow), dissect caudally along the dorsal roof of thorax cavity adjacent to the trachea using scissors (Fig. 6f; blue dashes). Also dissect any attachments of pericardial ligaments to the sternum and dorsal pleura to the thoracic wall. Then cut through the vena cava, the esophagus, and the thoracic aorta at the diaphragmatic hiatus and pull dorsally to extract all thoracic organs.

2. Following placement on a clean surface, examine the esophagus, larynx, trachea, pleura, apical, middle and caudal pulmonary lobes, heart, pericardium, and mediastinal and tracheobronchial lymph nodes (Fig. 6g). To access both bronchi and tracheobronchial lymph nodes (Fig. 6h; arrow), free the esophagus and the aorta from the caudodorsal mediastinum. Using scissors, incise the esophagus along its entire length and examine its wall, contents, and mucosal surface (Fig. 6i). Then, incise the trachea at the trachealis muscle from the larynx down to the bifurcation of the bronchi (Fig. 6j) and evaluate its mucosal surface and contents (foamy material, blood, inflammatory secretions).
3. Examine the pleural surface and palpate the lungs gently. At this point, evaluate the degree of lung collapse, the severity and extension of vascular changes (interstitial and alveolar edema, congestion, and hemorrhages), as well as the presence and distribution of areas of consolidation consistent with pneumonic processes. Cut through the lung lobes using scissors and evaluate the pulmonary parenchyma and airways (bronchi and bronchiole) (*see* **Note 13**).
4. Separate the heart from the pulmonary structures by cutting through the great vessels (Fig. 6k) at the base of the heart. Then, open the heart cutting crosswise the ventricles and atria with a knife. Alternatively, cardiac cavities can be opened using scissors by following the path of the blood flow through the heart. For that, incise with scissors into the right atrium through the cava vein, continue into the right ventricle following the interventricular septum, and finally open the pulmonary artery. Repeat the procedure accessing the left atrium through the pulmonary veins, continue into the left ventricle, and cut through the aorta. Examine the lumina of great vessels, the epicardium, the coronary groove, the atrioventricular and pulmonary valves, the interventricular and interatrial septum, the endocardial surfaces, and the papillary muscles (Fig. 6l). Carry out transverse sections of the ventricular walls and interventricular septa to assess the myocardium (*see* **Note 14**).

3.5 Evaluation of the Organs of the Abdominal Cavity

1. Continue by examining and removing organs from the abdominal cavity starting with the most accessible organ, the spleen. Examine the spleen "in situ" (Fig. 7a; black arrow) and then remove it (Fig. 7b) along with the splenic lymph nodes which

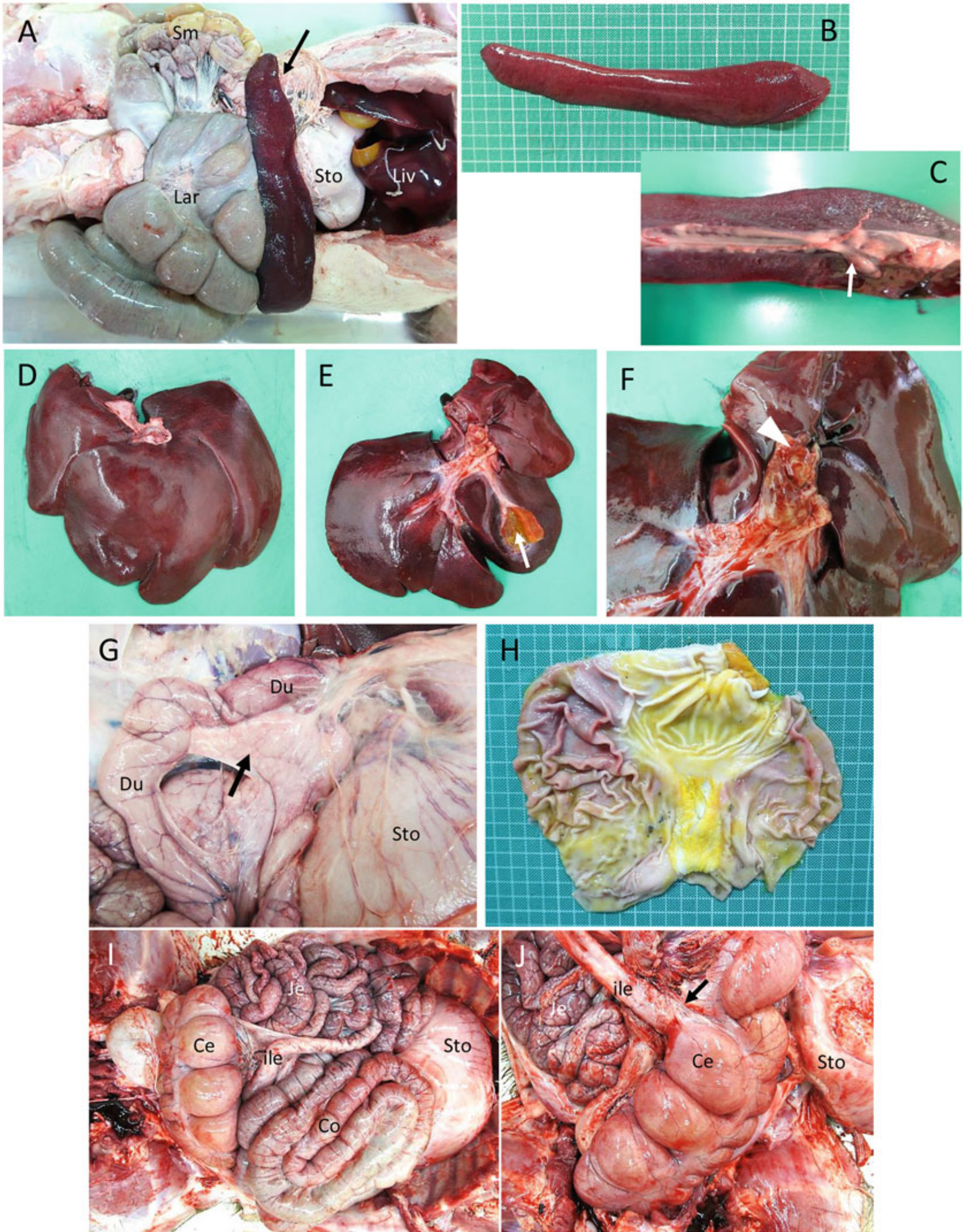

Fig. 7 (**a**) General view of the abdominal cavity spleen (arrow); (**b**) normal spleen; (**c**) normal splenic lymph nodes in the visceral surface of the spleen (arrow); diaphragmatic (**d**) and visceral surface (**e**, **f**) of the liver. Gall bladder (arrow). Normal gastrohepatic lymph nodes (arrowhead); (**g**) pancreas (arrow); (**h**) gastric mucosae of the stomach; (**i**, **j**) evaluation of the gastrointestinal tract in the abdominal cavity. Ileocecal valve (arrowhead). Stomach (sto), liver (li), small intestine (sm), large intestine (lar), duodenum (du), jejunum (je), ileum (ile), cecum (ce), colon (co)

are in the visceral surface of the organ (Fig. 7c; arrow). Once placed on a clean surface, slice the spleen to evaluate macroscopically the white and red pulp (*see* **Note 15**).

2. Remove the liver by dissecting the attachments to the diaphragm and the lateral ligaments. Gastrohepatic lymph nodes at the hepatic hilar can be removed along with the liver. On a table, evaluate the diaphragmatic (Fig. 7d) and visceral surface (Fig. 7e, f) of the liver focusing on size, color, and shape of the edges. Place visceral surface upside up, check gastrohepatic lymph nodes (Fig. 7f; arrowhead), and dissect the vena cava and its tributary veins into each hepatic lobe as well as the bile duct. Open the gall bladder with scissors and examine the mucosa, wall, and luminal contents (Fig. 7e; arrow). Finally, slice the liver to evaluate the hepatic parenchyma (*see* **Note 16**).
3. Examine "in situ" the gastrointestinal tract (Fig. 7a: stomach [sto], small intestine [sm], and large intestine[lar]). To minimize the spillage of the gastrointestinal contents, tie off the esophagus at the cardias of the stomach. Also tie off the rectum as close to the anus as possible. Dissect away the mesentery, lift the stomach and the intestines out of the abdomen, and place them on a table.
4. Locate the pancreas between the descending and the ascending duodenum (Fig. 7g; arrow; stomach [sto]; duodenum [du]) and remove it. Separate the stomach from the duodenum by cutting through the duodenum just after the pylorus. Examine externally the serosa of the stomach. Using an enterotome or round tip scissors, open the stomach following the greater curvature from the cardias to the pylorus. Evaluate the gastric wall, the gastric contents, and the thickness and color of glandular and non-glandular mucosae (Fig. 7h) (*see* **Note 17**).
5. At this point, we can spread out the intestine by cutting the mesenteric root. Alternatively, to conserve time, we can identify "in situ" sections of the small intestine (Fig. 7i: duodenum, jejunum [je], and ileum [ile]); locate the ileocecal junction as well as the ileocecal valve, a muscular valve that separate small and large intestine (Fig. 7j; arrow); and identify sections of the large intestine (Fig. 7i, j: cecum [ce], colon [co], and rectum). At the same time, locate and evaluate mesenteric lymph nodes that drain each intestinal section. Before opening, examine the serosal surface of each section of the intestinal regions. Using an enterotome or round tip scissors, open representative segments of all areas of the intestinal tract by cutting along the mesenteric border, avoiding damaging the mucosal surface. Examine the intestinal wall, the thickness and color of the mucosa, and the consistency, color, and smell of the contents. If required, collect fresh gastrointestinal contents in sterile containers or using swabs (*see* **Note 18**).

6. The organs of the urinary and female reproductive system will still remain in the abdomen. Evaluate "in situ" the kidneys, ureters, and urinary bladder. Also evaluate the renal lymph nodes (Fig. 8a; arrow) located medially to the renal hila and the adrenal glands (Fig. 8a; arrowhead) attached medially to the cranial pole of each kidney. Remove the kidney and evaluate the external renal cortex (Fig. 8b, c). Carefully, cut along the lateral border, remove the renal capsule using a pair of forceps, and examine externally the renal cortex (Fig. 8b). Then, using a knife, cut each kidney longitudinally in half and examine the renal cortex, medulla, and renal pelvis (Fig. 8d) (*see* **Note 19**).
7. To help with the removal of the urinary bladder and the genital tract in sows, the pubic symphysis can be dislocated using a saw or a bone cutter by carrying out cuts on each side of the pelvis. Remove the urinary bladder and examine the serosal surface. Then, open the urinary bladder with scissors incising from bladder neck and evaluate the mucosa, wall, and luminal contents (Fig. 8e). Before the incision, urine samples can be aspirated using sterile syringes and needles (*see* **Note 20**).

   Remove and examine the female reproductive system (ovaries, uterine horn, uterus, and vagina). Incise the uterine horn from the tip towards the body of the uterus and vagina. Evaluate the walls, mucosa surfaces, and contents.

3.6 Evaluation of the Head and Brain

1. Locate the atlanto-occipital joint (Fig. 8f; arrow). If required, obtain cerebrospinal fluid using sterile needles and syringes. Cut the soft tissues around the joint, incise into the vertebral canal at the atlanto-occipital joint level, and remove the head from the carcass (Fig. 8f; dashes). To facilitate handling the head, remove the ears as well as the skin and musculature especially around the masseters. At this point, the prosector has two options: (a) Remove the mandible and saw or split the head sagittally in two halves using a small axe or a cleaver by incising through the hard palate and skull base. Each half of the brain and spinal cord (Fig. 8g–j; arrowhead) may then be removed from the exposed cranial vault. Nasal cavity structures (Fig. 8g, h; arrows) will be also exposed to be evaluated by removing the nasal septa if necessary. (b) Secure the head in a head vise and carry out three main incisions (Fig. 9a). Firstly, medially to the right and left occipital condyles, cut by sawing ventrodorsally the occipital bone from occipital condyles until the nuchal crest (Fig. 9b, c). Then, cut by sawing caudocranially the parietal and frontal bones from the nuchal crests until the posterior commissure of each eye (Fig. 9d, e). Finally, cut transversely the skull along a line drawn between the posterior commissure of the eyes (Fig. 9f). Remove the cap of the cranial vault by prying with a mason's chisel or large screwdriver. A

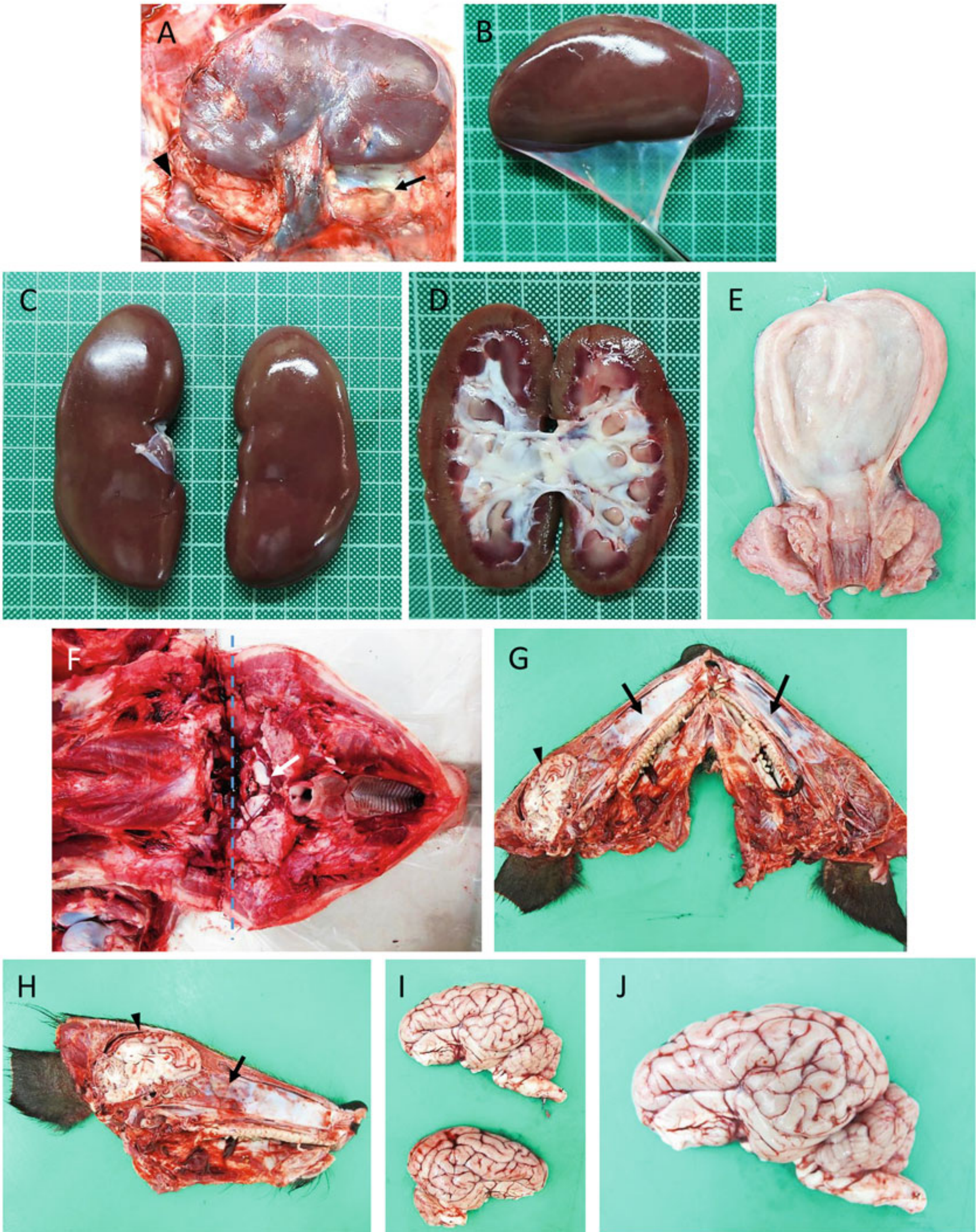

Fig. 8 (**a**) Evaluation of the kidney, renal lymph nodes (arrow), and adrenal gland (arrowhead) in the abdominal cavity; (**b**, **c**) evaluation of the renal capsule and renal cortex; (**d**) longitudinal dissection of the kidney and evaluation of the renal cortex, medulla, and renal pelvis; (**e**) dissection of the urinary bladder to evaluate the bladder neck, mucosa, wall, and luminal contents; (**f**) removal of the head from the carcass (dashes). Atlanto occipital joint (arrow); (**g**, **h**) incision through the hard palate and skull base to split the head sagittally. Evaluation of the brain (arrowhead) and nasal cavity structures (arrow); (**i**, **j**) normal brain and spinal cord

Fig. 9 Dissections to open the skull and evaluate the brain, spinal cord, and nasal cavity (**a**–**f**); evaluation of the brain and spinal cord before (**g**, **h**) and after removing (**i**) the meninges (dura mater). Dissection of the head by incising through the nasal cavity and the hard palate (**j**, **k**) to evaluate the nasal cavity structures (**l**)

mallet may be also required to facilitate this procedure (Fig. 9g).

2. Evaluate and remove the meninges (dura mater) with scissors (Fig. 9g–i). To extract the brain and spinal cord easily with minimal artifacts, place the head upside down on a table and,

working dorsocaudally, carefully cutting the olfactory peduncles, arteries, cranial nerves, and meninges that attach the brain to the cranial vault. The brain and spinal cord will be gently rested on the table as it is being dissected. For subsequent histopathological studies, the entire brain and spinal cord will be fixed in 4% formalin. After the period of fixation, the brain and spinal cord will be sliced following specific protocols to examine and sample for different areas (*see* **Note 21**).

3. Once the brain and spinal cord have been removed, saw the head sagittally by incising through the nasal cavity and the hard palate (Fig. 9j–k). Then split the head and evaluate the nasal cavity structures as described (Fig. 9l).

4 Notes

1. Throughout the necropsy or upon completion, the macroscopic findings observed should be recorded. In parenchymal organs, we should focus on the location of lesions (anatomical position regarding other organs), number and size of lesions, color changes (dark, light, brilliant, mottled, etc.), lesion shape (round, flat, nodular, lobular, etc.), percentage of area injured or affected regarding the total area of the organ, distribution (unilateral, bilateral, focal, multifocal, diffuse), consistency of the organ regarding its normal consistency (soft, firm, hard, gas-filled, friable, etc.), weight, and aspect of cut surfaces. In tubular organs, also examine the lumen diameter in relation to the wall, consistency and odor of contents, as well as changes in walls and lesions in mucosa.

   To evaluate the macroscopic lesions of pigs infected with ASF, it is recommended to follow one of the available macroscopic scoring protocols [10–12]. The scoring sheets should also record additional data such as the animal's ID, breed, sex, age, and body weight. Information regarding if the animal died or was euthanized (including the method of euthanasia), time of death, duration of illness/clinical course, or time since the animal was experimentally infected will also contribute to the evaluation and interpretation of lesions.

   As the necropsy is carried out, and once the macroscopic evaluation of specific organs and tissues is completed, tissue samples and fluids for different downstream studies should be collected. Waiting until the end of the necropsy to collect all the samples is not recommended.

2. During acute and subacute forms of ASF, animals usually display high viremia levels along with increase in temperature, changes usually correlated with lethargy, inappetence, reduced intake, and weight loss (Fig. 10a). These changes may affect

body condition especially in chronic forms of the disease caused by low virulent ASFV isolates where animals display recurrent transient pyrexia, stunting, and emaciation (Fig. 10b) and where the appearance of opportunistic concomitant pathogens, mainly bacteria, is frequent.

3. The presence of congested ocular mucosa with mucopurulent discharges is also a common finding in pigs that suffer acute forms of ASF (Fig. 10c).

4. The presence of bloodstained foamy material in nostril and mouth characteristic of alveolar edema (Fig. 10d) is associated with ASF clinical courses presenting with breathing difficulty (dyspnea), cough, increased pulse, and respiratory rate. During natural outbreaks, the presence of foamy material in the mouth and nose of animals that developed acute or subacute forms of ASF during the agonal period constitutes one of the most typical images of the disease.

5. In acute and subacute forms of ASF, animals may display initial mucoid diarrhea, sometimes with bloodstained mucus on the feces (Fig. 10e). As disease progresses, animals may display more severe watery diarrhea with fresh or blackened blood adhering to the tail, perianal area, and hocks (Fig. 10f).

6. In white pig breeds, vascular skin lesions characteristic of acute and subacute forms of ASF (erythema, cyanosis, petechial hemorrhages, and ecchymoses) are easily identifiable, being mainly observed in the tips of ears, periocular area, snout, distal limbs, tails, perianal area, scrotal sac, ventral chest, and abdomen (Fig. 10g–l). Cyanotic areas usually appear in these locations 24–48 h before death. Small foci of necrosis in the skin may appear in subacute forms of ASF. In clinical courses in which low virulence ASFV isolates are involved, and which may induce chronic forms of ASF, reddened or raised necrotic skin foci may be observed all over the body, being particularly frequent in areas that overlie bony protuberances. Such necrotic skin lesions are generally more frequent, larger, and more severe than those described in subacute forms of ASF (Fig. 11a–f).

7. Joint swelling due to arthritis and periarthritis is a characteristic lesion especially described in clinical courses caused by low virulent ASFV isolates (Fig. 11g–i). In these cases, there is an increase in synovial fluid, which may display different features that range from a serofibrinous (Fig. 11j, k) or serohemorrhagic (Fig. 11l) to purulent aspect (Fig. 11m) in more severe cases. The presence of subcutaneous edema and inflammatory changes in the structures around the joints are also usually described, as well as the presence of necrotic skin areas that may range from small foci to severe deep ulcers (Fig. 11n).

Fig. 10 (**a**) Acute ASF. Lethargic pigs huddling together; (**b**) chronic ASF pig showing poor body condition and emaciation; (**c**) ocular discharge and mucosa congestion; (**d**) bloodstained foamy material in nostril and mouth; (**e**) bloodstained feces (melena); (**f**) soiling and fecal encrusting around the rears, hocks, and tail indicative of poor consistency of stools or diarrhea; (**g**) skin erythema in the ears and dorsal skin areas; (**h**) severe erythema and cyanosis in the ears and snout; skin erythema, cyanosis, and hemorrhages in the abdomen (**i**), caudal skin areas (**j**), distal limbs (**k**), tail, perianal area, and scrotal sac (**l**)

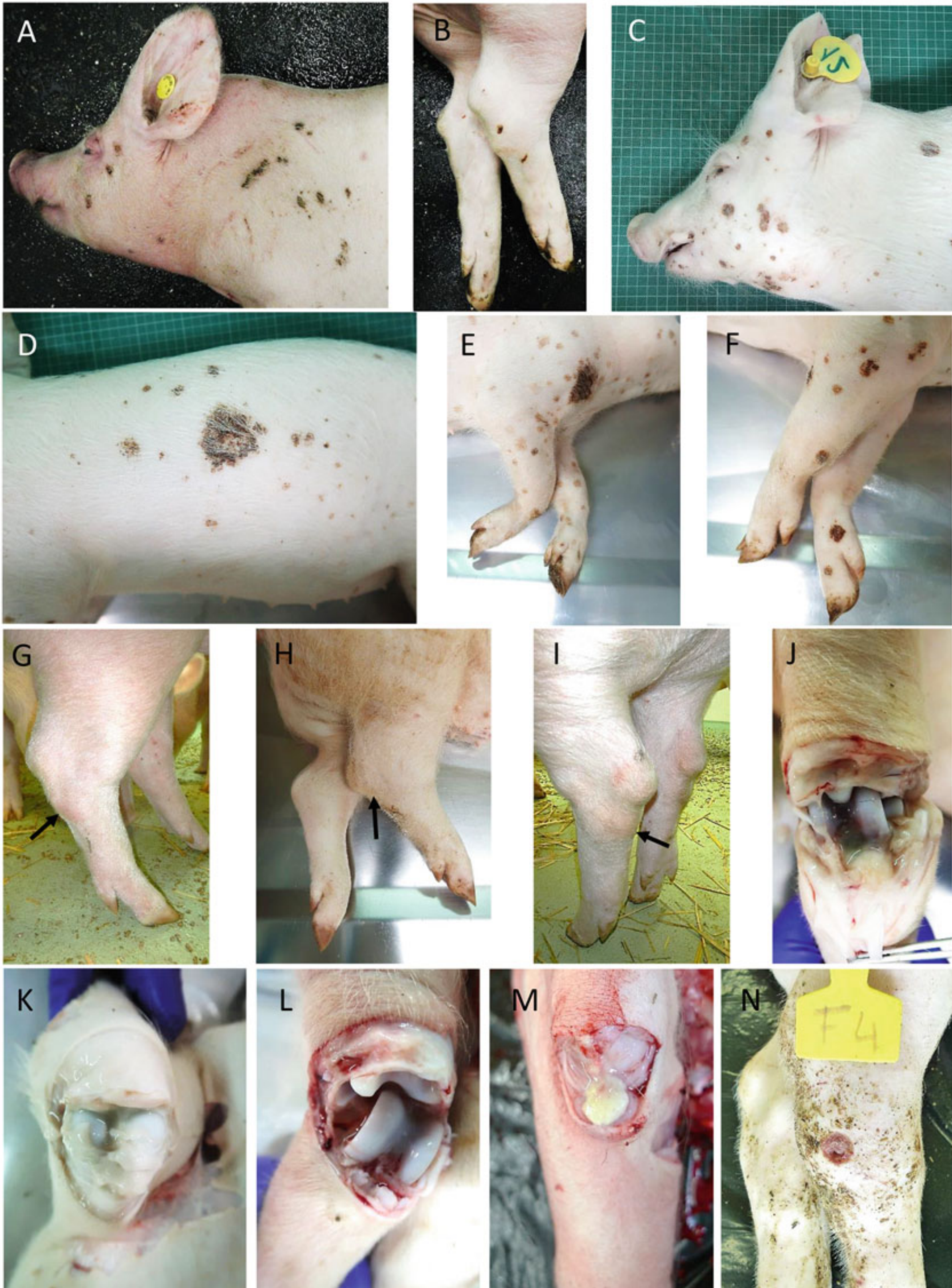

Fig. 11 (**a**–**f**) Reddened or raised necrotic skin foci observed in different body areas caused by low virulent ASFV isolates involved in subacute and chronic forms of ASF; (**g**–**i**) joint swelling (arrows) due to arthritis and periarthritis caused by low virulent ASFV isolates; increase of serofibrinous (**j**, **k**) or serohemorrhagic synovial fluid (**l**); purulent arthritis (**m**), deep skin ulcer on hock joint (**n**)

8. Subcutaneous and intermuscular edema (Fig. 12a, b), along with musculature hemorrhages (petechiae or ecchymoses) and hematomas, is present on both acute and subacute forms of ASF, although they are more severe and affect wider areas in subacute forms. During experimental infections, bleeding from injection sites (neck, ears) due to thrombocytopenia and vascular damage caused by the virus is frequent, being able to induce severe subcutaneous and intermuscular hemorrhages (Fig. 12c). In chronic forms, pale areas due to the necrosis of skeletal muscle are also observed especially in muscle masses of the limbs (Fig. 12d).
9. The vascular disorders in lymph nodes constitute, without doubt, the most representative changes in ASF. In acute forms, the lymph nodes show a mild to moderate increase in size, edematous aspect, color changes, and hemorrhages, which may become so severe that some lymph nodes appear as blood clots (hemorrhagic lymphadenitis). Hemorrhages can be present in both peripheral and internal areas of lymph nodes. The gastrohepatic, renal, and mesenteric lymph nodes (Fig. 12e, f) are often the most severely affected and, usually, the only ones with visible hemorrhagic lesions at early acute forms of ASF. Other lymph nodes like superficial inguinal, parotid, prescapular, submandibular, retropharyngeal, cervical, tracheobronchial, and mediastinal lymph nodes may also display lymphadenopathy along with less severe hemorrhagic lesions (petechiae and ecchymoses) in medulla and cortex.

   In subacute forms of ASF, most of the lymph nodes (and not just some target lymph nodes as renal, gastrohepatic, or mesenteric) appear markedly enlarged, edematous, and resembling blood clots (hemorrhagic lymphadenitis) (Fig. 12g–o). However, in chronic forms of ASF induced by low virulent ASFV isolates, lymph nodes do not display hemorrhagic changes. Instead, lymph nodes show a hyperplasia characterized by an increase in size, rubbery consistency, and diffuse white or tan color.
10. The thymus, as other lymphoid organs, is a target organ for ASFV. In acute and subacute forms of ASF, young animals may display different degree of thymus atrophy (Fig. 13a) as well as hemorrhages (petechial and ecchymoses) (Fig. 13b).
11. The tonsils play a key role during initial ASFV replication after infection also being one of the locations where the virus can be detected for a long time. The presence of erythema (Fig. 13c) or petechiae on the surface of the palatine tonsils is a common finding in acute and subacute forms of ASF. Necrotic areas in palatine tonsils, likely due to secondary bacterial infections, have also been described in natural chronic forms.

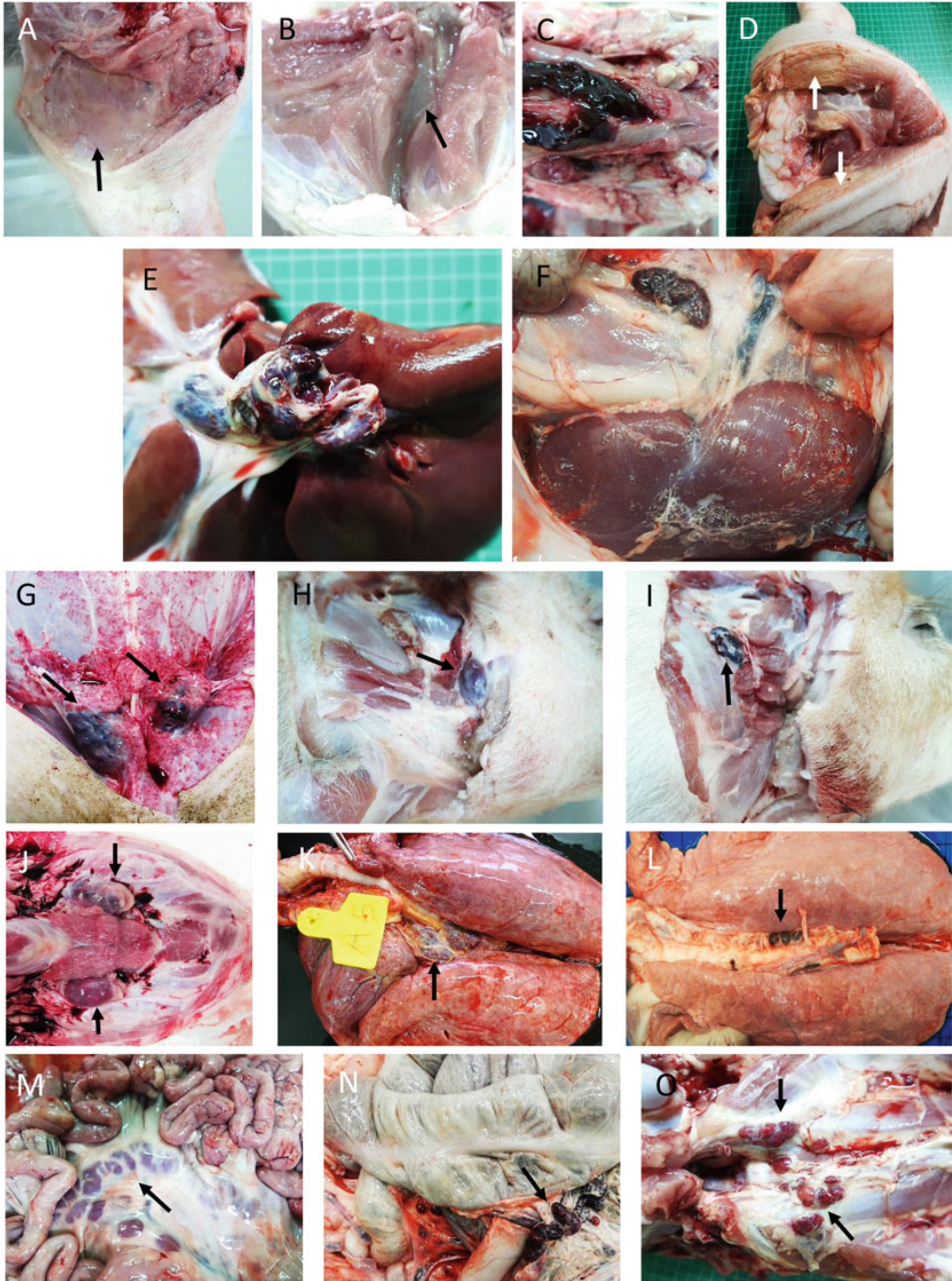

Fig. 12 Subcutaneous (**a**) and intermuscular edema (**b**) (arrows); intermuscular hemorrhages in the neck sampling site (**c**); pale areas (arrows) due to the necrosis of skeletal muscle (**d**); gastrohepatic (**e**) and renal lymph nodes (**f**) showing hemorrhagic lymphadenitis in pig with acute ASF; superficial inguinal (**g**), parotid (**h**), prescapular (**i**), submandibular (**j**), tracheobronchial (**k**), mediastinal (**l**), mesenteric (**m**), ileocecal (**n**), and deep inguinal lymph nodes displaying hemorrhagic lymphadenitis (arrows) in pigs with subacute ASF

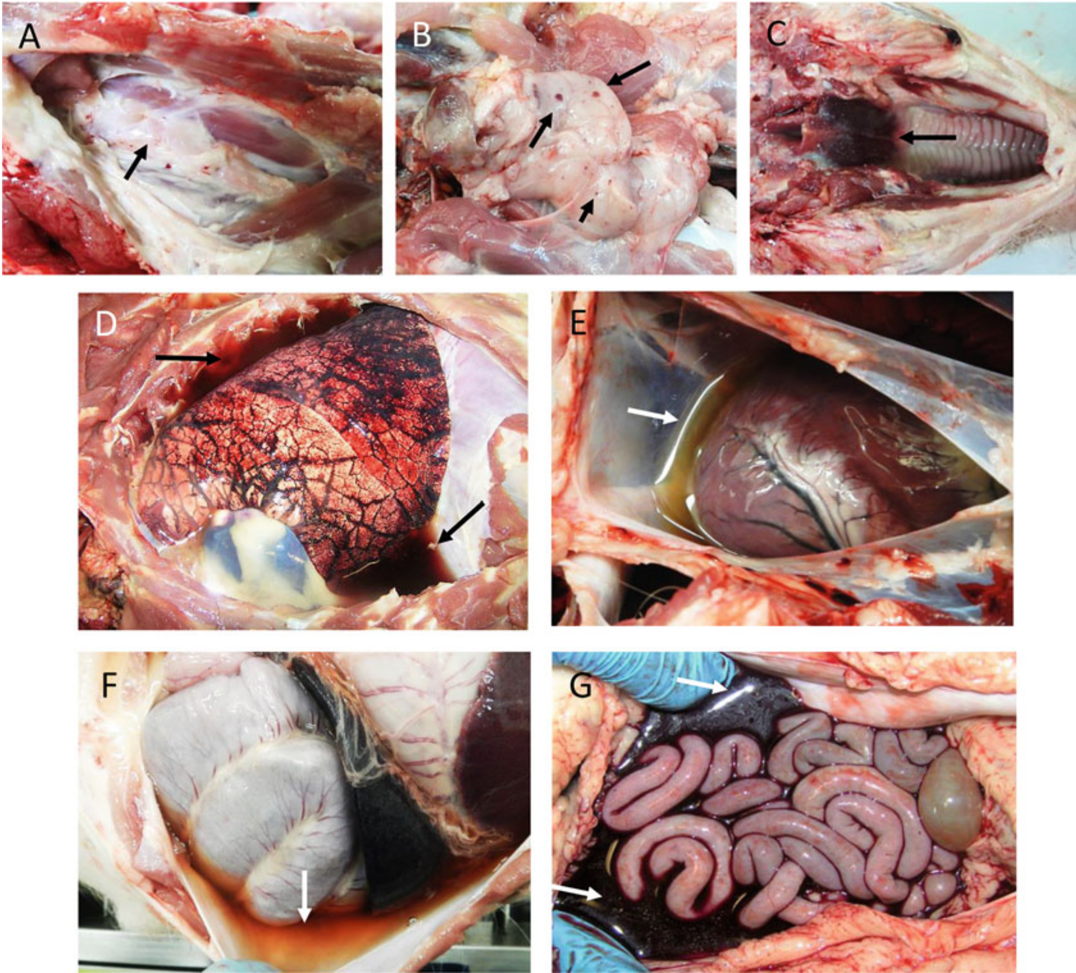

Fig. 13 Thymus atrophy (arrow) (**a**) and multifocal hemorrhages (arrows) in thymus (**b**); severe erythema in palatine tonsil (arrow) (**c**); fluids in body cavities in pigs with acute and subacute forms of ASF. (**d**) Severe hydrothorax (arrows) with reddish fluid (subacute ASF). Also observe the severe diffuse congestion, interstitial edema, as well as the severe hemorrhages that affect extensive areas of the pleura and pulmonary parenchyma; (**e**) moderate hydropericardium (arrow) with yellowish fluid (acute ASF); (**f**) severe ascites arrow with yellowish fluid (acute ASF); (**g**) severe ascites arrows with red-tinged fluid and blood clots hemoabdomen (subacute ASF)

12. Evaluate the changes of the pleural and pericardial contents within the thoracic cavity. In acute and, especially, subacute forms of ASF, hydrothorax with yellowish or red-tinged fluid (Fig. 13d) as well as hemothorax (blood clots within the thoracic cavity) may be present. Also, evaluate the possible presence of fibrin deposition on the surface of the pericardial sac and pleura or the existence of fibrinous pleural adhesions to the rib cage, the last one associated with the existence of concomitant pulmonary bacterial infections and mainly described in chronic forms induced by low virulent ASFV isolates.

The existence of hydropericardium with yellowish (Fig. 13e) or red-tinged fluid is a typical finding in acute and subacute forms of ASF. In severe subacute forms, hemopericardium (blood clots within the pericardial sac) may be also present. The presence of fibrin deposits on the surface of epicardium along with fibrinous pericarditis is associated with the existence of concomitant bacterial infections described in chronic forms of ASF. Check the level of lung collapse. If the lungs do not collapse after opening the thoracic cavity, it indicates an abnormal negative intrathoracic pressure at the time of death.

In the abdominal cavity, evaluate the presence and severity of ascites, constituted by transparent, yellowish (Fig. 13f), or red-tinged fluid (Fig. 13g). In severe subacute forms, hemoabdomen (blood clots floating in the fluid of the abdominal cavity) can be present (Fig. 13g).

13. In subacute forms, the presence of pleural cavity containing clear fluid can be observed. The absence of lung collapse may be caused by the accumulation of air (emphysema) in septa or alveoli, by the existence of circulatory disorders (thrombi, infarcts, hemorrhages, as well as congestion and edema affecting the alveoli or septa), or by the presence of inflammatory cells in alveoli (alveolar pneumonia), septa (interstitial pneumonia), airways (bronchitis or bronchiolitis), or pleura (pleuritis).

    In acute and subacute forms of ASF, the lungs usually show lack of collapse, rib impressions, diffuse congestion, distension of interlobular septa (interstitial edema) with presence of yellow gelatinous infiltrate (Fig. 14a, b), and alveolar edema with the presence of exudation of fluid and froth on cut surface along with foamy material (which may be bloodstained) in the trachea (Fig. 14c). Particularly in subacute forms, vascular changes are more evident and widespread, contributing to a marked distension of pulmonary parenchyma. Such a distension is due to the existence of a severe interstitial and alveolar edemas as well as the appearance of multifocal petechial hemorrhages (Fig. 14d), ecchymoses, and suggillations that may affect extensive areas of the pleura and pulmonary parenchyma, oozing abundant blood from cut surfaces (Figs. 13d and 14e, f). Also in subacute forms, areas of consolidation characteristic of pneumonia with focal or lobar extension are observed (Fig. 14g, h). On the other hand, in natural cases of animals that develop chronic forms of ASF, the presence of severe pneumonic lesions of different characteristics (fibrinous, purulent, necrotic) attributed to opportunistic pathogens, mainly bacteria, has been traditionally described.

14. In acute forms of ASF, the presence of small multifocal petechial hemorrhages and ecchymoses in the epicardium and

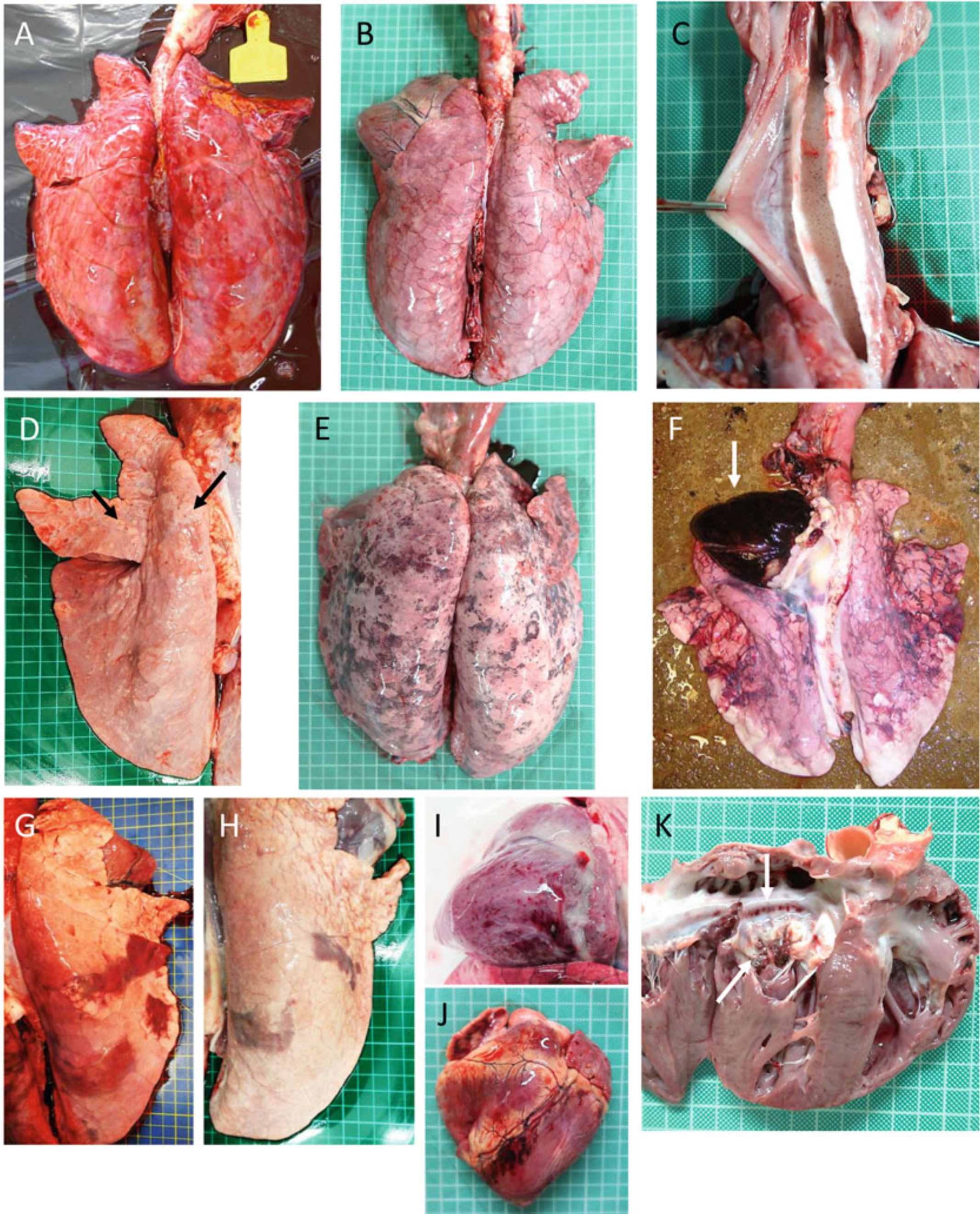

Fig. 14 Lungs from pigs with acute (**a**) and subacute ASF (**b**) displaying diffuse congestion, lack of collapse, and distension of interlobular septa (interstitial edema); (**c**) presence of moderate foamy material in trachea due to alveolar edema; (**d**) occasional presence of subpleural petechiae (arrows) (acute ASF); (**e**, **f**) non-collapsed lungs from pigs with subacute ASF showing diffuse congestion, interstitial edema as well as multifocal petechial hemorrhages and ecchymoses that affect extensive areas of the pleura and pulmonary parenchyma. Observe also the severe hemorrhages almost completely covering the epicardium (**f**; arrow); (**g**, **h**) multifocal areas of lung consolidation characteristic of pneumonia (subacute ASF); (**i**, **j**, **k**) presence of petechial hemorrhages and ecchymoses in the epicardium and endocardium (arrows) of pigs with subacute ASF

endocardium is frequent in the heart. However, it is in animals that develop subacute forms where the most severe coalescent interstitial hemorrhages (ecchymoses and suggillations) can be observed. Hemorrhages will be present in the epicardium (Fig. 14i, j), myocardium, and endocardium (Fig. 14k) up to the point of involving almost the totality of the cardiac surface (Fig. 14f; arrow).

15. The so-called hyperemic splenomegaly is one of the most significative lesions that is associated to ASF, although it is not always present. In some acute forms of ASF, the spleen extends across the whole width of the abdominal cavity from side to side. In such cases, the spleen shows a marked increase in size, rounded edges, friable consistency, dark red to black color, and intense bleeding after sectioning (Fig. 15a, b). The presence of petechiae in the capsule as well as small multifocal splenic infarcts in the margins may also be observed. In other cases where pigs have developed an acute or subacute form of ASF, the spleen shows normal appearance and size or may display mild increase in size without friable consistency (Fig. 15c, d). In some subacute forms, the existence of partial hyperemic splenomegaly affecting only small areas of the splenic parenchyma is also described (Fig. 15e, f).

    On the other hand, in chronic forms induced by low virulent ASFV isolates, vascular changes described above are not observed in the spleen. In these cases, the spleen shows a hyperplasic splenomegaly characterized by a mild to moderate increase in size, rubbery consistency, and light brown color.

16. Changes in the liver and gall bladder are more evident in subacute forms of ASF. While in acute forms the liver presents only mild hepatomegaly and congestion along with mild edema of the bile duct and gall bladder wall with occasional petechiae in mucosa, in subacute forms these vascular changes are much more severe. In these cases, the liver shows marked hepatomegaly, domed hepatic surfaces and firm consistency, and changes accompanied by diffuse congestion (Fig. 16a) with marked blood effusion after incision along with multifocal subcapsular petechiae and suggillations. A severe edema with wall distension is also observed in the gall bladder wall and bile ducts, changes that may be accompanied by a gelatinous edema affecting the hepatic hilum area (Fig. 16b, c). In addition, multifocal hemorrhages on the serosal and submucosal surfaces of the gall bladder are more severe and numerous (Fig. 16d–i). In severe subacute cases, blood clots are also observed on the mucosal surface.

17. Lesions in the stomach are mainly observed in pigs that develop subacute forms of ASF, although they are not frequent. Among

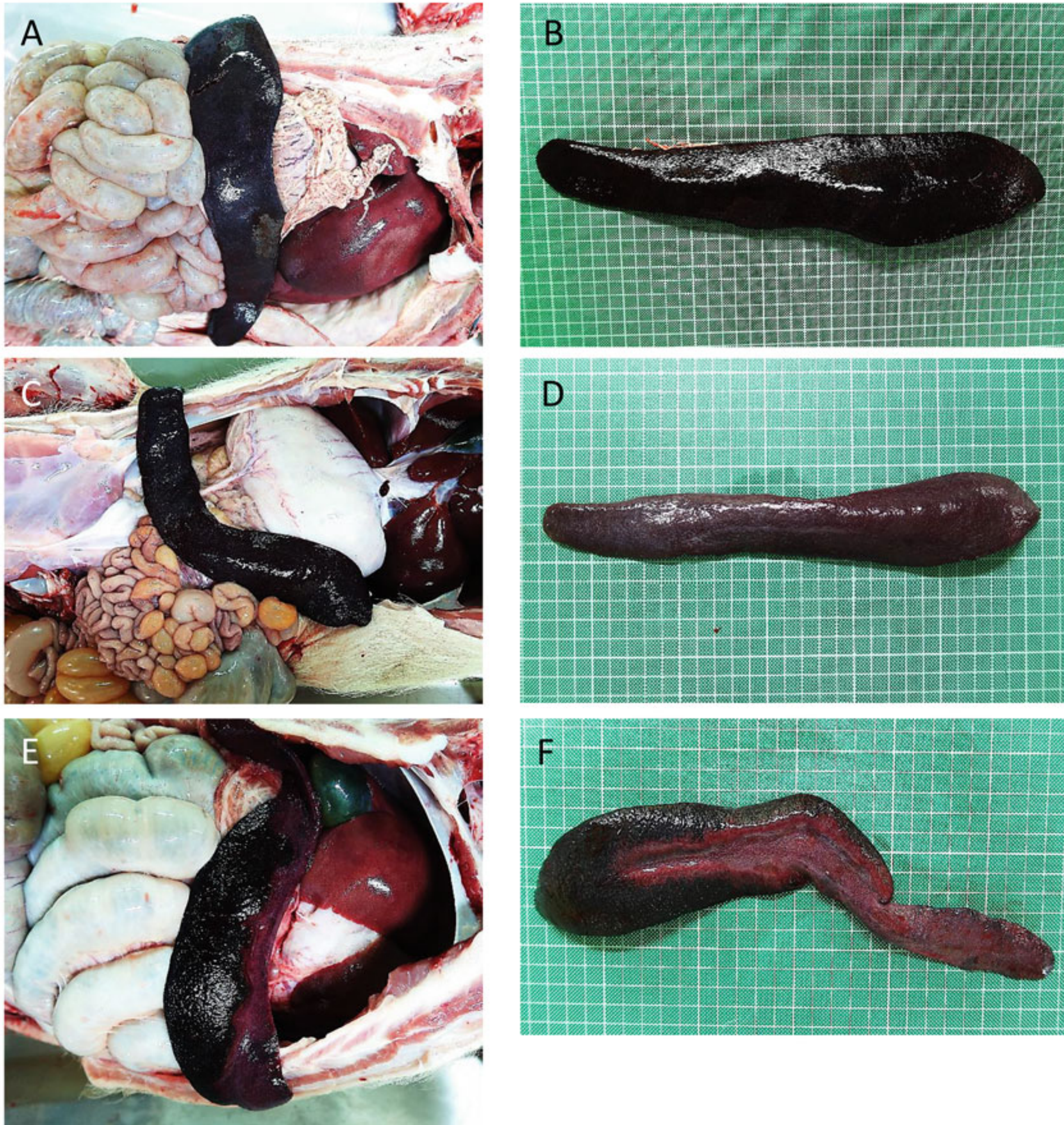

Fig. 15 (**a**) Spleen extending across the whole width of the abdominal cavity from side to side; (**b**) the spleen show a marked increase in size, rounded edges, friable consistency, and dark red to black color (hyperemic splenomegaly; acute ASF); (**c**, **d**) moderate hyperemic splenomegaly (acute ASF); (**e**, **f**) partial hyperemic splenomegaly affecting only some areas of the splenic parenchyma (subacute ASF)

them, petechiae, ecchymoses, and suggillations in serosal (Fig. 16j) and mucosal surfaces along with clotted blood digested (black) or fresh (red) mixed with stomach content are the most characteristic lesions. The stomach wall may be slightly thickened, while mucosal surfaces appear hyperemic and edematous (hemorrhagic gastritis) (Fig. 16k). Occasionally, erosions normally affecting the glandular mucosa may also be present (Fig. 16l).

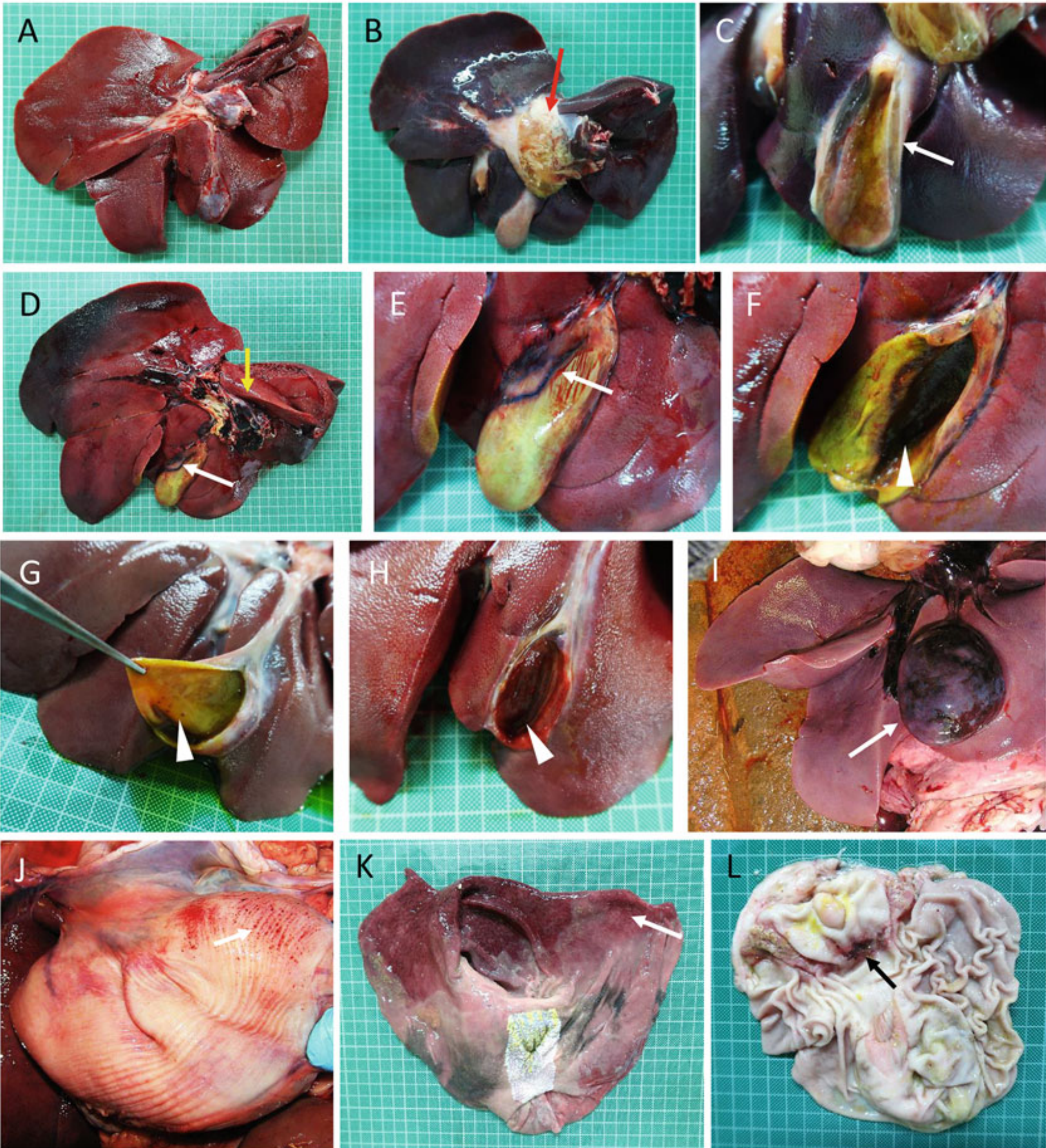

Fig. 16 Livers from pigs with subacute ASF. (**a**–**d**) Mild to moderate hepatomegaly and diffuse congestion; (**b**, **c**) severe edema in the hepatic hilum area (red arrow) and gall bladder wall showing wall distension due to edema (white arrow); (**d**–**i**) hemorrhages (petechiae and ecchymoses) on the serosa (white arrows) and submucosal surfaces (white arrowheads) of the gall bladders Hepatic lymph nodes with severe hemorrhagic lymphadenitis (yellow arrow); (**j**) multifocal petechiae (arrow) in the serosa of the stomach (subacute ASF); (**k**) stomach with hyperemic mucosal surfaces and submucosal petechiae (arrow) (subacute ASF); (**l**) focal ulcer (arrow) in the glandular mucosa of the stomach (subacute ASF)

18. Large segments of the small and large intestine show congestive changes and hemorrhagic lesions which are usually more severe in subacute forms of ASF. Intestinal loops appear distended. Multifocal petechial hemorrhages and ecchymoses are

usually observed in the serosa (Fig. 17a–c) along with thickened intestinal walls, while mucosal surfaces show different degrees of congestions, extensive hemorrhages (Fig. 17d–f), and bloody content (hemorrhagic enteritis) with medium consistency bloodstained feces or bloody liquid stool. Indicate specifically the segment affected by the inflammatory changes (duodenitis, jejunitis, ileitis, colitis, typhlitis, or proctitis).

Occasionally, the presence of petechiae and necrotic areas are observed in the pancreas of pigs that developed subacute forms of ASF.

19. The presence of circulatory disorders in the kidneys is considered as one of the hallmarks of ASF. In acute forms, focal small hemorrhages (Fig. 17g) along with mild congestive changes are usually observed in the renal cortex, medulla, and renal pelvis. In subacute forms, circulatory disorders may become severe and widespread extending through large areas of renal parenchyma (Fig. 17h). Although the renal capsule may detach easily from the renal cortex, it may present with plenty of petechiae, ecchymoses, and suggillations (Fig. 17i). The presence of moderate to severe perirenal edema of gelatinous consistency and yellowish in color is also a frequent finding (Fig. 17j). The kidneys may also display pelvic dilation, edema in the pelvis, diffuse severe congestion (generalized renal darkening), and severe interstitial hemorrhagic lesions of different size (petechiae, ecchymoses, and suggillations), which affect wide areas of the cortex, medulla, and renal pelvis (Fig. 17k–m). The presence of infarcts, especially in cortex (pale areas surrounded by a hyperemic border), may be occasionally observed.

    Evaluate the size of the adrenal glands and slice them. The normal ratio adrenal cortex-medulla should be roughly 1:2. Hemorrhages are occasionally observed in the inner cortical and medullary region.

20. In the urinary bladder, the vascular lesions may range from the presence of occasional petechial hemorrhages in the submucosa without any other circulatory disorder in ASF acute forms (Fig. 18a, b) to the appearance of gelatinous infiltrations in the urinary bladder wall as consequence of edema and the presence of widespread severe hemorrhages (petechiae, ecchymoses, and suggillations) in the submucosa and serosa along with blood clots covering large areas of the mucosal surface in ASF subacute forms (Fig. 18c–e). In sows, the existence of vascular lesions (hyperemia and hemorrhages) in the reproductive system is quite rare.

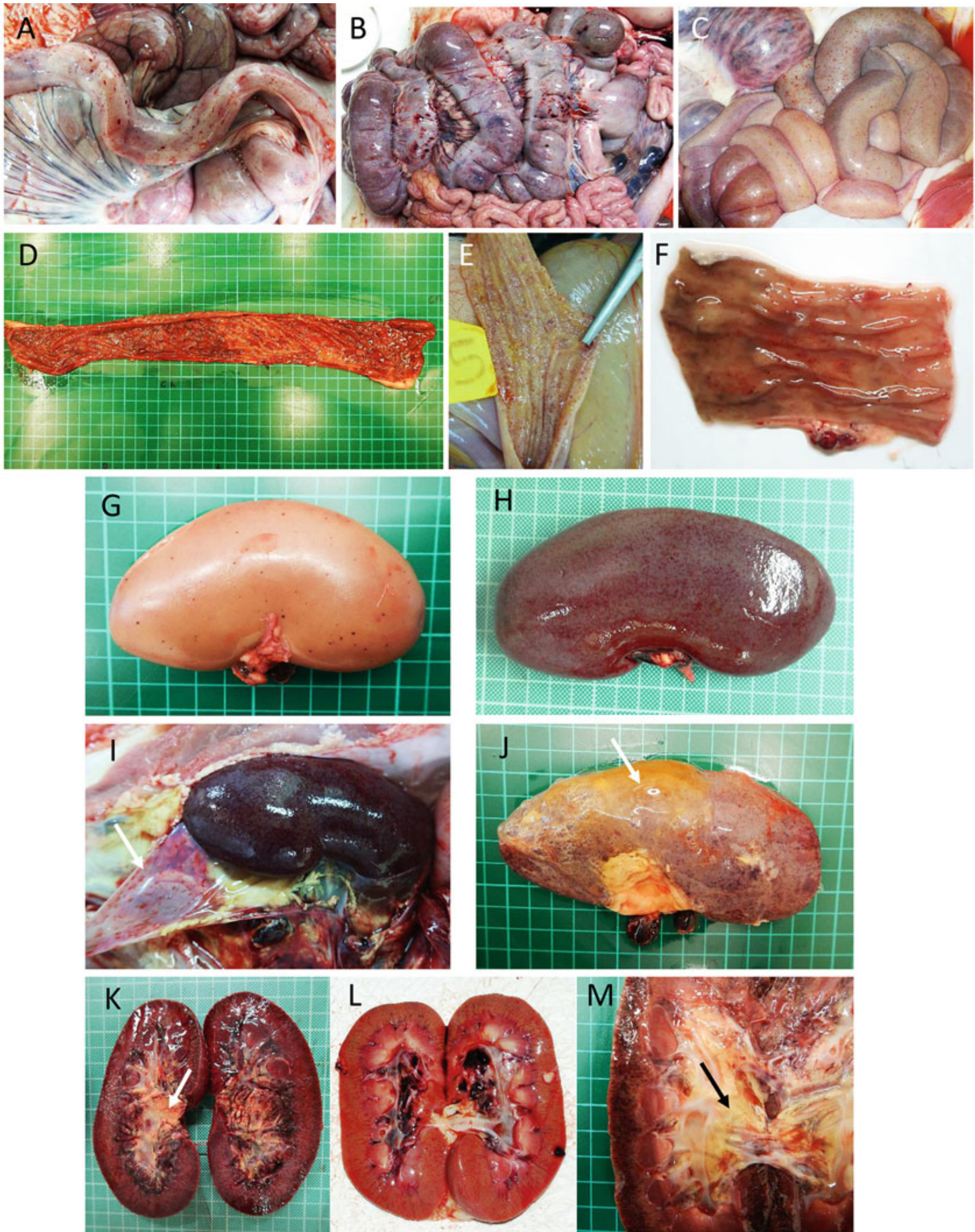

Fig. 17 Multifocal petechial hemorrhages and ecchymoses in the serosa of the ileum (**a**), colon (**b**), and jejunum (**c**) from pigs with subacute ASF; diffuse congestion and petechial hemorrhages in mucosal surfaces of jejunum (**d**), colon (**e**), and rectum (**f**) in pigs with subacute ASF; (**g**) petechiae in renal cortex (acute ASF); diffuse congestion and interstitial hemorrhages (petechiae, ecchymoses, and suggillations) in pigs with subacute ASF (**h**–**j**). Multifocal hemorrhages in renal capsule (**i**; arrow) and severe perirenal edema (**j**; arrow); (**k**–**m**) pelvic dilation and edema (arrows) along with congestion and severe interstitial hemorrhagic lesions in the cortex, medulla, and pelvis

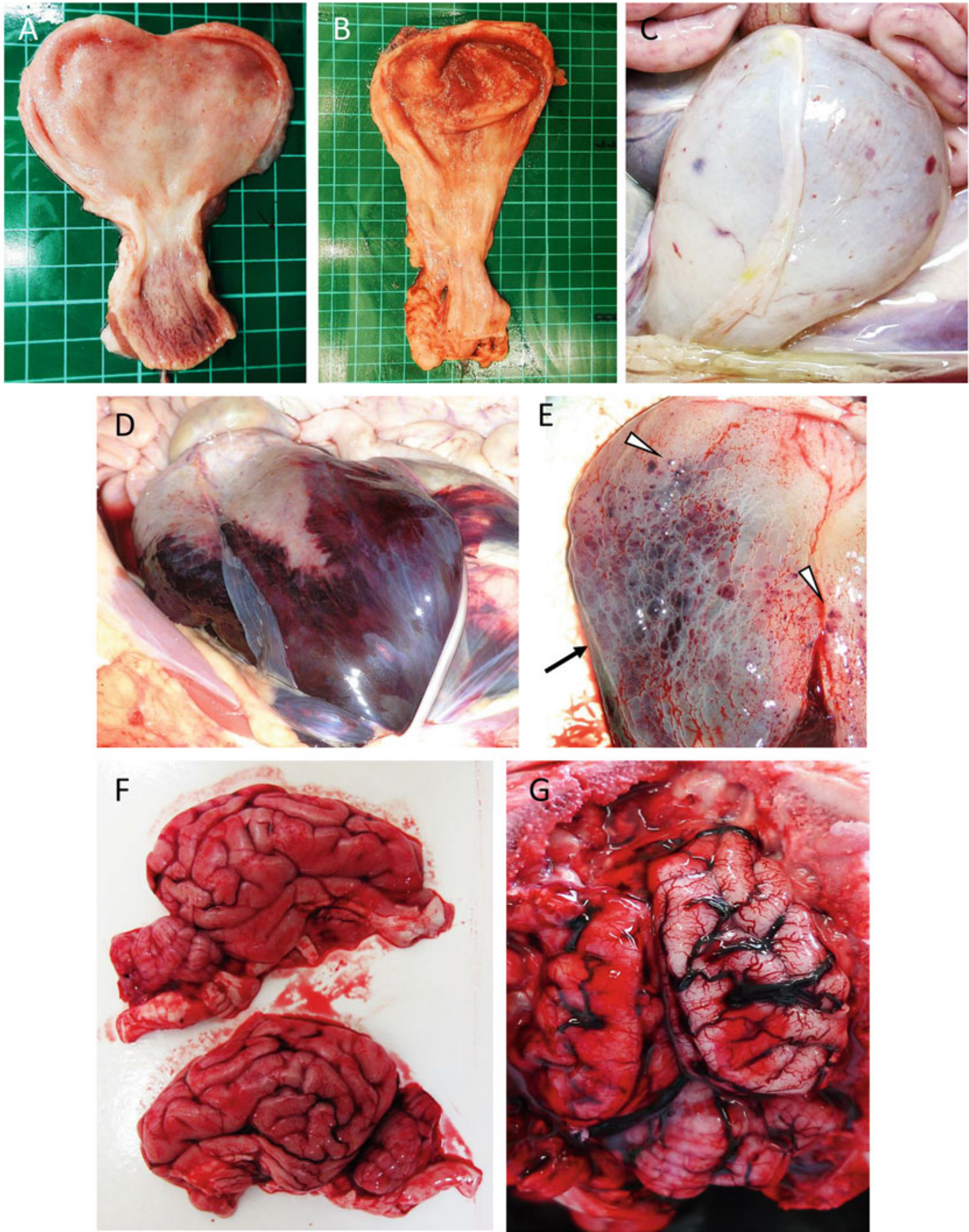

Fig. 18 (**a**, **b**) Urinary bladders showing mild to moderate congestion along with the presence of petechiae in mucosa (acute ASF); (**c**) urinary bladder from a pig with subacute ASF showing petechiae and ecchymoses in serosa; (**d**, **e**) urinary bladders from pigs with subacute ASF showing severe hemorrhages (petechiae, ecchymoses, and suggillations) in serosa and mucosa, edema in the urinary bladder wall (black arrow), and presence of blood clots on the mucosa surface (white arrowheads); (**f**) diffuse moderate congestion in the meninges (acute ASF); (**g**) diffuse severe congestion and severe hemorrhages in the meninges (subacute ASF)

21. During the macroscopic evaluations, lesions in the brain of pigs that suffer ASF are mild. Apart from moderate congestion in the meninges and choroid plexus in acute forms (Fig. 18f), no other gross lesions are usually observed. In subacute forms, vascular changes are slightly more marked, observing more severe congestive and edematous lesions as well as occasional petechiae and ecchymoses (Fig. 18g).

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Chapter 3

Preparation of Immunofluorescently Labeled Tissue Sections for Imaging at Low and High Magnifications in the Confocal Microscope

Jennifer Simpson and Philippa C. Hawes

Abstract

The confocal laser scanning microscope allows us to examine tissue sections in greater detail than a widefield fluorescence microscope. However, this requires samples to be better preserved than standard cryostat sections, which are not usually aldehyde-fixed. Thick sections (approximately 70 μm) of formaldehyde-fixed tissue can be cut using a vibrating microtome and subsequently labeled with primary and secondary fluorescent antibodies and/or fluorescent stains. When imaged in the confocal microscope, these samples allow us to collect high-resolution images, detailing the intracellular location of multiple proteins and structures. In this chapter, we describe the technique used to prepare vibrating microtome sections, using porcine tissue infected with African swine fever virus as an example. This technique can easily be applied to any animal tissue with any suitable combination of antibodies, depending on the hypothesis.

Key words African swine fever virus, Confocal microscopy, Vibrating microtome, Immunofluorescence labeling, Animal tissue sections, Aldehyde fixation

1 Introduction

When studying how viruses infect and replicate in host animals, investigating the cell biology of infection by microscopy is one of the most informative routes to follow. Immunofluorescence microscopy is popular because it allows researchers to confirm the presence of, and importantly identify the location of, specific proteins in cells. The ideal system in which to study virus–host cell interactions is in the host animal infected with a field strain virus, but this is often impractical, so cell culture models are regularly used with the general acceptance that they are representative of host animal cells.

Field strains of ASFV replicate poorly in commonly used cell lines, so an attenuated virus (Ba71v) was developed by Enjuanes et al. in 1976 [1] which infects Vero cells. Vero cells grow well in

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cell culture, and it is possible to infect a high proportion of these cells with Ba71v. In vitro infection systems utilizing immortalized cell lines from non-host species must be questioned, however, as there could be significant differences, at any stage, between infection in cell culture and in an animal. A comparison of infection in a monkey kidney epithelial cell line by an attenuated virus, with infection by a field strain in swine, must be considered to establish if cell culture infection is truly representative of infection in the host. Field strains of ASFV have a tropism for macrophages [2], so an infection system using primary macrophages isolated from pigs could potentially be a useful compromise between animal and cell culture models. Macrophages can be collected, purified, and plated onto coverslips before infection with field strains of the virus. While less artificial than using cell lines, infection with field strains of ASFV has achieved low infection rates in the past [3], thus making these systems difficult to interpret and time-consuming to optimize. Therefore, the Ba71v/Vero cell system has been used for many years to study the cell biology of infection in host cells using indirect fluorescence labeling and imaging in a widefield or confocal microscope [4–6]. However, for a more realistic representation of infection, a method of preparing tissue samples from host animals infected with a field strain virus for immunofluorescence microscopy is needed.

When preparing animal tissue for immunofluorescence studies, small blocks of the required organs are cut immediately postmortem and prepared for sectioning in either the cryostat or the vibrating microtome. For the more commonly used cryostat sectioning, fresh tissue blocks are placed in molds, surrounded by a liquid matrix which will hold the blocks in place during sectioning, and frozen in solid CO_2/ethanol slush at −80 °C. Blocks can then be stored indefinitely at −80 °C and, when convenient, 7–10 μm sections cut using a cryostat at −20 °C. Sections are collected onto glass slides and labeled at room temperature with relevant primary and fluorescent secondary antibodies before being imaged in a widefield or confocal microscope. This is a well-established method for preparing tissue sections so we will not discuss the details of the protocol here.

The advantage of using cryostat sections is that they provide the best opportunity for primary antibodies to recognize relevant antigens because samples have not been fixed with aldehyde fixatives. Aldehydes, most commonly formaldehyde for immunofluorescence, can alter protein structure so that antibodies may not bind as efficiently to antigens as they would in unfixed cells. Each antibody has a different sensitivity to fixation. In general, the majority of antibodies will still bind effectively to formaldehyde fixed antigens; however, some antibodies are particularly sensitive to fixation. It is not possible to predict whether an antibody will or will not be sensitive to fixation; each has to be tested individually. Therefore,

using unfixed tissue allows all antibodies the opportunity to bind to relevant and available antigens.

The disadvantage of using cryostat sections and unfixed samples is that tissue structure is not preserved. This may not be important if samples are to be imaged at low magnifications (e.g., using ×10 or ×20 lenses), but if higher resolution images are to be collected using a confocal microscope, the damage to the tissue structure is more obvious and limits the useful obtainable information.

An alternative method for the preparation of tissue sections for imaging in the confocal microscope uses a vibrating microtome rather than a cryostat to cut sections. This method needs planning into any experiment as the preparation starts immediately postmortem when small tissue blocks are cut from the organs of interest and immersed in aldehyde fixative. Sections are cut at room temperature in a vibrating microtome and labeled using the relevant primary and fluorescent secondary antibodies before being imaged in a confocal microscope. These sections are much thicker than those cut in a cryostat (70 μm compared to 7–10 μm), so it is not possible to image these in a standard widefield microscope; it has to be a confocal microscope. The advantage of this technique is that the tissue is well-preserved and it is possible to image organ structure (e.g., spleen) at low magnification (Fig. 1) and individual cells at higher magnifications (Fig. 2), but the disadvantage is that the antigenicity may be compromised for some antibodies. Regarding the duration of fixation, a balance has to be struck between preservation of structure and preservation of antigenicity. For this reason, samples are not stored in fixative; after fixation they are stored at 4 °C in PBS for a maximum of 4 weeks. After this time, the tissue will deteriorate.

This vibrating microtome technique makes it possible to acquire much higher resolution images compared with the cryostat technique, containing more detailed localization of labeled proteins comparable with images captured from equivalent infected cell cultures.

2 Materials

2.1 Chemical Reagents

1. 4% formaldehyde fixative (*see* **Note 1**).
2. Phosphate buffered saline (PBS).
3. 0.1% Triton X-100 in PBS.
4. 0.5% bovine serum albumin in phosphate-buffered saline (PBS/BSA).
5. Primary antibodies diluted in PBS/BSA.
6. Secondary antibodies diluted in PBS/BSA.

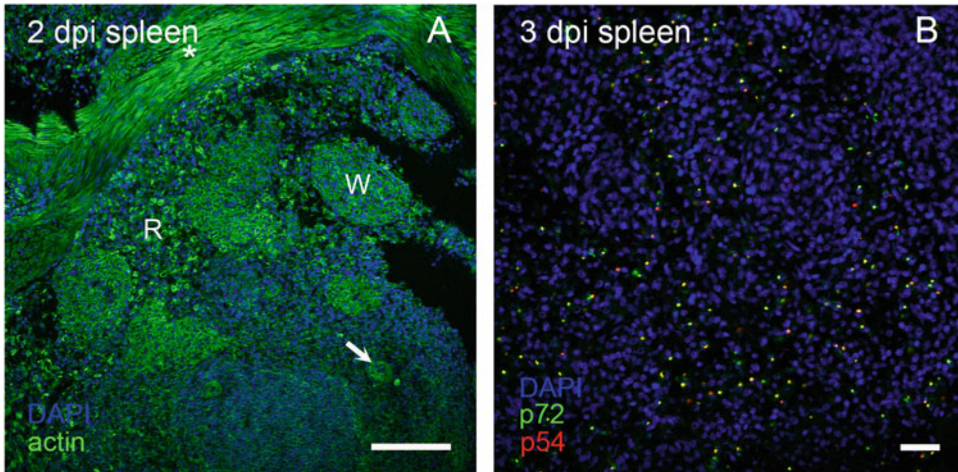

Fig. 1 Confocal imaging of ASFV-infected porcine spleen at low magnifications. Spleen tissue was collected from pigs killed at the times indicated, fixed, and processed for immunofluorescence labeling according to the method described here. Tissue from 2 days post infection (dpi) spleen contained low levels of viral protein, and the structure was still intact. (**a**) Actin staining (green) illustrated the gross structure of the spleen. The white pulp (W) contains mainly B cells, and the red pulp (R) contains mainly macrophages. Endothelial cells of capillaries are identifiable (arrow), and the whole spleen is protected by a layer of connective tissue (*). (**b**) At 3 dpi the red pulp contained many infected cells, indicated by co-localization of two ASFV antibodies, anti-p72 (green) and anti-p54 (red). Scale bar (**a**) = 100 μm, (**b**) = 50 μm

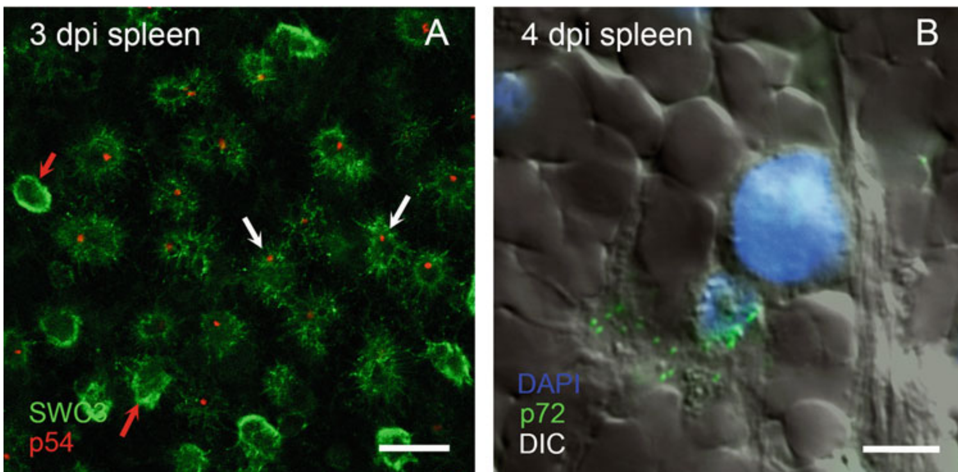

Fig. 2 Confocal imaging of ASFV-infected porcine spleen at higher magnifications. (**a**) Individual infected cells are clearly visible, labeled with ASFV anti-p54 antibody (red), and exhibit classic macrophage shape. SWC3 antibody indicates two populations of myeloid cells, macrophages (white arrows) and another uninfected cell type (red arrows). (D) DIC image of an infected cell at high magnification surrounded by red blood cells in 4 dpi spleen shows discrete bumps in the cell cytoplasm which correspond to p72 labeling (green). Scale bar (**a**) = 30 μm, (**b**) = 5 μm

7. DAPI 1:5000.
8. Phalloidin (optional).
9. Ultrapure water.
10. Mounting medium.

2.2 Hardware

1. Blunt forceps.
2. Marker pen for labeling Sterilin pots.
3. Thin paint brushes, with most bristles removed.
4. Ceramic dimple dishes.
5. Vibrating microtome with associated accessories (*see* **Note 2**).
6. Hot box/incubator set to 37 °C.
7. Black plastic/rubber sheet.
8. Confocal microscope.

2.3 Consumables

1. 20 mL Sterilin pots.
2. Petri dishes.
3. Disposable scalpels.
4. Disposable 1 mL Pasteur pipettes.
5. Filter paper.
6. Superglue.
7. Appropriate blades for vibrating microtome.
8. Parafilm®.
9. 24-well cell culture plate.
10. Pipette with tips for preparing antibodies.
11. 1.5 mL microcentrifuge tube for preparing antibodies.
12. 5 mL plastic screw top jars for preparing antibodies/DAPI/ phalloidin.
13. Glass microscope slides.
14. Hybridization gene frames (if cutting by hand).
15. Appropriately sized coverslips.
16. Clear nail varnish.

3 Methods

Ensure all relevant animal ethics protocols and licenses are put into place and are up to date. If you are not licensed to undertake veterinary procedures, ensure organs are removed postmortem by the relevant license holder and passed to you for processing.

3.1 Collecting Samples for Vibrating Microtome Sectioning

In the postmortem room:

1. Before starting, ensure your work area is ready with clean Petri dishes for cutting tissue, fresh scalpels for each tissue, labeled 20 mL Sterilin pots containing 10 mL fixative (*see* **Note 1**) and relevant waste receptacles.
2. Relevant organs should be removed from the animal immediately postmortem by the license holder and handed to you. In a clean Petri dish base or upturned lid, cut the tissue into 5 mm^3 pieces, taking into account the orientation if necessary, for example, if cutting skin epithelium. Do this as quickly as possible.
3. Place one or two (no more) blocks into a labeled Sterilin pot containing formaldehyde fixative. Gently shake to ensure blocks are surrounded by free-flowing fixative. Repeat for every required organ. Keep work area clean and clear and dispose of waste according to local regulations.
4. Once all samples have been collected, transfer the Sterilin pots to the relevant lab and leave in a fume hood at room temperature for a total of 3 hours (*see* **Note 3**). If possible, place on a rotator or shaker at low speed.
5. Meanwhile, label fresh Sterilin pots and put 10 mL PBS into each one.
6. After fixation, carefully remove each tissue block from the fixative with blunt forceps and place into a Sterilin pot containing PBS. Do not put more than two blocks into each Sterilin. Dispose of the used formaldehyde solution according to local regulations.
7. Samples can be stored for a limited period at 4 °C before sectioning (*see* **Note 4**).

3.2 Cutting with a Vibrating Microtome

1. Prepare the microtome and work area before handling samples. Lay the specimen plate by the side of the microtome and fill the cutting chamber with cold PBS.
2. Remove the Sterilin containing the sample from the fridge. If the sample needs trimming: fill a Petri dish with cold PBS and cut to a reasonable size using a scalpel (about 5 mm^3).
3. Apply a small droplet of superglue to the center of the specimen plate, and then working quickly using forceps, pick up the sample, touch it gently on blotting paper for 1–2 seconds, and then place it in the superglue droplet. It will only take a few seconds for the sample to bond.
4. Load the specimen plate into the PBS in the specimen chamber in the correct orientation with reference to the microtome blade. Lock in place.

5. Move the blade into position, ensuring correct alignment so that the blade is parallel to the leading edge of the sample block.
6. Set the microtome controls to cut 70 μm sections, with the correct cutting speed/frequency (*see* **Note 5**) and set the microtome running. In our experience, some soft tissues do not cut easily in the microtome. In these circumstances, cut sections by hand under PBS, as thinly as possible, using a fresh scalpel blade. These sections will be thicker than 70 μm; however, they will still be suitable for imaging.
7. Once a section is cut, use a small paintbrush to remove it from the chamber and place it into a fresh, labeled Sterilin or, if you are going to continue with the labeling procedure immediately, a ceramic dimple dish containing PBS.
8. Continue until all sections have been collected. Reset the microtome controls so that the blade is out of the way, remove the specimen plate from the chamber, and use a fresh razor blade to carefully remove the tissue block (and superglue remnants) from the plate and put it back into the original Sterilin pot containing PBS. This can be stored in the fridge for use again if necessary (*see* **Note 4**).
9. If wanting to cut another sample, dry the specimen plate before repeating the process from Step 2.
10. If finished, dispose of any tissue remnants according to local guidance, discard the PBS from the specimen chamber, and rinse all accessories with water. Allow to dry.
11. Turn off the vibrating microtome according to local protocols and clear work area.
12. Sections can either be labeled straight away (preferable) or stored at 4 °C in PBS and labeled within 1 or 2 days.

3.3 Immunofluorescence Labeling

This procedure is to be carried out either by moving tissue sections through droplets of reagents laid out on a hydrophobic surface, for example, Parafilm®, or in a 24-well cell culture plate by removing and replacing reagents as necessary. If using the droplet method, tissue sections can be moved using a small paintbrush with most of its bristles removed. If using a 24-well cell culture plate, samples can be agitated gently during incubation steps. *Do not allow the tissue sections to dry out at any stage in the procedure*. During incubations, cover droplets with a large Petri dish lid, or use the cell culture plate lid. Using a black plastic/rubber background makes it easier to see the sections when in the droplets or 24-well cell culture plates.

Controls (*see* **Note 6**): it is important to include all relevant controls every time a labeling procedure is carried out. The minimum controls needed are:

- No first antibody control (NFA) – leave samples in PBS/BSA while experiments are in primary antibody. This tests the specificity of the primary antibody.
- Double labeling controls – to test for cross reactivity of the primary antibodies across species, e.g., use a rabbit secondary antibody with a mouse primary antibody and vice versa.

1. Permeabilize sections with 0.1% Triton X100 in PBS at room temperature for approximately 60 minutes (*see* **Note 7**).
2. Block nonspecific binding with PBS/BSA overnight in a moist chamber to reduce the risk of evaporation. The easiest way to do this is to place a couple of pieces of damp filter paper into a Petri dish lid, with a hydrophobic surface on top, e.g., Parafilm® or a rubber pad, and place the sections into droplets on top of this. Put the Petri dish base over the droplets as a lid and leave at room temperature.
3. The next morning, place the tissue sections in 150–200 μl primary antibody droplets (NFA controls to be placed into PBS/BSA) laid out on Parafilm® or in a 24-well cell culture plate, inside a 37 °C chamber for approximately 90 minutes. Make sure the droplets or dishes are covered to avoid evaporation (*see* **Note 8**).
4. At room temperature, wash the sections in PBS either by laying out ten droplets on Parafilm® and moving the tissue sections between droplets for approximately 2 minutes each or by removing the primary antibody from the relevant well in the cell culture plate and replacing with 500 μl PBS three times for approximately 5 minutes each wash.
5. Repeat Step 3 with species-specific secondary fluorescent conjugate for approximately 90 minutes.
6. Repeat Step 4 (for phalloidin staining, *see* **Note 9**).
7. To stain the nuclei and DNA, wash for approximately 30 minutes in 1:5000 DAPI (in water) using either the droplets on Parafilm® or the 24-well cell culture plate method.
8. Wash in ultrapure water for a few minutes using either the droplet or 24-well cell culture plate method.
9. While the sections are in water, place a small drop of an aqueous mounting medium, e.g., Vectashield®, on a glass slide (*see* **Note 10**).
10. Using a dissecting microscope, carefully orientate the tissue section in mounting medium and gently lower an appropriately sized glass coverslip over the tissue section to avoid air bubbles forming. (For hand-cut sections, *see* **Note 11**).

11. Seal the coverslips with clear nail varnish (if using hybridization gene frames for hand-cut tissue, glass coverslips adhere to them and do not need sealing) and allow to dry.
12. Samples are ready for viewing in the confocal microscope. These samples are not suitable for viewing in a standard wide-field fluorescence microscope because of the thickness of the sections.

4 Notes

1. Make up 4% formaldehyde solution in phosphate-buffered saline using paraformaldehyde powder, with gentle heating and stirring until the solution is clear. Do not use formalin to make up fixative as this contains additives such as methanol.
2. Several models of vibrating microtome (sometimes referred to as a vibratome) are available to purchase, e.g., the 7000smz-2, 5100mz-Plus, or the Leica VT1000.
3. The 3-hour fixation time in this protocol is largely based on the practicalities of how long it takes to move tissue between our postmortem rooms and the relevant labs for subsequent processing. Three hours also allows enough time for the fixative to fully penetrate small tissue blocks, so it should be considered the optimum fixation time. The longer tissue is in fixative unnecessarily, the more likely it is that antigens will become damaged by protein cross-linking. However, the practicalities of physical location, ethics protocols (particularly in the case of human tissue), and/or high containment protocols may make it necessary to fix samples for longer. If this is the case, then these constraints take priority, and it has to be accepted that antigenicity may suffer.
4. Formaldehyde protein–protein cross-linkages are not permanent, so that when samples are removed from fixative and stored in PBS, these linkages gradually start to break down. It is therefore important to cut, label, and image samples as soon as possible after fixation. Tissue blocks can be stored for up to 4 weeks with minimal noticeable degradation; however, the sooner they can be post-processed the better, to give the best opportunity of recording an accurate result from valuable tissue.
5. The frequency and speed of the blade can be altered depending on the instrument used and the type of sample. This can be optimized depending on local conditions.
6. As with any experiment, it is essential that a range of controls are built into the experimental design. This is particularly true if

the primary and secondary antibodies are being used for the first time on vibrating microtome sections. If it is possible to include positive control tissue, then this is preferable; however, it is not always feasible. Negative labeling controls are the minimum control required, with the "no first antibody" being the most important. Tissue sections are prone to autofluorescence so negative controls are essential to prove primary antibodies are specific. Take extra time to carefully consider what controls are necessary for each experiment.

7. The minimum droplet size is considered to be 150–200 μl. This is also the minimum volume to be used in 24-well plates. This is the volume used for primary and secondary antibodies; however for other, not so precious, reagents, e.g., PBS, PBS/BSA or Triton X-100, larger volumes can be used. Larger volumes of approximately 500 μl are preferable for wash steps.
8. As with other immunofluorescence protocols, it is acceptable to double label concurrently if both primary antibodies are raised in different species (or are different subclasses), e.g., when using a mouse monoclonal antibody and a rabbit polyclonal antibody. Mix the antibodies so that the combined concentrations are correct for each individual antibody. For example, if antibody A should be used at 1:1000 and antibody B at 1:500, prepare 1000 μl of antibody A, and add 2 μl of antibody B.
9. Using the actin stain phalloidin provides a useful gross morphological overview of a section at low magnifications. To use, incubate directly conjugated phalloidin (e.g., Alexa Fluor™ 488 Phalloidin) made up at 1:25 in PBS for 10 minutes during the last PBS wash after the secondary antibody incubation.
10. Choose the correct mounting medium depending on the secondary antibody used. Antifade reagents in mounting media work particularly well with different fluorophores, e.g., when using Alexa Fluor™ fluorophores, Vectashield® is particularly good at avoiding or reducing bleaching induced by the lasers during imaging. In our experience, the non-setting Vectashield® without DAPI is the best to use when carrying out this protocol. DAPI is introduced during a separate staining step prior to mounting.
11. If mounting hand-cut sections, use hybridization gene frames to contain the mounting medium and compensate for the extra thickness of the sample. They can be sealed with 22 mm^2 glass coverslips.

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Chapter 4

Primary Macrophage Culture from Porcine Blood and Lungs

Lynnette C. Goatley, Rachel Nash, and Christopher L. Netherton

Abstract

Primary cultures represent the most reliable method to isolate and propagate field isolates of African swine fever virus (ASFV). Within the pig ASFV predominantly targets the reticuloendothelial system for replication; therefore, primary macrophage cell cultures are commonly used to isolate, propagate, and study the virus life cycle in the laboratory. In this chapter we will describe methods for the direct isolation of pulmonary alveolar macrophages by lung lavage and the culture of monocyte-derived macrophages from pig blood. We also include a method for the positive selection of CD14+ monocytes as a source for monocyte-derived macrophages from pig blood using microbeads.

Key words African swine fever, Virus, Monocyte, Macrophage, Peripheral blood mononuclear cells, Buffy coat

1 Introduction

African swine fever virus (ASFV) productively infects macrophage cells in the domestic pig, and field isolates of the virus have been successfully propagated in in vitro cultures of such cell types since the early 1960s [1]. ASFV has also been successfully adapted to grow in continuous cell lines derived from pigs [2] and African green monkeys [3, 4]; however, such adaption leads to significant genome alterations [4–7] and attenuation. ASFV has been successfully propagated in MA-104 clone 1 cells [8]; however, both these and other cell lines derived from African green monkey fall under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). More recently promising, continuous macrophage cell lines have been developed that support the growth of ASFV [9, 10] which may ultimately replace the use of primary cultures. Until these macrophage lines are more widely used and validated, the 60 years plus experience with primary cultures makes them an ideal choice for propagating virus and has the added advantage of being a well-tested model for studying host–pathogen interactions in vitro. Although ASFV is generally

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considered stable after short-term culture in primary macrophages, repeated passage can also lead to attenuation [2, 11–13]. Here, we describe the culture of monocyte-derived macrophages from peripheral blood mononuclear cells (PBMCs) from blood and pulmonary alveolar macrophages (PAMs) from porcine lungs. Obtaining blood samples has the advantage that they can be carried out without euthanizing a pig; however, the number of leukocytes is usually low, and approximately only 10% of the populations are monocytes. Monocytes need to be differentiated into monocyte-derived macrophages (3–5 days) before being susceptible to ASFV. In contrast, lung lavages produce large numbers of leukocytes which consist of approximately 90% macrophages. Both PBMCs and alveolar macrophages can be successfully frozen with very little loss of viability of cells and with no loss of virus susceptibility. Primary macrophage cultures derived from bone marrow have also been used extensively for isolating and propagating ASFV, and a method for this is contained within Chap. 5.

2 Materials

All plasticware should be tissue culture grade and sterile. All reagents and buffers should be tissue culture grade, and solutions should be sterilized by autoclaving or filtration. Maintain sterility by handling samples in a biological safety cabinet according to good laboratory practice.

1. Biological safety cabinet.
2. Polypropylene conical tubes (50 mL).
3. Centrifuge (capable of spinning 50 mL conical tubes).
4. Pipette gun.
5. Serological pipettes (10 or 25 mL).
6. Aerosol-resistant filter tips.
7. Phosphate-buffered saline, divalent cation free (PBS).
8. Distilled water.
9. Red blood cell lysis buffer: 155 mM ammonium chloride, 12 mM sodium hydrogen carbonate, 0.1 mM ethylenediaminetetraacetic acid (*see* **Note 1**).
10. Hemocytometer.
11. 0.4% (w/v) trypan blue.

2.1 Bronchoalveolar Lavage

1. Pig lungs (*see* **Note 2**).
2. Porcine alveolar macrophage (PAM) media: Roswell Park Memorial Institute 1640 (RPMI 1640) media supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine or

glutamax, 25 mM HEPES, 100 μg/mL penicillin, 100 U/mL streptomycin (*see* **Note 3**).
3. Sterile glass beaker, volume dependent on the size of the pig.
4. Clamp stand.
5. Clamping scissor or tweezer to firmly clamp the trachea to allow the lungs to be suspended upright.

2.2 Blood-Derived Macrophages Using Dextran

1. Porcine blood (*see* **Note 4**).
2. Anticoagulant (*see* **Note 4)**: Any one of EDTA (10% ethylenediaminetetraacetic acid tripotassium salt dihydrate in water), heparin (200 U/mL), sodium citrate (3.2% in water).
3. Dextran solution: 6% (w/v) dextran from *Leuconostoc* spp. (M_r ~ 100,000) and 0.85% (w/v) sodium chloride in water.
4. Water bath set at 37°C.
5. Blood macrophage media: Dulbecco's modified Eagles media (DMEM) supplemented with 30% porcine serum (*see* **Note 5**), 2 mM glutamine or glutamax, 25 mM HEPES, 100 μg/mL penicillin, 100 U/mL streptomycin.

2.3 Blood-Derived Macrophages Using Density Gradient Centrifugation

1. Porcine blood (*see* **Note 4**).
2. Anticoagulant (*see* **Note 4)**: Any one of EDTA (10% ethylenediaminetetraacetic acid tripotassium salt dihydrate in water), heparin (200 U/mL), sodium citrate (3.2% in water).
3. Histopaque-1077/1083 or Ficoll 1.077/ 1.084.
4. Water bath set at 37 °C.
5. Blood macrophage media: Dulbecco's modified Eagles media (DMEM) supplemented with 30% porcine serum (*see* **Note 5**), 2 mM glutamine or glutamax, 25 mM HEPES, 100 μg/mL penicillin, 100 U/mL streptomycin.

2.4 Selection of CD14 + Monocytes and Macrophages

1. Human CD14+ microbeads and LS columns.
2. MACS buffer: Ca/Mg-free PBS, 2% heat inactivated FBS, and 2 mM EDTA.
3. MACS separator.

3 Method

3.1 Bronchoalveolar Lavage

1. Remove the whole lungs and attached trachea from the pig postmortem; the heart may be left attached; however, if it is removed, care must be taken to ensure the lungs are not cut while doing so. Inspect the lungs to make sure they are healthy and intact. In addition, the trachea should be sealed with a small plastic bag and tape or cable ties to prevent blood

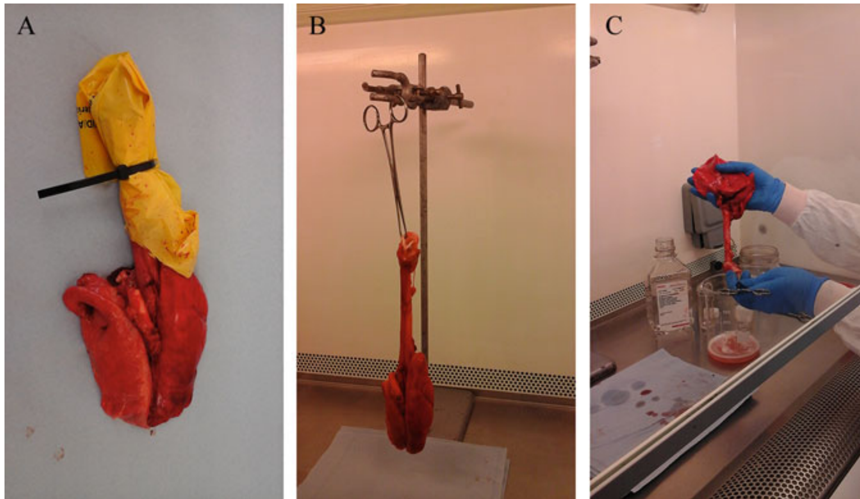

Fig. 1 Preparing macrophages from lung lavage. (**a**) A plastic bag covering the trachea opening to prevent the ingression of blood into the lungs. (**b**) Lungs suspended from clamp scissors and clamp stand. To suspend the lungs, attach the clamp scissors to one side of the top of the trachea and place the scissor clamp onto the arm of the clamp stand. (**c**) Decanting lavage from the lungs into a beaker

ingression and to provide a measure of sterility (Fig. 1A). Place the lungs in a suitable container for transfer to the laboratory; for a 4-to 6-week-old pig, we use reusable rubber-sealed glass jars.

2. Open the container within a biological safety cabinet, wipe down the lungs using tissue to remove any excess blood, and remove the bag. Hang the lungs by attaching the clamp scissors to one side of the top of the trachea and place the scissor clamp onto the arm of the clamp stand (Fig. 1B).
3. Fill the lungs with PBS either using serological pipettes or by decanting from a 50 mL conical tube (*see* **Note 6**). Do not over fill the lungs or they will start to disintegrate; we commonly use 25 mL of PBS in very small lungs (from a 4-week-old pig) and 50 mL in larger lungs. This volume can be increased if using older pigs. In order to dislodge the alveolar macrophages, gently massage the lungs, making sure that all surfaces are covered.
4. Remove the lungs from the apparatus and decant into a glass beaker (Fig. 1C). If the lungs contain a lot of blood or blood clots, the lungs could be compromised. Discard this first lavage and repeat the wash. If this is still contaminated, discard the lungs.
5. Reattach the lungs to the apparatus and repeat Steps 3 and 4 until the decanted fluid is clear.
6. Transfer lung lavage from beaker to 50 mL conical tubes.

7. Centrifuge the lung lavage (400 *g*, 10 min, room temperature).
8. Discard the supernatant and resuspend cell pellets by knocking the tubes together. Add fresh PBS to half of the tubes and pipette up and down to fully resuspend the cells and then add resuspended cells to the remaining tubes.
9. Repeat wash Step 8 (this will progressively concentrate the cells into fewer and fewer tubes).
10. Inspect tubes for red blood cell contamination. If there are relatively few red blood cells, then these may be ignored, particularly if the cells are going to be frozen. Moderate contamination can be removed in Step 11; otherwise go to Step 12 (*see* **Note** 7).
11. Add 5 mL red blood cell lysis buffer to cell pellet and pipette to resuspend the cells, incubate at room temperature for 5 min, and then add 40 to 45mL PBS and mix (*see* **Note 8**). Centrifuge at 400 *g*, 10 min, at room temperature.
12. Wash cells with PBS one last time and then resuspend in 25 mL PBS. Take 100 μl of resuspended cells, add to 800 μl PBS and 100 μl trypan blue solution, and mix. Transfer to a hemocytometer and count clear cells (dead cells will take up the trypan blue dye) and determine cell concentration.

    Cell concentration = (number of cells counted × dilution factor) ÷ volume counted. For a typical hemocytometer, the volume of one of the big squares is 0.1 μl for a 0.1 mm depth chamber or 0.2 μl for a 0.2 mm depth chamber (*see* **Note 8**). Multiply the cell concentration by the volume of PBS the cells are resuspended in to derive the total number of cells.
13. Centrifuge at 400 *g*, 10 min, at room temperature and decant the PBS. To use the cells immediately, resuspended in PAM media at 1×10^6 cells/mL and plate out at 5×10^5 cells/cm^2 in tissue culture grade multi-well plates or flasks.

    If freezing for later use, then resuspend at either 2.5×10^7 cells/mL or 5×10^7 cells/mL (depending on the number of cells obtained and the number of cells which will be required when thawed out). We routinely use freezing medium containing 10% dimethyl sulfoxide (DMSO, molecular grade) and 90% fetal bovine sera (FBS) and transfer to a freezing container and store overnight at −80 °C. If storing in liquid nitrogen, transfer within 48 h (*see* **Note 10**).
14. Once cells have adhered, change media and begin experiment (*see* **Notes 11** and **12**).

3.2 Culture of Blood-Derived Macrophages Using Dextran

1. Collect blood into tubes containing an anticoagulant (final concentration in blood/coagulant mix of 4 U/mL heparin, or 0.1% EDTA, or 0.32% sodium citrate) or use vacutainer tubes which have a coating of these solutions on the inside of the tubes.

2. Transfer blood to a biological safety cabinet and transfer to a 50 mL tube, add equal volume of 6% dextran solution, mix by inversion, and then incubate at room temperature for approximately 30 min or until the red blood cells have sedimented.
3. Carefully remove all the liquid above the sedimented red blood cells and transfer to a new tube and concentrate the leukocytes by centrifugation (400 *g*, for 10 min, at 4 °C) (*see* **Note 13**).
4. Discard the supernatant and inspect the pellet for contaminating red blood cells. If there is heavy contamination, add 5 mL of red blood cell lysis buffer per tube, resuspend the pellet, and incubate at room temperature for 5 min. Add 40 to 45 mL PBS (*see* **Note 8**). Centrifuge at 400 *g*, for 10 min, at 4 °C.
5. Wash the cells with PBS at least two more times (three washes in total).

   Resuspend cells in blood macrophage media. Take 100 μl of resuspended cells and add to 800 μl PBS and 100 μl trypan blue solution. Count clear cells and determine cell concentration.

   Cell concentration = (number of cells counted × dilution factor) ÷ volume counted. For a typical hemocytometer, the volume of one of the big squares is 0.1 μl for a 0.1 mm depth chamber or 0.2 μl for a 0.2 mm depth chamber (*see* **Note 9**).
6. Plate at least ten times the number of cells you eventually require, i.e., for a 24-well plate, aim for 5×10^5 macrophages, therefore plate 5×10^6 cells at a concentration of 5×10^6 cells/mL (*see* **Note 14**).
7. Leave at least for 2 h or overnight and then discard the media and non-adherent cells and replace with the same volume of media (~0.5 mL per cm^2) (*see* **Note 15**).
8. Cells will be susceptible to ASFV after 3 days of culture.

3.3 Culture of Blood-Derived Macrophages Using Density Gradient Centrifugation

1. Add 15 mL room temperature Histopaque or Ficoll-Paque to 50 mL tubes and overlay with pig blood diluted 1:1 with PBS.
2. Centrifuge the overlaid tubes at 1000 *g* for 30 min at room temperature with brake off.
3. Collect the buffy coat between the two layers using a pipette into a new 50 mL tube and top up with PBS.
4. Centrifuge at 400 *g* for 10 min at room temperature.
5. Discard the supernatant and inspect the pellet for contaminating red blood cells. If there is heavy contamination, add 5 mL of red blood cell lysis buffer per tube, resuspend the pellet, and incubate at room temperature for 5 min. Add 40 to 45 mL PBS (*see* **Note 8**). Centrifuge at 400 *g*, for 10 min.

6. Wash the cells with PBS at least two more times (three washes in total). Resuspend cells in blood macrophage media. Take 100 μl of resuspended cells and add to 800 μl PBS and 100 μl trypan blue solution. Count clear cells and determine cell concentration. Cell concentration = (number of cells counted × dilution factor) ÷ volume counted. For a typical hemocytometer, the volume of one of the big squares is 0.1 μl for a 0.1 mm depth chamber or 0.2 μl for a 0.2 mm depth chamber (*see* **Note 9**).
7. Plate at least ten times the number of cells you eventually require, i.e., for a 24-well plate, aim for 5×10^5 macrophages, therefore plate 5×10^6 cells at a concentration of 5×10^6 cells/mL (*see* **Note 14**).
8. Leave at least for 2 h or overnight and then discard the media and non-adherent cells and replace with the same volume of media (~0.5 mL per cm^2) (*see* **Note 15**).
9. Cells will be susceptible to ASFV after 3 days of culture.

3.4 Selection of CD14 + Monocytes and Macrophages

1. Resuspend PBMCs in PBS and add human CD14 microbeads at a concentration of 10 μl per 1×10^7 PBMCs.
2. Incubate for 15 min at room temperature and then wash the cells by adding 10–20 × labeling volume of MACS buffer. Centrifuge at 400 *g*, for 10 min, at 4 °C and resuspend at 2×10^8 cells/mL in MACS buffer.
3. Prepare a LS column by placing in the magnetic field of the MACS separator and adding 3 mL of MACS buffer and allowing the liquid to flow through the column.
4. Add the cells to the top of the column and allow to pass through the column.
5. Wash the column three times with 3 mL of MACS buffer.
6. Remove the column from the magnetic field and add 5 mL of MACS buffer. Use the plunger supplied with the column to firmly flush out the CD14+ cells.
7. Wash the cells by adding 5 mL of MACS buffer and centrifuging at 400 × *g*, for 10 min, at 4 °C. Repeat wash and resuspend in PBS or media.
8. Take 100 μl of resuspended cells and add to 800 μl PBS and 100 μl trypan blue solution. Count clear cells and determine cell concentration. Dilute the cells to required concentration for downstream protocol.

4 Notes

1. Ethylenediaminetetraacetic acid (EDTA) is poorly soluble in water except at pH >10. Either prepare a stock solution (0.5 M) and dilute or use of the potassium or sodium salts.
2. We normally collect the lungs from pigs between 4 and 8 weeks old, from a high health farm that has been weaned from their mother. The pigs need to be old enough to have developed immune systems (piglets rely on maternal antibodies in the first weeks until their own immune system matures), but larger animals make manipulation of the lungs cumbersome. Chapter 2 describes methods to remove the lungs from pigs during necropsy.
3. Addition of an antimycotic such as amphotericin B may be beneficial if contamination with fungus is commonly observed.
4. We have not observed any differences in the results obtained from blood prepared with heparin, EDTA, or sodium citrate as an anticoagulant. Blood may be collected into commercially available vacuum tubes coated with any one of the listed anticoagulants or into tubes or bottles containing any one of them.
5. Heterologous porcine serum can cause nonspecific hemadsorption which can be problematic if the blood macrophages are to be used for virus isolation. If possible use autologous serum or screen several batches prior to carrying out your experimental work.
6. It is important not to add the PBS too quickly here, and use of a 50 mL tube can help reduce the flow rate. If the PBS is not filling the lungs, then it is possible that the trachea has become stretched or there is an air pocket. These can be alleviated by gently lifting the base of the lungs when pouring. Air pockets can also be prevented by massaging the trachea or by dispensing PBS directly into the trachea using a serological pipette. Note that if the lungs have been damaged during their removal, then PBS may leak out; if one lobe has been damaged, it may be possible to tie off this lobe and recover sufficient cells from the other.
7. Heavy contamination with red blood cells is indicative of a potentially poor quality prep. Cultures derived from such preparations may be suitable for virus isolation but are less likely to be useful for more sensitive applications such as measuring immune responses or generating recombinant viruses.
8. Red blood cell lysis buffer is a more reliable method to remove red blood cells for inexperienced users; however, “flashing” with water can yield good results. Rather than adding lysis

buffer, simply place cells on ice, add ice cold water, mix briefly, and then immediately top up the tube with 40 to 45 mL PBS.

9. We routinely use hemocytometers for cell counting, but this could be substituted with an automated system if it is available in the lab.
10. While storage in liquid nitrogen is the best practice for long-term storage of most cell types, we have stored alveolar macrophages at −80 °C for up to a year without detectable degradation in sensitivity to African swine fever virus.
11. Typically we leave cells overnight, but macrophages and monocytes adhere quickly, and it is likely this could be shortened.
12. We routinely screen our PAMs for susceptibility to ASFV infection before an experiment as each pig is unique. Occasionally a prep is not susceptible and is discarded. Also, if the cells are to be used for more in-depth analysis (such as immune responses during an infection), further preliminary screening must be carried out to gauge the suitability of the cells for this analysis.
13. The ultimate goal of this method is to isolate monocytes which make approximately 10% of porcine blood mononuclear cells. This method is a relatively simple way to isolate porcine blood leukocytes. An even simpler approach would be to prepare a buffy coat of leukocytes by centrifuging blood diluted 50:50 with PBS. The buffy coat could then be washed and counted as from Step 4 of 3.2.
14. We use 30% porcine sera to differentiate blood-derived monocytes into macrophages; however, growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) can also be used.
15. Monocytes are isolated from leukocytes by virtue of their adherence to plastic.

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Chapter 5

Isolation of Porcine Bone Marrow Cells and Generation of Recombinant African Swine Fever Viruses

Anusyah Rathakrishnan, Ana Luisa Reis, Katy Moffat, and Linda K. Dixon

Abstract

Genetic manipulation of ASFV has been increasingly used not only for the development of live attenuated vaccines but also as an indispensable tool to further our understanding of the virus–host interactions. Here we present methods for isolation of porcine bone marrow cells and purification of recombinant ASFV using both chromogenic and fluorescent reporters. We also describe in detail a newly developed method to purify genetically modified ASFV using fluorescence-activated cell sorting (FACS).

Key words Porcine bone marrow cells, ASFV, FACS, Single-cell isolation, Recombinant virus, Titration

1 Introduction

African swine fever virus (ASFV), the sole member of the family *Asfarviridae*, is a large DNA virus with 150–167 genes, half of which remain uncharacterized [1]. ASFV causes African swine fever, a disease with lethality rates reaching 100% in pigs and wild boar following infection with virulent isolates [2]. The target cells for ASFV in nature are mainly porcine monocytes and macrophages. Macrophages derived from pig blood, bone marrow, and lung lavages have been used in vitro to isolate ASFV and can be used in laboratory for propagation, titration, and manipulation [3–8].

Presently, no licensed vaccines exist for ASF, mainly due to the gap in knowledge regarding ASFV–host interactions [9, 10]. A few approaches have been used to develop an ASFV vaccine, and one of the faster routes is via targeted gene(s) deletion of virulence factors and immune response inhibitory genes [11, 12]. Examples of genes that were deleted from the ASFV genome include B119L [13–15], DP71L [16, 17], DP96R [14, 15, 18], DP148R [15, 19], EP153R [20–24], EP402R [24–29], and I177L [30] and genes from multi-gene families (MGF) [15, 31–39]. These deletions have varying

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ranges in terms of safety in pigs and protection against a virulent challenge. Moreover, the deletion of the same gene from different genotypes of ASFV does not guarantee the same effect, astoundingly seen in the case of DP148R, when deleted from genotype I, Benin isolate; 100% protection was afforded with a homologous challenge [19], while the same deletion from a genotype II isolate was not attenuated [15]. This highlights a need for further research into functional gene assays which require the genetic manipulation of ASFV.

Here, we describe a method to generate recombinant virus starting with guidelines to design transfer plasmids right through to growing a final stock. Within this method are protocols for the isolation of porcine bone marrow cells (PBMs) and the titration of ASFV virus stocks using hemadsorption assay which can be used routinely in both the diagnostic and research labs for growing and characterizing ASFV isolates, not just recombinants. We also describe a method to obtain a purer population of PBMs without granulocytes, platelets, and red blood cells (RBC) via density gradient. These purified PBMs can be kept in −80 °C for long-term storage minimizing the number of pigs used. We also describe the conventional methodology of producing recombinant ASFV, via homologous recombination and limiting dilution. The conventional method is a long process, which can take up to 3 months to produce a single gene-deleted ASFV. Recently, we published a method that utilizes fluorescent-activated cell sorting (FACS) to isolate single recombinant ASFV-infected cells which is then purified with a combination of single cell sorting and limiting dilutions [40].

2 Materials

All plasticware should be tissue culture grade and sterile. All reagents and buffers should be tissue culture grade, and solutions should be sterilized by autoclaving or filtration. Maintain sterility by handling samples in a biological safety cabinet according to good laboratory practice.

1. Biological safety cabinet (BSC).
2. Polypropylene conical tubes (50 mL).
3. Centrifuge (capable of spinning 50 mL conical tubes).
4. Pipette gun.
5. Serological pipettes (10 or 25 mL).
6. Aerosol-resistant filter tips.
7. Phosphate-buffered saline, divalent cation free (PBS).

2.1 Generating Transfer Plasmid and Diagnostic Primers

1. Transfer plasmid either generated by traditional cloning or commercially synthesized (*see* **Note 1**).
2. Primers designed to discriminate wild type from recombinant virus by endpoint PCR.
3. Primers designed for Sanger sequencing of recombination sites.

2.2 Porcine Bone Marrow Cells Preparation

1. Primary porcine bone marrow cells (PBMs) media: Earle's Balanced Salt Solution (EBSS) supplemented with 10% heat-inactivated pig sera, 1% penicillin-streptomycin (10,000 U/mL), and 1% HEPES solution, 1 M, pH 7.0–7.6.
2. Bone cutters.
3. Scalpel blades with handles.
4. Petri dishes.
5. Forceps.
6. 1 L conical flasks.
7. Funnel with muslin cloth (sterile).
8. PBS wash: PBS supplemented with 1% FBS and 1% penicillin-streptomycin.
9. 3% acetic acid, glacial.
10. Hemocytometer or automated cell counting system.
11. Bench-top centrifuge.
12. 50 mL polypropylene tubes.
13. Freezing containers.
14. 2-propanol.
15. −80 °C freezer.

2.3 Porcine Bone Marrow Cell Purification

1. Histopaque-1077/1083 or Ficoll-Paque 1.077/ 1.084 (*see* **Note 2**).
2. 1× Red blood cell (RBC) Lysis Buffer: Dilute commercially available 10× RBC Lysis Buffer in sterile distilled water. Prepare fresh.
3. Purified PBM media: Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with GlutaMAX, 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin (10,000 U/mL), and 100 ng/mL recombinant pig CSF1-Fc.
4. Freezing medium: 90% FBS, 10% DMSO.

2.4 Production of Recombinant ASFV by Limit Dilution

1. Porcine alveolar macrophages (PAMs) media: RPMI 1640 Medium supplemented with GlutaMAX, 10% pig sera, 1% penicillin-streptomycin (10,000 U/mL), and 1% HEPES.

2. Spontaneously immortalized wild boar cell line, WSL-R media [41]: ZB28 medium made up of 50% Ham's F12 Nutrient Mix medium, 50% Iscove's Modified Dulbecco's Medium (IMDM), supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, and 1% glutamine.
3. OptiMEM.
4. Appropriate method to detect expression of reporter gene, either
   - (a) Inverted fluorescence microscope if using fluorescent marker such as mNeonGreen.
   - (b) X-Gluc solution: 100 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) in DMSO if using β-glucuronidase as a reporter.
   - (c) X-Gal solution: 100 mg/mL (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) in DMSO if using β-galactosidase as a reporter.
5. Transfection reagent
   - (a) If using non-primary cells: *Trans*IT-LT1 (Mirus Bio) transfection reagent.
   - (b) If using primary cells: *Trans*IT-X2 (Mirus Bio) or other similar reagents suitable for primary cells.
6. 0.05% Trypsin-EDTA.
7. Sterile reagent reservoirs.

2.5 Production of Recombinant ASFV by FACS

1. Porcine alveolar macrophages (PAMs) media: RPMI 1640 Medium supplemented with GlutaMAX, 10% pig sera, 1% penicillin-streptomycin (10,000 U/mL), and 1% HEPES.
2. Spontaneously immortalized wild boar cell line, WSL-R media [41]: ZB28 medium made up of 50% Ham's F12 Nutrient Mix medium, 50% Iscove's Modified Dulbecco's Medium (IMDM), supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, and 1% glutamine.
3. OptiMEM.
4. Inverted fluorescence microscope.
5. FACS machine with the capability of sorting live-infected cells (e.g., FACSAria Fusion cell sorter in a biological safety cabinet, with DIVA8 acquisition software).
6. FACS buffer: PBS, 5 mM EDTA, 25 mM HEPES, and 1% FBS (*see* **Note 3**).
7. Cell strainer with 70 μm mesh.

2.6 ASFV Titration

1. Primary porcine bone marrow cells (PBMs) media: Earle's Balanced Salt Solution (EBSS) supplemented with 10% heat-

inactivated pig sera, 1% penicillin-streptomycin (10,000 U/mL), and 1% HEPES solution, 1 M, pH 7.0–7.6.

2. Tissue culture grade flat bottomed 96-well plates.
3. Deep well sterile plates.

2.7 Titration by Fluorescence

1. Inverted fluorescence microscope.
2. PFA: 4% paraformaldehyde in PBS.
3. PBS + 1% BSA: 1% (w/v) bovine serum albumin (BSA) diluted in PBS.
4. 0.2% Triton X-100: 0.2% Triton X-100 in PBS.
5. Anti-ASFV antibody (e.g., against p30 or p72 viral proteins).
6. Species-specific fluorescent secondary antibody.

3 Methods

All steps described below should be performed in Class II Safety Cabinet unless mentioned otherwise. All centrifugation steps are at room temperature, unless mentioned otherwise.

3.1 Transfer Plasmid and Primer Design

Detailed subcloning, primer design, and endpoint PCR protocols are outside the scope of this method. Here we give general guidelines to the design of the plasmids and primers.

1. Transfer plasmids containing a reporter marker gene under the control of an ASFV gene promoter to drive expression in ASFV-infected cells. Conventionally reporter genes coded for enzymes such as β-galactosidase (β-Gal) or β-glucuronidase (GUS) can be assayed by color change of a substrate such as X-Gal or X-Gluc, respectively. More recently, genes coding for fluorescent proteins have been used as reporter genes. Examples of ASFV promoters that have been used include promoters for late phase expressed B646L/VP72, or the early to late phase immunogenic CP204L/VP30 [40, 42, 43]. The B646L/VP72 promoter used is approximately 39 bp in length, while the CP204L/VP30 promoter is around 50 bp [40, 42]. The reporter gene and promoter are flanked by 500–1000 bp left and right flanking regions of gene to be deleted (Fig. 1) (*see* **Note 4**). Transfer plasmids can be cloned; however, as a faster option, these can be synthesized commercially (*see* **Note 1**).
2. Design pairs of PCR primers to anneal to an internal portion of the genetic loci targeted for deletion. These should only produce a PCR product in wild type virus. If the genetic loci being targeted are relatively small, then pairs of primers can be

Fig. 1 Schematic diagram of a transfer plasmid used for producing recombinant ASFV. A transfer plasmid should contain the left and right flanking regions of the gene(s) to be deleted or mutated. Between these sites, a reporter gene under the control of an ASFV promoter should be inserted, as an example, the mNeonGreen fluorescent protein is shown. The LoxP sites are optional and are particularly useful for removing the reporter genes, especially in situations where additional deletion or mutations are required in the virus, and the options of reporter genes are limited. The gene maps were copied from SnapGene® Viewer software and modified on Adobe® Illustrator® CS6

designed to span the target site which will allow the discrimination of wild type and recombinant virus by the size of the PCR product.

3. Design sequencing primers to anneal at least 100–200 bp outside of the flanking regions of the recombination site so that any abnormalities can be detected. In addition, whole genome sequencing can be performed to detect abnormalities outside of the target region (see Chap. 16).

3.2 Isolation of Porcine Bone Marrow Cells

1. Primary porcine bone marrow cells (PBMs) are extracted from the long leg bones of 4-week-old outbred pigs (*see* **Note 5**).
2. In a biosafety cabinet, clean the bones with a scalpel blade to remove all periosteum (membrane that covers the bone) and cartilage.
3. Expose bone marrow by chopping the cleaned bones with a bone cutter into fragments around 1 cm long.
4. Transfer bone fragments into a conical flask containing 500 mL PBS supplemented with 1% FBS and 1% penicillin-streptomycin (four bones/flask).
5. Incubate in a shaking water bath at 37 °C for 1.5 to 2 h.
6. Using a sterile funnel with muslin cloth, filter the bone suspension.
7. Transfer cell suspension into 50 mL tubes.
8. Centrifuge at 350× *g* for 5 min.
9. Discard supernatant, add 10 mL PBS wash, and pool the resuspended PBMs.
10. Centrifuge again at 350× *g* for 5 min.
11. Discard supernatant and resuspend all cells in 50 mL complete EBSS.
12. Add 100 μl cells to 900 μL 3% acetic acid for cell counting (*see* **Note 6**).
13. PBMs can be seeded at 1.0–1.6 × 10^7 cells/mL in complete EBSS (*see* **Note 7**) and incubated in a 37 °C incubator, with 5% CO_2 for 2–4 days (*see* **Note 8**).

3.3 Porcine Bone Marrow Cell Purification

1. Dilute isolated PBMs with PBS at the ratio of 1:2 to 1:4. Mix by inverting tube several times or via a pipette.
2. Add 15 mL room temperature Histopaque or Ficoll-Paque to 50 mL tubes.
3. Gently overlay with 30 mL of the diluted PBMs atop the Histopaque or Ficoll-Paque.
4. Centrifuge the overlaid tubes at 1000× *g* for 30 min at room temperature with brake off (*see* **Note 9**).
5. Collect the buffy coat into a new 50 mL tube and top up with PBS (*see* **Note 10**).
6. Centrifuge at 400× *g* for 10 min.
7. Discard supernatant and add 5 mL 1× RBC Lysis Buffer to lyse remaining red blood cells.
8. Incubate at room temperature for 5 min.
9. Top up with PBS and centrifuge again as in Step 6.
10. This is then followed by two rounds of PBS wash and centrifugation at 400× *g* for 10 min.
11. The purified PBMs can be either
    (a) Used directly after cell count where cells are maintained in complete RPMI at 2–5 × 10^6 cells/mL.
    (b) Or resuspended in freezing medium and kept in freezing containers containing 2-propanol at −80 °C for 24–48 h. The cells are then stored at −80 °C for future use.

3.4 Production of Gene-Deleted Recombinant African Swine Fever Viruses by Limit Dilution

Handle ASFV only in approved laboratories as described in the OIE Terrestrial Manual at OIE BSL 3+ or BSL 4 [44]. All ASFV work should adhere to appropriate biocontainment guidelines.

1. Seed purified PBMs, PAMs, or WSL-R cells (*see* **Note 11**) in 24-well cell culture plates. Plate 500 μL of either WSL-R cells at 1 × 10^5 cells/mL, PAMs at 1 × 10^6 cell/mL, or purified PBMs at 2–5 × 10^6 cells/mL.
2. Culture WSL-Rs and PAMs for 1 day and purified PBMs for 2 days.
3. Calculate the amount of parental virus to be used at varying multiplicities of infection (MOI) and dilute in appropriate medium (*see* **Note 12**).
4. Remove old medium from cells and add 250 μL virus prep per well.
5. Optional: Centrifuge plates at 600× *g* for 1 hour at 26°C (*see* **Note 13**).
6. Incubate plates at 37 °C, with 5% CO_2 for 3 hours.

7. Prepare transfection mixture 30 min before the end of the 3-hour incubation time according to the manufacturer's instructions. The transfection mixture should contain the transfer plasmid, OptiMEM, and the transfection reagent.
8. Add 250 μL of fresh warm medium onto infected cells.
9. Add 50 μl transfection mixture to appropriate wells.
10. Optional: Centrifuge plates at 600× *g* for 1 hour at 26°C (*see* **Note 14**).
11. Incubate plates at 37 °C, with 5% CO_2.
12. Two to three days after beginning step 11, seed PBMs into at least ten 96-well plates, at 5×10^6 cells/mL for purified cells or at 1.6×10^7 cells/mL for non purified cells with complete medium, 100 μL per well.
13. Four to five days after the initial infection/transfection, identify infected/transfected wells by either addition of chromogenic substrates or observing expression of fluorescent proteins. Either
    - (a) If using β-glucoronidase or β-galactosidase expression as a marker, defrost X-Gluc or X-Gal stock solution, respectively. Dilute the defrosted stock 1:200 in complete medium and then add 140 μL of diluted X-Gluc or X-Gal to each well of the 24-well plates (for an approximate final concentration of 100 μg/mL). Leave for up to 2 days to observe color change (*see* **Note 15**).
    - (b) Observe 24-well plates with inverted microscope and identify wells expressing fluorescent protein.
14. Harvest recombinant viruses from the 24-well plates by gently scraping cells with a tip and collecting both supernatant and cells into tubes.
15. Prepare serial dilutions of the recombinant viruses by diluting 100 μL of harvested virus into 100 mL of complete medium to prepare a starting dilution of $1{:}10^3$. Use this starting dilution to prepare 50 mL of the $1{:}10^4$ dilution (*see* **Note 16**). Remaining harvested virus can be stored at 2–8°C for several weeks or at –80°C indefinitely.
16. Remove the old medium from the 96-well plates and replace with 100 μL fresh media. Then add 100 μl per well of both dilutions (5 plates per dilution).
17. Three to five days later, identify recombinant viruses by either addition of chromogenic substrates (to a final concentration of 100 μg/mL) or observing expression of fluorescent proteins as in step 13.

18. Harvest the positive wells from the plates, selecting those from the higher dilution if present and evaluate via PCR for correct deletions and absence of contaminating parental virus. Repeat the limiting dilution process (**steps 14–16**) until pure recombinant virus without contaminating parental virus is obtained.

3.5 Production of Gene-Deleted Recombinant African Swine Fever Viruses By Fluorescent-Activated Cell Sorting

Handle ASFV only in approved BSL 3+ or BSL 4 laboratories as described in the OIE Terrestrial Manual [44]. All ASFV work should adhere to appropriate biocontainment guidelines. Note that Steps 1 to 11 of this protocol are identical to the previous limit dilution method (*see* Sec. 3.4).

1. Seed purified PBMs, PAMs, or WSL-R cells (*see* **Note 11**) in 24-well cell culture plates. Plate 500 μL of either WSL-R cells at 1×10^5 cells/mL, PAMs at 1×10^6 cell/mL, or purified PBMs at $2–5 \times 10^6$ cells/mL. Seed wells for uninfected, infected but untransfected controls in addition to at least one plate for each recombinant you plan to make.
2. Culture WSL-Rs and PAMs for 1 day and purified PBMs for 2 days.
3. Calculate the amount of parental virus to be used at varying multiplicities of infection (MOI) and dilute in appropriate medium (*see* **Note 12**).
4. Remove old medium from cells and add 250 μL virus prep per well.
5. Optional: Centrifuge plates at $600 \times g$ for 1 h at 26 °C (*see* **Note 13**).
6. Incubate plates at 37 °C, with 5% CO_2 for 3 h.
7. Prepare transfection mixture 30 min before the end of the 3-hour incubation time according to the manufacturer's instructions. The transfection mixture should contain the transfer plasmid, OptiMEM, and the transfection reagent.
8. Add 250 μL of fresh warm medium onto infected cells.
9. Add 50 μl transfection mixture to appropriate wells.
10. Optional: Centrifuge plates at $600\times g$ for 1 h at 26 °C (*see* **Note 14**).
11. Return 24-well plates to incubator.
12. Seed 100 μl of purified PBMs per well into five 96-well plates at 5×10^6 cells/mL in complete RPMI (*see* **Note 17**). Culture for 2 to 3 days, remove media, and replace with 100 μl of fresh media before beginning Steps 13 through 24.

Live cell sorting requires careful preparation to avoid stressing the cells. From this point onwards, unless stated otherwise, cells

should be kept cold throughout the experiment, and centrifugation should be at minimal speed, ~300× *g*.

13. 36 to 60 h after the initial infection/transfection harvest recombinant viruses from the 24-well plates. Depending on whether you have used purified PBMs, PAMs, or WSL-Rs either
    (a) For PBMs or PAMs, gently scrape cells and transfer along with supernatant into tubes and keep on ice.
    (b) For WSL-R cells: Transfer supernatant into tubes and keep on ice. Wash cells with 500 μL of PBS three times. Trypsinize cells at 37 °C until cells are dislodged. Add complete ZB28 medium to detached WSL-R and transfer to the same tube as supernatant.
14. Centrifuge tubes at 300× *g*, for 10 min at 4 °C.
15. Discard supernatant and wash cells with PBS. Centrifuge tube at 300× *g*, for 10 min at 4 °C.
16. Repeat wash three times to ensure removal of extracellular viruses (*see* **Note 18**).
17. After the final wash and centrifugation, discard supernatant and add appropriate amounts of FACS Buffer to resuspend the cells at 3×10^5 cells/mL. Keep samples on ice.
18. Pass cells through a 70 μm cell strainer prior to cell sorting (*see* **Note 19**).
19. Sort single positive cells with a FACS equipped with the lasers necessary to excite and detect your fluorescent proteins (405 nm (violet), 488 nm (blue), 561 nm (yellow green), and 640 nm (red) lasers) (*see* **Note 20**).
20. Setup gates for FACS using the uninfected controls
    (a) First, cells are gated based on cell size/granularity (SSC-A vs. FSC-A) and singlets (SSC-H vs. SSC-A) (Fig. 2a, b). The FSC threshold was set to 5 K, to detect debris to be excluded during live cell sorting.
    (b) Autofluorescent cells, identified as being dual positive for BP filters violet 450/40 and blue 530/30, should be excluded, predominantly in primary cells. PBMs, whether with or without RBCs, are often highly autofluorescent. These cells should be excluded when gating. See Fig. 3c, g.
    (c) Virus-infected-transfected cells are then identified as single positive for GFP or RFP or double positive ($RFP^{+}GFP^{+}$) if purifying viruses with the two markers (Fig. 2c) (*see* **Note 21**).

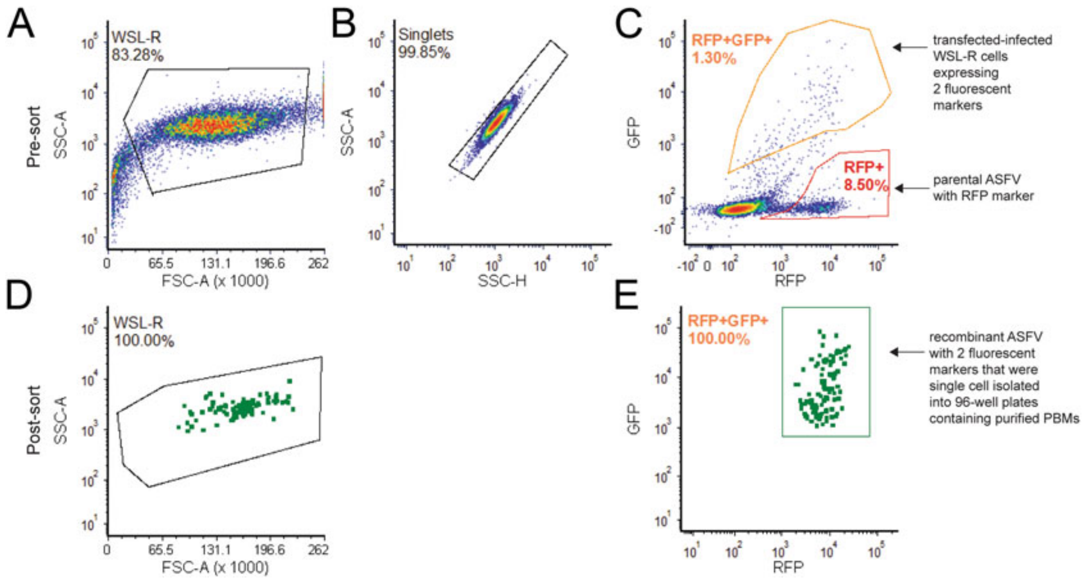

Fig. 2 Identification and isolation of single cells after homologous recombination event. WSL-R cells were infected with a parental ASFV containing a red fluorescent reporter gene (RFP) and transfected with a transfer plasmid containing a green fluorescent reporter gene (GFP). After 48 h, cells are trypsinized, washed, and subjected to FACS for identification (**a**–**c**) and, thereafter, single-cell isolation (**d**, **e**). The flow cytometry profiles include Panel A, SSC-A vs FSC-A for total cells; Panel B, SSC-A vs. SSC-H to obtain singlets; Panel C, blue BP 530/30 nm-A vs. yellow-green (YG) BP 582/15 nm-A to capture the intended recombinant ASFV-infected/transfected cell subpopulation, now positive for both RFP and GFP. A post-sort phase is shown, where Panel E represents total cells that were single cell sorted into 96-well plate containing purified PBMs, and each dot in panel F represents an individual RFP^+GFP^+ cell that was sorted. The percentages in each panel reflect the percentage of cells within that gate relative to the parent gate

21. Sort positive cells with an 85 μm nozzle at 45 psi and in single cell precision mode (*see* **Note 22**). Figure 2d, e shows an example of single sorted cells. Figure 2d shows the cells' size and granularity, whereas in Fig. 2e, each dot represents an individual cell and the respective intensity of green and red fluorescence.
22. Collect recombinant virus-infected cells at single-cell density into individual wells of 96-well microplates containing cultured purified PBMs prepared in Step 12.
23. Sterilize the flow cytometer between sorts with 2.3% sodium hypochlorite at a flow rate of 5, for 5 min, as recommended by OIE [45].
24. Incubate microplates at 37 °C, 5% CO_2 for 4–5 days.
25. Monitor cultures for expression of fluorescent markers under an inverted fluorescent microscope. Identify and mark wells positive for fluorescent marker(s) of interest.

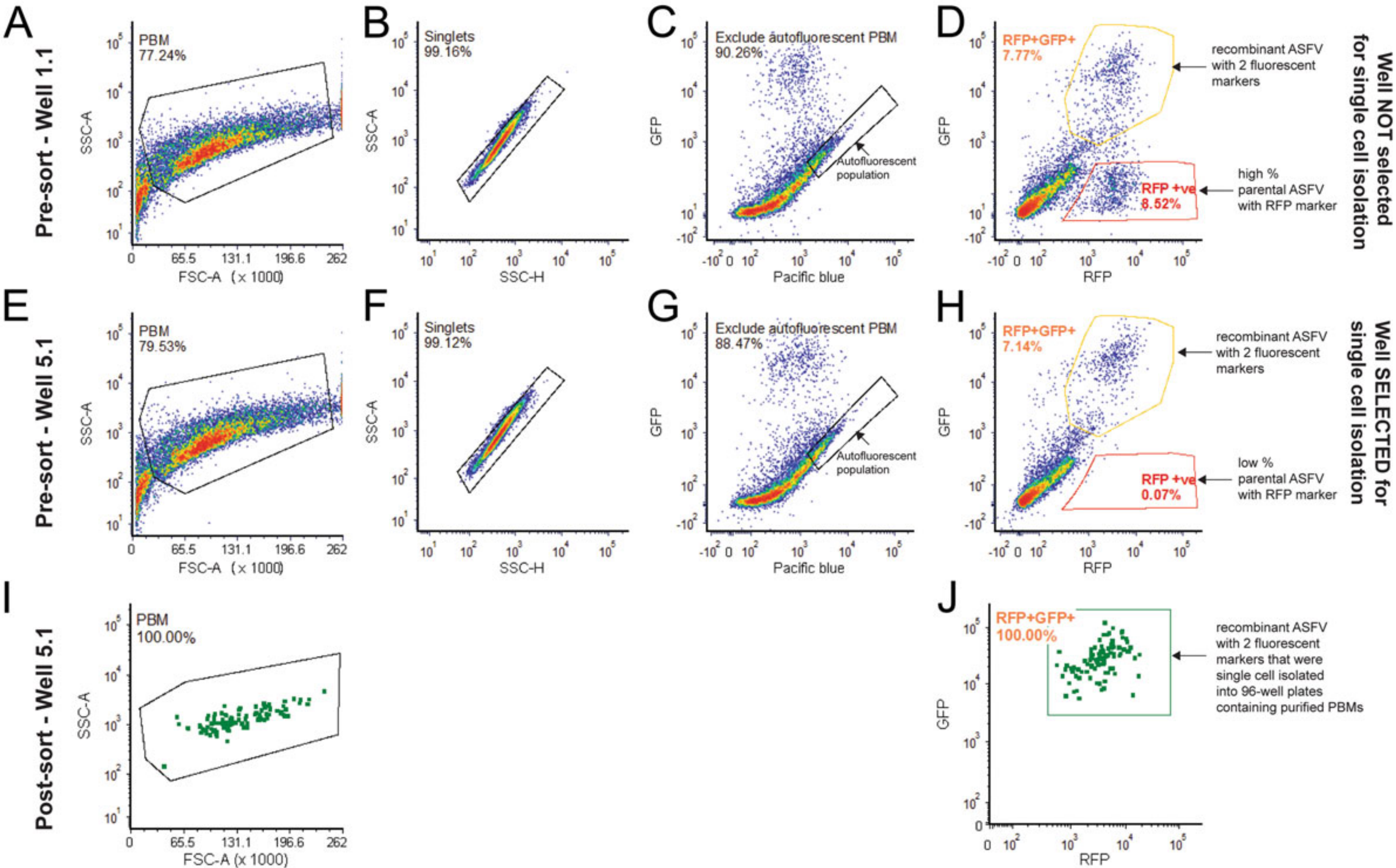
A
Pre-sort - Well 1.1
PBM 77.24%
SSC-A
FSC-A (x 1000)
B
Singlets 99.16%
SSC-A
SSC-H
C
Exclude autofluorescent PBM 90.26%
Autofluorescent population
GFP
Pacific blue
D
RFP+GFP+ 7.77%
RFP +ve 8.52%
GFP
RFP
recombinant ASFV with 2 fluorescent markers
high % parental ASFV with RFP marker
Well NOT selected for single cell isolation
E
Pre-sort - Well 5.1
PBM 79.53%
SSC-A
FSC-A (x 1000)
F
Singlets 99.12%
SSC-A
SSC-H
G
Exclude autofluorescent PBM 88.47%
Autofluorescent population
GFP
Pacific blue
H
RFP+GFP+ 7.14%
RFP +ve 0.07%
GFP
RFP
recombinant ASFV with 2 fluorescent markers
low % parental ASFV with RFP marker
Well SELECTED for single cell isolation
I
Post-sort - Well 5.1
PBM 100.00%
SSC-A
FSC-A (x 1000)
J
RFP+GFP+ 100.00%
GFP
RFP
recombinant ASFV with 2 fluorescent markers that were single cell isolated into 96-well plates containing purified PBMs

Fig. 3 Purification of recombinant ASFV via single-cell isolation using FACS. After isolation of recombinant ASFV, the virus must be further purified to remove contaminating parental virus. Here, an example is given for purification of RFP^+GFP^+ recombinant ASFV, to remove parental ASFV which has only the RFP reporter. Cells from individual wells identified as positive for RFP^+GFP^+ under a fluorescent microscope are harvested, washed, and subjected to FACS. (**a**–**d**) and (**e**–**h**) represents two individual wells, 1.1 and 5.1, respectively as containing RFP^+GFP^+ cells. The biparametric flow cytometry profiles for the two wells include Panels A and E, SSC-A vs. FSC-A for total cells; Panels B and F, SSC-A vs. SSC-H to obtain singlets; Panels C and G, blue BP 530/30 nm-A vs. violet BP 450/40 nm-A to exclude autofluorescence cells; Panels D and H, blue BP 530/30 nm-A vs. yellow-green (YG) BP 582/15 nm-A to capture the RFP^+GFP^+ cell subpopulation (recombinant virus) and also to identify the parental virus (RFP^+). In both wells, the percentage of RFP^+GFP^+ cell population is similar (7.7% and 7.1%); however when observing the RFP^+ parental ASFV, there is a higher percentage of parental virus in well 1.1 (**d**) compared to well 5.1 (**h**). In this case, only well 5.1 was selected for further purification and single cell sorted into plates containing PBMs (**i**, **j**). The gating for the recombinant ASFV subpopulation can be adjusted to be more stringent to capture the brighter selection of cells for single-cell isolation. The percentages in each panel reflect the percentage of cells within that gate relative to the parent gate

26. Harvest the wells individually 4 to 5 days after the single-cell isolation, by gently scraping wells with a tip and collecting both cells and supernatant into a sterile tube containing 500 μL PBS.
27. Centrifuge tubes at 300× *g* for 5 min, 4 °C.
28. Discard supernatant and wash cells with PBS. Centrifuge tube at 300× *g*, for 5 min at 4 °C. Repeat wash three times to ensure removal of extracellular viruses.
29. Upon the last wash, add 250 μL of FACS Buffer and resuspend cells. Keep samples on ice.
30. Repeat Steps 20 to 27 (*see* **Note 23**)
31. Single-cell isolation is repeated until a pure recombinant virus is obtained which can evaluated by PCR using diagnostic primers (*see* **Note 24** and **Note 25**).

3.6 Propagation of ASFV in PBMs and Titrations

1. Add appropriate amounts of ASFV to flasks containing either PBMs, purified PBMs, or PAMs. Keep flask in 37 °C, 5% CO_2 incubators for 4–5 days.
2. After 4–5 days, harvest the virus by gently detaching remaining cells with a cell scraper. Transfer into an appropriate tube.
3. Centrifuge at 1000× *g* for 10 min at 4 °C.
4. For extracellular ASFV alone, transfer all supernatant into another tube, and discard pelleted cellular debris. Aliquot virus and store in −80 °C.
5. For both extracellular and intracellular ASFV (*see* **Note 26**), transfer supernatant into another tube, leaving behind approximately 2–5 mL of supernatant. Keep the supernatant tube cold.
6. Gently vortex the tube containing the cell pellet to dislodge cells.
7. Freeze-thaw pellets three times.
8. Centrifuge at 1000× *g* for 10 min at 4 °C.
9. Transfer the supernatant into the tube containing the rest of the supernatant. Mix by inverting. Brief pulse-centrifuge to remove the droplets from the cap. Aliquot and store in −80 °C.

3.7 ASFV Titration

3.7.1 Hemadsorption Test

The hemadsorption (HAD) test is the most common titration method for ASFV. This method described as early as 1960 by Malmquist and Hay can be used to titrate hemadsorbing ASFV isolates, based on the adherence of pig RBCs to infected macrophages and the formation of rosettes [3].

1. Plate out 100 μl per well of PBMs (from Method 3.2) into 96-well plates at 1–1.6 × 10^7 cells/mL in complete EBSS and incubate at 37 °C, with 5% CO_2 for 2–4 days. One plate is sufficient for three titrations.

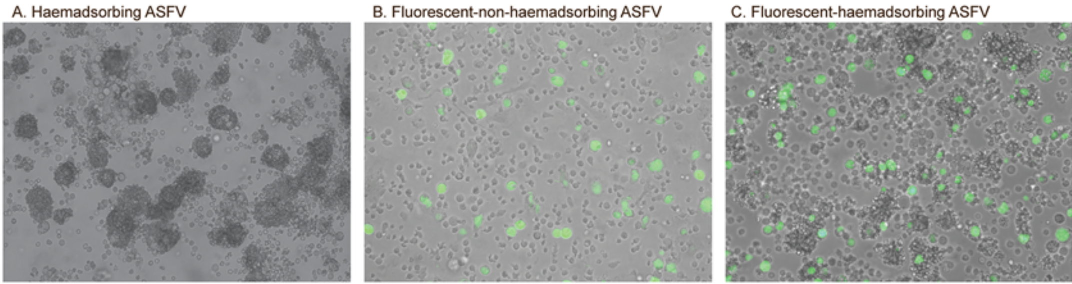

Fig. 4 Representative microscopy images of porcine bone marrow macrophages infected with wild type ASFV (Panel A), a recombinant non-hemadsorbing ASFV expressing mNeonGreen (Panels B), or a recombinant hemadsorbing ASFV expressing mNeonGreen (C). Note rosettes of red blood cells surrounding cells in Panels A and C, but not B

2. Prepare tenfold serial dilutions of test samples (blood, cell culture virus, etc.) in a 96-deep-well plate, from 1:10 to 1: 10^8 in complete EBSS. Include one serial dilution without a sample to act as a negative control. Each sample should be titrated in at least quadruplicate, so 50 μL of sample serially diluted into 450 μL complete EBSS is appropriate (*see* **Note 27**).
3. Remove old medium from plates containing PBMs. Immediately add 100 μL of serially diluted samples onto cells with a multichannel pipette.
4. Incubate plates at 37 °C, with 5% CO_2 for 2–5 days.
5. Examine plates under a light microscope for formation of rosettes (Fig. 4a) (*see* **Note 28** and **Note 29**).
6. Mark the wells containing rosettes and calculate the endpoint titers in HAD_{50}/mL using the Spearman and Kärber method. For this calculation to be valid, a dilution where all of the wells were positive for HAD and a row where all of the wells were negative are required. If not, repeat the titration adjusting the initial dilution so that the dilution series ranges from $1{:}10^3$ to $1{:}10^{10}$, for example.
7. Titer ($Log_{10}HAD_{50}$) = – (a + 0.5 – Σ_p). Where a is the highest dilution for which all of the wells show rosettes and Σ_p is the fraction of positive wells at the highest dilution plus the fraction of wells which are less than 100% positive.

   For example, in the case shown in Table 1, Σ_p = 1.75. Therefore, the titer in 100 μl = – (–5 + 0.5–1.75) = 6.25. To determine the titer per milliliter, multiply the result by 10. Therefore, the final titer of the example shown in Table 1 would be 7.25 $Log_{10}HAD_{50}$/mL.

Table 1
Example titration results to illustrate Spearman–Karber endpoint calculation

Dilution	Infected wells	Fraction infected (p)	Total infected (Σp)
−5 (1:10^5)	4/4	1.0	1.75
−6 (1:10^6)	2/4	0.5	
−7 (1:10^7)	1/4	0.25	
−8 (1:10^8)	0/4	0	

3.7.2 Fluorescence Test

1. Plate out 100 μl per well of PBMs (from Method 3.2) into 96-well plates at 1–1.6 × 10^7 cells/mL in complete EBSS and incubate at 37 °C, with 5% CO_2 for 2–4 days. One plate is sufficient for three titrations.
2. Prepare tenfold serial dilutions of test samples (blood, cell culture virus, etc.) in a 96-deep-well plate, from 1:10^1 to 1:10^8 in complete EBSS. Include one serial dilution without a sample to act as a negative control. Each sample should be titrated in at least quadruplicate, so 50 μL of sample serially diluted into 450 μL complete EBSS is appropriate (*see* **Note 27**).
3. Remove old medium from plates containing PBMs. Immediately add 100 μL of serially diluted samples onto cells with a multichannel pipette.
4. Incubate plates at 37 °C, with 5% CO_2 for 2–5 days.
5. Remove the supernatant and wash cells once with PBS.
6. Fix cells in 4% PFA for 20–60 min.
7. Wash with PBS three times.
8. Permeabilize cells with 0.2% Triton X-100 for 5 min at room temperature.
9. Wash with PBS three times.
10. Prepare the primary ASFV antibody by diluting the antibody in PBS + 1% BSA. Add onto cells and incubate at room temperature for 30–60 min.
11. Wash with PBS three times.
12. Prepare the secondary antibody (e.g., species-specific Alexa Fluor 488) in PBS + 1% BSA. Add onto cells and incubate at room temperature for 30–60 min. Keep plates in dark.
13. Wash with PBS three times.
14. Visualize under an inverted fluorescent microscope for fluorescent wells. Mark the last positive wells. Calculate the titers using the Spearman and Kärber method as described in Steps 6 to 8 of Method 3.7.1 using the number of fluorescent wells

in place of those displaying rosettes. The titers should be reported as $TCID_{50}$/mL rather than HAD_{50}/mL.

4 Notes

1. Generating a transfer plasmid from scratch by traditional cloning methods may take months. Generating plasmids containing combinations of reporter genes in combination with different ASFV promoters with multiple cloning sites either side can help speed up the process for future cloning efforts. However, if the lab budget is available, it is a lot simpler to pay for the commercial synthesis of your insert and flanking regions. It is important to note that inserts for many fluorescent markers are significantly shorter than traditional enzymatic markers (~800 bp compared to 2000 to 3000 bp) which will offer a significant cost saving. Bear in mind that any synthesized insert containing an ASFV open reading frame is likely to come under legislation designed to control dual-use technology. Import and export licenses may be required depending on your jurisdiction and that of the company you are purchasing the sequence from.
2. The numbers 1077/1.077 and 1083/1.084 represent the densities of either 1.073 or 1.084 $\pm$ 0.001 g/mL. Both work equally well with pig bone marrow cells.
3. Use Ca^{2+}/Mg^{2+} free PBS to reduce cell aggregation. EDTA is added to prevent cell adhesion. HEPES can improve buffering capacity.
4. When deleting a particular ASFV gene, always remember to check the flanking genes. This is to avoid accidentally removing the adjacent ORF, or the promoters and transcription termination sequences of the adjacent genes.
5. The euthanizing of pigs should be performed in accordance with the regulated procedures approved by the licensing authorities, for example, the UK Home Office. Sedation of pigs followed by an overdose of barbiturates is the procedure we use.
6. 3% glacial acetic acid lyses red blood cells to facilitate the counting of white blood cells. If a high number of cells are obtained, it might be necessary to further dilute the cell suspension beyond 1:10.
7. Typically, we obtain 1.0–3.0 $\times$ 10^8 PBMs/mL. Depending on the pig, this number may vary.
8. The cells are usually used in 2–4 days to allow for leukocyte maturation such that cells become susceptible to ASFV infection.

9. Brake is turned off to avoid remixing of separated layers by rapid deceleration.
10. The sample will be separated into four different layers. Red blood cells are at the bottom most layer, followed by granulocytes and the Ficoll/Histopaque layer, while the topmost layer contains plasma. Sitting in the interface of the Ficoll/Histopaque and the plasma/buffer layer is the buffy coat containing mononuclear cells and low-density, slow sedimenting particles, e.g., platelets.
11. Primary macrophages derived from PBMs and PAMs are difficult to transfect. As an alternative, WSL-R cells, which are ASFV susceptible [8, 41, 46] and relatively easier to transfect, can be used in the first step of homologous recombination.
12. MOI can typically range from 1 to 10.
13. This centrifugation step has been shown to increase infection efficiency.
14. This centrifugation step has been shown to increase transfection efficiency.
15. The length of time for marker expression to be clearly visible will depend on the proportion of recombinant virus to wild type virus in each well. If there is only recombinant virus in the well, then the color change may take as little as an hour; however, a high proportion of contaminating wild type virus will mean this will take longer.
16. The range of useful dilutions for purifying recombinants can vary between $1{:}10^3$ and $1{:}10^5$ depending on the batch of cells and the particular mutant you are trying to create. As the number of passages increases. the dilutions should also increase. For example, in the second passage, the dilutions should range between $1{:}\ 10^5$ and $1{:}10^7$.
17. For the first round of sorting, we use five 96-well plates for each recombinant we are generating. For later rounds this can be reduced to three as required.
18. The cell sorter cannot discriminate against virus particles so it is important to remove them to prevent them being sorted into PBMs.
19. This is to remove any cell clumping and generate a single-cell suspension.
20. Expression of a green fluorescent reporter (e.g., mNeonGreen) in infected-transfected cells can be excited with a 488-nm laser (blue) and detected through a blue 530/30 BP filter, whereas a red fluorescent protein (e.g., TagRFP-T) in infected cells can be excited with a 561 nm laser (yellow green) and detected through a yellow/green 582/15 BP filter. The high violet

450/40 BP signal is used to gate out autofluorescent cells which is substantial in primary macrophages.

21. If the parental virus has a fluorescent marker, choose wells that have minimal amount of parental virus. For example, to purify a recombinant ASFV with two fluorescent markers (e.g., $RFP^{+}GFP^{+}$), where the parental virus has only a red fluorescent protein (RFP^{+}) (see Fig. 3), once they are gated by size (Fig. 3a, e), by single cells (Fig. 3b, f), and the autofluorescent cell population excluded (Fig. 3c, g), gate the cells using both fluorescent channels (Fig. 3d, h). Figure 3 represent PBMs from two different wells. The percentage of RFP^{+} only cells (parental virus) is 8.52% in Fig. 3a–d, while in Fig. 3e–h, this percentage is 0.07%. Only well 5.1 (Fig. 3e–h) is selected for further purification via single-cell isolation and is shown in Fig. 3i, j. This method of selection allows for speedier purification since it reduces the amount of contaminating parental virus.
22. These settings reduce the stress on live cells while sorting.
23. Stringency of gating can be increased to capture the correct subpopulation. In Fig. 3i, j, infected PBMs from well 5.1 were sorted at higher stringency, where only cells expressing high levels of RFP and GFP were single-cell sorted.
24. A gradual increase in the percentages of the total PBM population expressing the fluorescent protein(s) of interest and the number of wells containing these fluorescent marker(s) can be observed over the rounds of single-cell sorting. We have previously obtained pure recombinant ASFV after three rounds of single-cell sorting.
25. Single-cell isolation can also be combined with limiting dilutions especially if the opportunity of using the FACS is limited. In this case, five 96-well plates, one at $1{:}10^{6}$, two at $1{:}10^{7}$, and two at $1{:}10^{8}$, are our standard approach for a new recombinant and can be modified in terms of precise dilutions and number of plates to suit.
26. Both intracellular mature virions and extracellular enveloped virions are infectious [47].
27. Complete EBSS contains pig serum, and mixing this directly with pig blood can cause the first dilution to rapidly turn into a gel. Prepare the first two dilutions in PBS if this occurs, and immediately prepare the second dilution (1:100) from the first (1:10). Don't prepare a series of 1:10 dilutions and then use a multichannel to prepare the further dilutions. This problem is unlikely to occur with dilutions from $1{:}10^{3}$ to $1{:}10^{8}$. If measuring low titer experimental samples, then you can start with neat sample and then serial dilute down to $1{:}10^{7}$. This is unlikely to work for blood or homogenized tissues.

28. The number of RBCs in a given batch of PBMs can vary, and if it is difficult to see rosettes, then it can be helpful to shake the plate either by manually tapping the side of the plate or using a rotary shaker. For both biosafety reasons and the avoidance of accidental contamination, then plates should be covered with clear adhesive plate film before doing this.
29. If titrating a recombinant virus expressing a fluorescent protein, then instead of using a light microscope to observe and record rosettes, use a fluorescent microscope to observe and record fluorescent cells (Fig. 4b). Due to the autofluorescent nature of macrophages, a negative control titration is essential. Titers should be recorded as $TCID_{50}$/mL rather than HAD_{50}/mL.

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Chapter 6

Laboratory Diagnosis and Quantification of African Swine Fever Virus Using Real-Time Polymerase Chain Reaction

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Abstract

Real-time polymerase chain reaction (PCR) for the detection of African swine fever virus (ASFV) is the tool of choice for the diagnostic laboratory and is a robust and easily scalable method for the researcher analyzing viral replication both in vitro and in vivo. In this chapter, we describe protocols for both quantitative real-time polymerase chain reactions (qPCR) and non-quantitative real-time polymerase chain reactions (real-time PCR) for the detection of African swine fever virus genome in a range of samples.

Key words African swine fever, Diagnosis, Real-time PCR, qPCR, TaqMan®

1 Introduction

African swine fever virus (ASFV) diagnosis in the field is problematic due to the non-specific nature of the clinical signs of the disease, and therefore laboratory analysis is required to confirm suspect cases. Real-time polymerase chain reaction (PCR) is the method of choice for diagnosis due to its rapidity, robustness, and the ability to perform assays at scale when compared to traditional virus isolation. In addition, real-time PCR can be successfully performed on poor quality samples from which it would not be possible to isolate live virus, which is a significant advantage for passive surveillance of wild boar carcasses. As well as the "go-to" assay for the diagnostic laboratory, qPCR can also be used to replace, or complement, virus titration for researchers studying virus replication both in vitro and in vivo. Standard guidance for confirmatory diagnosis of ASFV suggests the use of two antigen detection assays targeting separate parts of the viral genome, and we present modified King et al. [1] and Fernández-Pinero et al. [2] methods currently used in our laboratory (*see* **Note 1**) which have been extensively validated through proficiency tests and are

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recommended in the OIE terrestrial manual [3]. We also describe two methods to quantify the number of genome copies present within a sample.

2 Materials

All plasticware should be certified free of DNase and RNase as well as DNA-free.

1. Separate work areas for master mix preparation and template addition, preferably biological safety cabinets or PCR cabinets.
2. Real-time PCR instrument.
3. Calibrated pipettes (volumes as appropriate).
4. Freezer −20 °C.
5. Refrigerator +4 °C.
6. Chilled rack.
7. Centrifuge (capable of spinning PCR plates).
8. Deep well plates.
9. Optical reaction plates and seals.
10. Nucleic acid-degrading reagent.
11. Microcentrifuge tubes (0.5–2 mL, any manufacturer).
12. Samples known to contain ASFV DNA (*see* **Note 2**), negative control samples (*see* **Note 3**), and test samples.
13. Nuclease-free water (store at approximately 22 °C).
14. Real-time PCR Master Mix (store as indicated by the manufacturer; *see* **Note 4**).
15. Aerosol-resistant filtered tips.

2.1 Real-Time PCR Assay

1. Primers and probes (Tables 1 and 2) should be diluted to the indicated working concentration using nuclease-free water and stored in aliquots at −20 °C. Once defrosted, aliquots should be stored at +4 °C for a maximum of 6 months. New batches of primers and probes should be evaluated against existing batches and should not deviate by more than one cycle threshold (Ct) value.

2.2 qPCR Assay

1. Primers and probes (Table 3) should be diluted to the indicated working concentration using nuclease-free water and stored in single-use aliquots at −20 °C.
2. Either high-quality plasmid DNA containing the sequence of the ASFV B646L/p72 gene prepared using commercial kits, diluted to 10^{10} copies/mL using nuclease-free water and

Table 1
Real-time PCR primers and probes for modified King et al. method

Primer	Oligo name	Sequence (5′ – 3′)	Working concentration (μM)
Forward	ASFV sense	CTGCTCATGGTATCAATCTTATCGA	20
Reverse	ASFV anti-sense	GATACCACAAGATCRGCCGT	20
Probe	ASFV TaqMan	FAM-CCACGGGAGGAATACCAACCCAGTG-QSY	10

Table 2
Real-time PCR primers and probes for modified Fernández-Pinero et al. method

Primer	Oligo name	Sequence (5′ – 3′)	Working concentration (μM)
Forward	ASF-VP72-F	CCCAGGRGATAAAATGACTG	20
Reverse	ASF-VP72-R	CACTRGTTCCCTCCAACCGATA	20
Probe*	ASF-VP72-P1	FAM-TCCTGGCCRACCAAGTGCTT-QSY	10

*UPL#162 is no longer commercially available and hence the probe in this assay has been replaced with that specified in Table 2

Table 3
King et al. method used for quantification of ASFV DNA in samples using the qPCR assay

Primer	Oligo name	Sequence (5′ – 3′)	Working concentration (pmol/μl)
Forward	ASFV sense	CTGCTCATGGTATCAATCTTATCGA	50
Reverse	ASFV anti-sense	GATACCACAAGATCRGCCGT	50
Probe	ASFV TaqMan	FAM-CCACGGGAGGAATACCAACC CAGTG-TAMRA	5

stored in single-use aliquots at −20 °C (*see* **Note 5**); or a sample of high titer ASFV (*see* **Note 6**).

3. An additional laboratory work area which is separate from the master mix preparation area, preferably a biological safety cabinet or PCR cabinet, should be used for the handling of plasmid DNA (*see* **Note 7**).

3 Method

3.1 Real-Time PCR

1. Isolate DNA from samples using a commercial kit suitable for either manual or robotic extraction as required (*see* **Note 8**). Nucleic acid extraction and testing of samples and known positive and negative controls should be performed in duplicate.
2. Clean PCR work areas using DNA-degrading reagent as per manufacturer's instructions.
3. Prepare the real-time PCR master mix according to the volumes outlined in Tables 4 and 5. Centrifuge all tubes before opening to minimize aerosol production. Ensure that a sufficient volume of master mix is prepared to allow testing of the required number of samples, and include an allowance of 5–10% to allow for pipetting discrepancies. Prepare reagents in a microcentrifuge tube and mix using a vortex. If intending to use an eight-channel multichannel pipette to aliquot the master mix, evenly distribute the mix between eight wells in a column of a 96-well plate or small reservoir.
4. Master mixes should be kept cold, either on ice or with a cooled rack, and shielded from direct sunlight.
5. Pipette 18 μl of real-time PCR master mix using a single or multichannel pipette as appropriate into optical reaction plates (*see* **Note 9**).
6. Add 2 μl of extracted nucleic acid to each well of the plate according to a defined plate layout. Great care should be taken to avoid the creation of aerosols and other potential routes of contamination. Cover each well of the PCR plate containing the reaction components firmly with a plate sealer.
7. After adding the nucleic acid to the optical PCR plate, the extraction plate can be stored at −20 °C or +4 °C depending on its further use.
8. Centrifuge the plate using a PCR plate spinner, or centrifuge to bring the reaction mix constituents to the bottom of the well and to eliminate all air bubbles.
9. Run samples on a real-time PCR instrument using the thermal profiles indicated in Table 6 (*see* **Note 10**).
10. If a Ct value occurs in more than one negative control well, this could indicate contamination, and therefore the samples must be re-extracted and assayed using the appropriate real-time PCR assay. A Ct value in a single negative control should be assessed by a technical manager or equivalent to decide if the test can be passed.

Table 4
Real-time PCR master mix composition for modified King et al. method

Reaction component	1 reaction (μl)
2 × real-time PCR master mix	10
Forward primer	0.4
Reverse primer	0.4
Probe	0.5
Nuclease-free water	6.7
Total volume	18

Table 5
PCR master mix composition for modified Fernández-Pinero et al. method

Reaction component	1 reaction (μl)
2 × real-time PCR master mix	10
Forward primer	0.4
Reverse primer	0.4
Probe	0.2
Nuclease-free water	7.0
Total volume	18

Table 6
Example thermal profile

Stage of real-time PCR	Temperature	Duration	Number of cycles
Taq activation/denaturation	95 °C	10 min	1 cycle
PCR	95 °C 60°C[a]	15 s 60 s	40 cycles

[a]Data collection stage

11. Positive control Ct values should be within the mean of the positive control Ct ±2 Ct as derived from previous data (recorded using a Shewhart chart or similar). In exceptional cases a single positive control Ct within ±3 Ct may be acceptable if all the other positive control Ct values are within ±2 Ct of the mean of the previous data. Otherwise the real-time PCR should be repeated.

12. Samples are considered positive if the Ct generated by the instrument (with or without manual setting of the threshold by the analyst) is less than 35. Ct values greater than 35 are considered to be inconclusive, and both the sample extraction and real-time PCR should be repeated. In order to confirm a suspect case, samples should have a positive Ct value in both the modified King et al. and Fernández-Pinero et al. assays.

3.2 Quantitative Real-Time PCR

1. Isolate DNA from samples using a commercial kit suitable for either manual or robotic extraction as required (*see* **Note 8**). Nucleic acid extraction and testing of samples and known positive and negative controls should be performed in duplicate or triplicate.

   Quantification of samples can be performed using tenfold serial dilutions of a plasmid containing the ASFV VP72 gene or using titrated ASFV. If using a virus stock as the standard for quantification, prepare a tenfold serial dilution using the same medium used to culture the virus.

2. Clean PCR work areas using degrading reagent as per manufacturer's instructions (*see* **Note 7**).
3. Prepare qPCR master mix according to the volumes outlined in Table 7. Ensure that a sufficient volume of master mix is prepared to allow testing for the required number of samples, and include an allowance of 5–10% for pipetting discrepancies. Prepare reagents in a microcentrifuge tube, mix using a vortex, and centrifuge to collect the contents at the bottom of the tube. Master mixes should be kept cold, either on ice or in a fridge, and shielded from direct sunlight until aliquoted. If intending to use an eight-channel multichannel pipette to aliquot the master mix, evenly distribute the mix between eight wells in a column of a 96-well plate or small reservoir.

Table 7
King et al. method used for quantification of DNA in samples using the qPCR assay

Reaction component	1 reaction (μl)
2 × real-time qPCR master mix	10
Forward primer	0.5
Reverse primer	0.5
Probe	1.0
Nuclease-free water	5
Total volume	17

4. Optical reaction plates containing the master mix should be kept cold either on ice or with a cooled rack.
5. If using plasmid standards, prepare tenfold serial dilutions from a working stock of plasmid containing B646L/Vp72 sequence (10^{10} copies/mL) in phosphate-buffered saline (PBS) or nuclease-free water. Mix each dilution well either using a vortex and centrifuging the tubes or by pipetting gently to prevent aerosolization of the diluted plasmid. This should be prepared in a separate area to that used to prepare the master mix, and great care must be taken to ensure that cross-contamination of the other laboratory areas does not occur.
6. Pipette 17 μl of real-time qPCR master mix using a single or multichannel pipette as appropriate into optical reaction plates (*see* **Note 9**).
7. Transfer plate to a second work area, and add 3 μl of extracted nucleic acid into duplicate wells of the plate according to a defined plate layout. After adding the nucleic acid to the optical PCR plate, the extraction plate can be stored at −20 °C or +4 °C depending on its further use. Great care should be taken to avoid the creation of aerosols and other potential routes of contamination.
8. If using standards add 3 μl of diluted plasmid to duplicate wells of the plate according to a defined plate layout. Great care should be taken to avoid the creation of aerosols and other potential routes of contamination.
9. Cover each well of the PCR plate containing the reaction components firmly with a plate sealer.
10. Centrifuge the plate using a PCR plate spinner, or centrifuge to bring the reaction mix constituents to the bottom of the well and to eliminate all air bubbles.
11. Run samples on a real-time PCR instrument using the thermal profiles indicated in Table 6.
12. If a Ct value occurs in more than one negative control well, this could indicate contamination. If one or more negative controls from the extraction are positive, repeat the extraction and qPCR. If one or more qPCR negative controls are positive, repeat the qPCR assay.
13. Calculate the average Ct value for standards, controls, and samples, and plot the average Ct values of the standards against copy number. The efficiency of the qPCR can then be calculated using the slope of this line with the formula below.

$$\text{Efficiency (\%)} = \left(10^{(-1/\text{Slope})} - 1\right) \times 100.$$

The efficiency should be between 90 and 110% and if wanting to compare between plates should be within a range of 10%, e.g., all plates for a given experiment should have an efficiency of between 92 and 102%. Plates outside of this range should be repeated. *See* **Note 11** and **Note 12**.

14. If the plate has passed and is within the required efficiency range, then the copy number of each sample can be calculated using the standard curve and the formula. *See* **Note 11**.

$$y = ax + b$$

where

$y =$ average Ct value of the sample

$a =$ slope of the line of the standard curve

$x = \log_{10}$ (copy number of the sample)

$b =$ y intercept of the standard curve

This will be the copy numbers of ASFV genomic DNA in 1 μl of the extracted sample (3 μl extracted sample and 3 μl of standard in each PCR). This value will then need converting back to the amount in the original sample which will depend on how much of the original sample was extracted and into what volume that sample was extracted into.

4 Notes

1. While this method is an accurate reproduction of that currently used in the non-vesicular disease laboratory at the Pirbright Institute, the extensive quality assurance, control systems, and associated standard operating procedures required by an accredited diagnostic laboratory are beyond the scope of this protocol.
2. This could be positive samples tested previously either from samples obtained from a reference laboratory, virus grown in primary cell cultures, or samples from infected animals.
3. We routinely use commercial pig serum as a negative control, but other samples from naïve animals are acceptable, or tissue culture media could be substituted if analyzing virus replication in vitro.
4. Different real-time PCR instruments may require different master mixes, for example, some instruments require the inclusion of ROX as a passive dye control.
5. Copy numbers of a given plasmid can be calculated by using the following formula.

$$\text{Copy number} = \left(\text{mass DNA} \times \left(6.022 \times 10^{23}\right) \div \left(\text{plasmid length} \times \left(1 \times 10^{9}\right) \times 650\right)\right.$$

where

The mass of DNA in nanograms

6.022×10^{23} is Avogadro's number

Plasmid length in base pairs

650 is the average molecular mass of a single DNA base pair

For the plasmid described in the King et al. assay, there are approximately 2.58×10^{11} molecules of the plasmid in 1 μg.

6. If using a virus standard, then the higher the titer, the greater the dynamic range of the qPCR assay. If analyzing a set of samples across multiple plates, then it is crucial there is sufficient sample for all of the plates that will be run in that set. A virus standard can also substitute for the positive control.
7. Use of plasmid in a diagnostic setting should be avoided at all costs [4]. In a research setting, great care should be exercised to avoid contamination and at a minimum plasmid should be prepared in an area that is spatially separated. Plasmid DNA is very difficult to eliminate; therefore, plasmid preparation should be performed by experienced/skilled operators. Ideally, plasmid standards would be used in an area which can be decontaminated by UV light.
8. We routinely use QIAamp viral RNA kits (Qiagen) for manual extraction of ASFV DNA and MagMAX CORE nucleic acid purification kit (Thermo Fisher Scientific) for robotic extraction from a variety of different matrices. In-house testing at Pirbright has shown that the lysis buffers contained in both of these kits are effective at inactivating ASFV infectivity (>4 log kill). Kits from other manufacturers are likely to be just as effective. Elute samples in the lowest volume acceptable for the kit in order to maximize the chance of minimal amounts of genome being detected.
9. Use a 96-well plate or a cold 96 plate rack to help stabilize the optical plate when loading master mix and samples.
10. Thermal profiles are unique to the real-time PCR instrument and reaction kit, and a typical thermal profile we use on both the Applied Biosystems 7500 fast and Agilent MxPro is shown in Table 6 as an example.
11. Most real-time PCR machines are able to calculate average Ct values, slope of the line, and real-time PCR efficiencies and convert Ct values to copies/mL automatically.
12. Common reasons for real-time PCR efficiencies to fall outside of the range 90–110% are uncalibrated pipettes, pipetting errors, and reusing freeze-thawed standards.

Acknowledgments

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Chapter 7

Quantification of ASFV DNA and RNA in *Ornithodoros* Soft Ticks

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Abstract

Molecular biology methods are highly sensitive to detect the genome of pathogens and to study their biology. Polymerase chain reaction (PCR) and reverse transcription followed by a polymerase chain reaction (RT-PCR) permit the detection of the presence and the replication of African swine fever virus in soft ticks. Here, we described our techniques to detect and quantify DNA and RNA of African swine fever virus in soft ticks including a housekeeping gene of soft ticks as internal control.

Key words PCR, RT-PCR, Soft tick, *Ornithodoros*, African swine fever virus

1 Introduction

The most common molecular approach to study ASFV is the DNA detection using PCR. Due to the high resistance of the virus and its DNA, this technique is a gold standard for the diagnosis. Furthermore, this approach is more sensitive than viral titration/isolation. However, working with RNA is more precise for ASFV biology study, including gene expression, replication kinetics, and detection of replicating virus. In this chapter, we described our methodologies to detect and study ASFV in different soft ticks from *Ornithodoros* genus using different approaches on purified DNA and RNA with a simplex or duplex PCR.

2 Materials

2.1 Solutions and Materials Used for both RNA and DNA Extraction

All reagents and plasticware should be DNase- and RNase-free.

1. Refrigerated microcentrifuge that can be rotated at 16,000 × *g* (*see* **Note 1**).

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2. Sodium hypochlorite 2.6%.
3. Sterile phosphate-buffered saline solution (PBS).

2.1.1 Solutions and Materials Used for Crushing Ticks

1. One steel bead of 3 mm and one steal bead of 4 mm per tick.
2. Tissue homogenizer (*see* **Note 2**).
3. 1.5 or 2 mL polypropylene tube.

2.1.2 Solutions and Materials Used for Tick Dissection

1. Sterile blade and pliers.
2. Binocular loupe.
3. Sterile PBS.

2.2 DNA Extraction

1. DNA extraction kit capable of generating high-quality DNA (*see* **Note 3**).

2.2.1 Duplex Real-Time PCR

1. Primers and probes listed in Table 1.
2. 2× PCR master mix (*see* **Note 4**).
3. DNase/RNase free water.
4. 10× ASF specific primer-probe mix: 6 μM VP72 forward primer, 6 μM VP72 reverse primer, 3 μM VP72 probe in DNase/RNase-free water.
5. Vortex.
6. Real-time PCR machine.
7. Optical plates or strips and lids suitable for model of real-time PCR machine (typically 96-well plate format).

2.2.2 Universal Probe Library Real-Time PCR

1. Primers and probe listed in Table 2.
2. 2× PCR master mix (*see* **Note 5**).
3. DNase/RNase-free water.
4. Vortex.

Table 1
Primers used for duplex qPCR

Target	Primers	Sequence	Concentration used for PCR	Size of amplicon
VP72	Forward	5’-TGC TCA TGG TAT CAA TCT TAR CG-3’	0.6 μM	159 bp
	Reverse	5’-CCA TGG GTT GGT ATT CCT C-3’	0.6 μM	
	Probe	5’-FAM-TTC CAT CAA AGT TCT GCA GCT CTT-TAMRA-3’	0.3 μM	
β-actin	Forward	5’-CGG TAT TGC CGA CCG TAT GC-3’	0.6 μM	140 bp
	Reverse	5’-GCT GGA AGG TGG ACA GGG AG-3’	0.6 μM	
	Probe	5’-HEX-CGA GAG GAA GTA CTC CGT CTG G-BHQ1-3’	0.2 μM	

Table 2
Primers used for UPL PCR. R: correspond to A or G

Target	Primers	Sequence	Concentration used for PCR	Size of amplicon
VP72	Forward	5′- CCC AGG RGA TAA AAT GAC TG-3’	0.6 μM	68 bp
	Reverse	5′- CAC TRG TTC CCT CCA CCG ATA-3’	0.6 μM	
	Probe	5′- 6FAM-GG CCA GGA-dark quencher dye-3’	0.3 μM	

Table 3
Primers used for conventional RT-PCR

Target	Primers	Sequence	Concentration used for RT-PCR	Size of amplicon
VP72	Forward	5’-TGC TCA TGG TAT CAA TCT TAR CG-3’	0.4 μM	159 bp
	Reverse	5’-CCA TGG GTT GGT ATT CCT C-3’	0.4 μM	
β-actin	Forward	5’-CAGGTGATCACCATCGGC-3’	0.4 μM	308 bp
	Reverse	5’-GAGGCCAGGATGGAGCC-3’	0.4 μM	

5. Real-time PCR machine.
6. Optical plates or strips and lids suitable for model of real-time PCR machine (typically 96-well plate format).

2.3 RNA Extraction

1. Sterile scissors.
2. Mortar and pestle.
3. Guanidinium thiocyanate-phenol-chloroform extraction chemical (TRIzol™; *see* **Note 6**).
4. Vortex.
5. Chloroform isoamyl alcohol 25:24:1.
6. DNase (*see* **Note 7**).
7. A commercial kit compatible with TRIzol™ lysate (*see* **Note 8**).

2.3.1 Conventional RT-PCR for RNA Detection

1. Primers and probes listed in Table 3.
2. 1% agarose gel.
3. DNA ladder (*see* **Note 9**).
4. Plasmid coding for VP72 gene of ASFV and β-actin of soft ticks.

5. DNA amplification kit (*see* **Note 10**).
6. One-step RNA reverse transcription and cDNA amplification kit (*see* **Note 11**).

2.3.2 Real-Time RT-PCR for RNA Detection and Quantification

1. Primers and probes listed in Table 1.
2. One-step RNA reverse transcription and cDNA amplification kit (*see* **Note 12**).
3. DNase/RNase-free water.

3 Methods

DNA and RNA extraction are important steps. Nucleic acid quality and purity are very important for the success of detection of ASFV in ticks. In the literature, two methodologies are described for DNA extraction from ticks. The first one is extraction using phenol-chloroform [1], and the second is extraction using commercial kits [2–10]. The second is the most frequently used. Commercial kit provided reproducible and highly pure DNA, avoiding the use of phenol. However, only one methodology has been described for RNA detection from soft tick samples [11]. In this chapter, we described our protocol using a commercial kit and also TRIzol™ reagent.

3.1 Tick Sample Preparation for DNA Extraction

DNA can be extracted from whole ticks or dissected organs. For both cases, washing step is important to remove external DNA contaminant.

3.1.1 Preparation for Whole Tick DNA Extraction

1. Wash ticks 5 s in sodium hypochlorite 2.6% and then three times in sterile PBS at room temperature.
2. Transfer ticks into a new tube with 200 μL of sterile PBS, one bead of 3 mm, and one bead of 4 mm.
3. Crush ticks by homogenizing at 25 Hz for 3 min.
4. Add 800 μL of sterile PBS to the homogenate. Vortex thoroughly.
5. Clarify homogenate by centrifugation (5000 × *g*, 2 min).
6. Supernatant can be directly used for DNA extraction step or stored at −80 °C.

3.1.2 Preparing Tick Organs for DNA Extraction

1. Wash ticks for 5 s in sodium hypochlorite 2.6% and then three times in sterile PBS at room temperature.
2. Dissect ticks with sterile blade and pliers. To prevent the organs from drying out, dissect the ticks in PBS. A binocular loupe is recommended for this step.

3. Place organs into a new tube with 200 μL of sterile PBS, one bead of 3 mm, and one bead of 4 mm.
4. Crush organs by homogenizing at 25 Hz for 3 min.
5. Lyse homogenates in lysis buffer using the lysis protocol of the High Pure PCR Template kit (Protocol "Isolation of nucleic acids from mouse tail"). Briefly, add 200 μL of lysis buffer and 40 μL of proteinase K to the homogenates, and then incubate for 4 h at 55 °C.
6. The homogenate can then be used in the DNA extraction step or stored at −80 °C.

3.2 DNA Extraction

1. Extract DNA from 200 μL of homogenate obtained from whole ticks or from all of the homogenate obtained for the larvae stage (200 μL) or tick organs according to manufacturer's instructions (*see* **Note 3**).
2. Elute DNA with 100 μL of elution buffer.
3. DNA can be immediately used for qPCR or stored at −20 °C or −80 °C. DNA is also ready to use for next-generation sequencing (NGS).

3.3 Real-Time PCR for DNA Detection and Quantification

Two different protocols can be used for DNA detection: (i) a duplex PCR using specific primers and probes to detect VP72 gene and actin of soft ticks and (ii) a simplex PCR using degenerated primers and probe (UPL#162) from the Universal Probe Library from Roche Life Science.

3.3.1 Duplex PCR

A duplex real-time PCR was developed for ASFV DNA detection in soft tick, in combination of soft tick β-actin gene as internal control [9]. This duplex qPCR was used for ASFV detection and quantification in *O. moubata*, *O. erraticus*, and *O. verrucosus* [8, 9]. It was efficient to detect ASFV in ticks presenting very low or undetectable viral titer on porcine alveolar macrophages and on different tick stages (larvae, nymph, and adults) (Fig. 1) and on dead ticks (*see* **Note 12**, Fig. 2). For ASFV detection, a conserved region between all viral strains is targeted [12], and for β-actin a conserved fragment is also targeted [13]. Some results of qPCR are presented in Fig. 1a (VP72 copy number) and 1B (β-actin copy number). These results were obtained from infected ticks at 2 months post-infection (PI) presented in our previous article [8].

1. Prepare the PCR master mix according to the volumes outlined in Table 4 (*see* **Note 13**). Ensure that a sufficient volume of master mix is prepared to allow testing of the required number of samples and include an allowance of 5–10% to allow for pipetting discrepancies. Prepare reagents in a microcentrifuge tube and mix using a vortex.

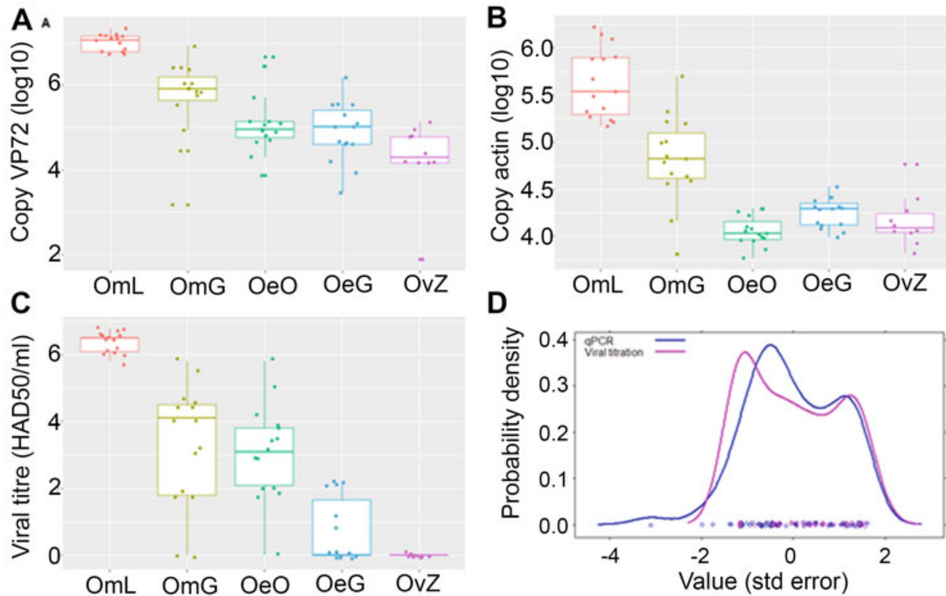

Fig. 1 Results of VP72 (**a**) and β-actin (**b**) at 2 months post-infection. Viral titer of those ticks is also shown (**c**). The probability density between the distribution of qPCR and viral titer results (**d**). OmL, *Ornithodoros moubata*-Liv13/33; *OmG O. moubata*-Georgia2007/1, OeO *O. erraticus*-OurT88/1, OeG *O. erraticus*-Georgia2007/1, OvZ *O. verrucosus*-Ukr12/Zapo

2. Pipette 20 μl of the master mix into each tube and then add 5 μl of DNA extract to the master mix. Run on real-time PCR machine using the program shown in Table 5 (*see* **Note 14**).

3.3.2 UPL PCR

A real-time PCR is available for ASFV DNA detection in soft ticks using degenerated primers and probe (UPL#162) from the Universal Probe Library from Roche Life Science and the LightCycler 480 Probes Master kit (Roche Applied Science) [14]. However, duplex with β-actin presented different results for ASFV DNA when compared to simplex PCR, and that was depending on the tick species. For a better duplex, it's preferable to use the real-time PCR presented above [9]. Nevertheless, the UPL PCR is faster than the other and can be used as a simplex real-time PCR.

1. Prepare the PCR master mix according to the volumes outlined in Table 6. Ensure that a sufficient volume of master mix is prepared to allow testing of the required number of samples and include an allowance of 5–10% to allow for pipetting discrepancies. Prepare reagents in a microcentrifuge tube and mix using a vortex.
2. Pipette 20 μl of the master mix into each tube, and then add 5 μl of DNA extract to the master mix. Run on real-time PCR machine using the program shown in Table 7 (*see* **Note 15**).

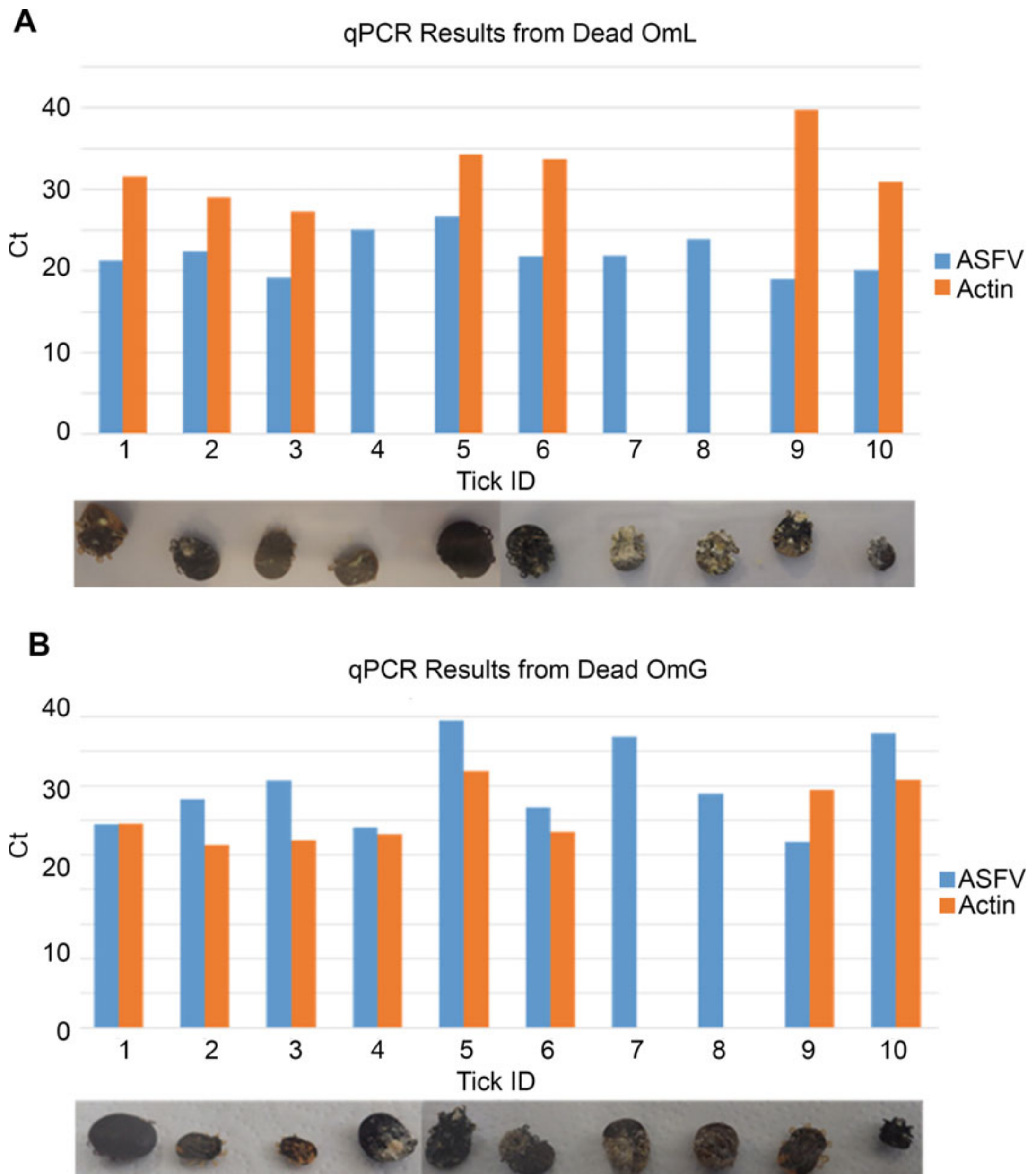

Fig. 2 qPCR results from ASFV and β-actin detection in *Ornithodoros moubata* infected by the Liv13/33 strain (**a**) and *Ornithodoros moubata* infected by the Georgia2007/1 strain (**b**). Ct: cycle threshold. Photos of the state of degradation are also shown in the bottom of the figure

3.4 Tick Sample Preparation for Total RNA Extraction

1. Wash ticks for 5 s in sodium hypochlorite 2.6% and then three times in sterile PBS at room temperature.
2. Place ticks in a new 2 mL tube containing 300 μL TRIzol™. Cut the tick with sterile scissors and then crush in a mortar and pestle.

Table 4
Mix for Duplex qPCR

Component	Volume for a reaction of 25 μL (= 1 reaction)	Final concentration
PCR master mix (2×)	12.5 μL	1×
ASF specific primers-probe mix (10×)[a]	2.5 μL	1×
β-actin forward primer	0.5 μL	0.6 μM
β-actin reverse primer	0.5 μL	0.6 μM
β-actin probe	0.5 μL	0.2 μM
DNase/RNase-free water	3.5 μL	1×
DNA	5 μL	1×

[a]This mix contains 6 μM of each primer and 3 μM of probe used for ASFV VP72 detection, as previously described

Table 5
PCR program for duplex qPCR (Pereira de Oliveira et al., 2019)

Temperature (°C)	Time of incubation	Number of cycle
50 °C	2 min	1
95 °C	10 min	1
95 °C	1 min	45
60 °C	1 min[a]	

[a]Fluorescence acquisition at the end of the cycle

Table 6
Mix for simplex PCR to detect ASFV

Component	Volume for a reaction of 30 μL (= 1 reaction)	Final concentration
PCR master mix (2×)	15 μL	1×
VP72 forward primer	0.6 μL	0.6 μM
VP72 reverse primer	0.6 μL	0.6 μM
VP72 probe	0.3 μL	0.1 μM
DNase/RNase-free water	11.5 μL	1×
DNA	2 μL	1×

3. Add 700 μL of TRIzol™ to the homogenate and vortex thoroughly.
4. Store sample at −80 °C.

Table 7
PCR program for UPL PCR

Temperature (°C)	Time of incubation	Number of cycle
95 °C	5 min	1
95 °C	20 secs	45
60 °C	30 secs[a]	

[a]Fluorescence acquisition at the end of the cycle

3.5 RNA Extraction

Pre-cool microcentrifuge to 4 °C before starting the RNA extraction.

1. Centrifuge 300 μL of TRIzol™ homogenate (12,000 × *g*, 5 min, 4 °C).
2. Transfer the supernatant to a new 2 mL tube.
3. Incubate the supernatant at room temperature for 5 min.
4. Add 60 μL of chloroform-isoamyl-alcohol (25:24:1) to the supernatant into a new tube and then mix well.
5. Incubate the mixture at room temperature for 3 min.
6. Centrifuge the tube to separate organic and aqueous phases (12,000 × *g*, 15 min, 4 °C).
7. Transfer the upper aqueous phase to a 2 mL tube.
8. Use an aliquot of the aqueous phase for RNA extraction following the manufacturer's protocol (*see* **Note 8**).
9. Treat extracted RNA (*see* **Note 15**) with DNase following the manufacturer protocol (*see* **Note 7**), and then stop the enzymatic reaction by adding 150 μL of TRIzol™.
10. Re-extract RNA following the manufacturer protocol.
11. RNA can be directly used for RT-PCR or stored at −80 °C.

3.5.1 Conventional RT-PCR for RNA Detection

A RT-PCR was developed to detect the expression of VP72 gene of ASFV and β-actin of soft ticks. Although this RT-PCR has only been validated for *Ornithodoros moubata*, it likely will work for other *Ornithodoros* tick species, provided further validation. To detect these RNAs, a one-step RT-PCR is used, consisting of the reverse transcription directly followed by the PCR in the same reaction tube.

1. Prepare the one-step RT-PCR master mix according to the volumes outlined in Table 8. Ensure that a sufficient volume of master mix is prepared to allow testing of the required number of samples, and include an allowance of 5–10% to allow for pipetting discrepancies. Prepare reagents in a microcentrifuge tube and mix using a vortex.

Table 8
Mix for RT-PCR. Primer mix contains 10 μM of forward and reverse primers

Component	Volume for a reaction of 25 μL (= 1 reaction)	Final concentration
One- Step ToughMix (2X)	12.5 μL	1×
qScript XLT One-Step RT (25X)	1	1×
Primers mix (10 μM)	2 μL	0.4 μM (for each primer)
DNase/RNase-free water	7.5 μL	1×
RNA	2 μL	1×

Table 9
PCR program for RT-PCR

Temperature (°C)	Time of incubation	Number of cycle
48 °C	20 min	1
94 °C	3 min	1
94 °C	30 secs	40
60 °C	1 min	
72 °C	7 min	1

2. Pipette 23 μl of the master mix into each tube, and then add 2 μl of RNA extract to the master mix. Run on real-time PCR machine using the program shown in Table 9.

3.5.2 Real-Time RT-PCR for RNA Detection and Quantification

A duplex RT-qPCR was developed for ASFV RNA detection in soft tick, in combination with soft tick β-actin gene as internal control. This duplex RT-qPCR was used for ASFV detection in *O. moubata*. It was efficient to detect VP72 expression in male and female but not tested on nymph stages.

1. Prepare the one-step RT-PCR master mix according to the volumes outlined in Table 10 (*see* **Note 13**). Ensure that a sufficient volume of master mix is prepared to allow testing of the required number of samples, and include an allowance of 5–10% to allow for pipetting discrepancies. Prepare reagents in a microcentrifuge tube and mix using a vortex.
2. Pipette 23 μl of the master mix into each tube, and then add 2 μl of RNA extract to the master mix. Run on real-time PCR machine using the program shown in Table 11 (*see* **Note 16**).

Table 10
Mix for duplex RT-qPCR

Component	Volume for a reaction of 20 μL (= 1 reaction)	Final concentration
qScript XLT One-Step RT-qPCR ToughMix (2×)	10 μL	1×
VP72 forward primer	0.4 μL	0.6 μM
VP72 reverse primer	0.4 μL	0.6 μM
VP72 probe	0.6 μL	0.3 μM
β-actin forward primer	0.4 μL	0.6 μM
β-actin reverse primer	0.4 μL	0.6 μM
β-actin probe	0.4 μL	0.2 μM
DNase/RNase-free water	5.4 μL	1×
RNA	2 μL	1×

Table 11
PCR program for duplex RT-qPCR

Temperature (°C)	Time of incubation	Number of cycle
48 °C	20 min	1
95 °C	1 min	1
95 °C	1 min	45
60 °C	1 min[a]	

[a]Fluorescence acquisition at the end of the cycle

4 Notes

1. A centrifuge without a refrigerated program can be used for DNA extraction.
2. We have used the Star-beater homogenizer successfully, but this could be substituted for any machine that can homogenize at 25 Hz for 3 min.
3. For DNA extraction, we used High Pure PCR Template kit (Roche Life Science), but other commercial kit or method can be used, as explain in Subheading 3. The most important is to obtain a high purity DNA.
4. For the duplex qPCR assay, we use the PCR master mix from the QuantiTect™ Multiplex PCR Kit (Qiagen).
5. For the UPL qPCR assay, we use the PCR master mix from the from the LightCycler 480 Probes Master kit (Roche).

6. Our method has been validated with TRIzol™; other guanidinium thiocyanate-phenol-chloroform extraction reagents may be equally effective.
7. In our protocol of RNA extraction, we use the highly efficient TURBO™ DNase (Invitrogen) to remove DNA contaminant.
8. To extract RNA, chloroform isoamyl alcohol 25:24:1, TURBO™ DNase (Invitrogen) and the Direct Zol RNA Kit (Zymo Research) are recommended. This protocol requires two extractions per sample. Therefore a 100 reaction kit will be sufficient for 50 samples. Other commercial kit or methods can be used [11]. The most important is to obtain a high pure RNA without any DNA contamination.
9. We used the GeneRuler 100 bp (Thermo Scientific as DNA ladder for conventional PCR.
10. For DNA detection by real-time PCR, we routinely use QuantiTect™ Multiplex PCR Kit (Qiagen), but another PCR can be performed using the LightCycler 480 Probes Master kit (Roche Applied Science).
11. For RNA detection by reverse transcription PCR (RT-PCR), we use qScript™ XLT One-Step RT-PCR Kit (Quanta BioSciences). For RNA detection by real-time RT-PCR, we use qScript™ XLT One-Step RT-qPCR ToughMix® (Quanta BioSciences).
12. Quantitative PCR is more sensitive than viral titer as shown in Fig. 1c, d. These results show that we can detect low amount of ASFV DNA in infected ticks while viral isolation/titration failed to detect the virus. Furthermore, our protocol still works on dead soft ticks as shown in Figs. 2 and 3. Depending on the state of degradation of the tick, β-actin is not always detected.
13. This PCR can be used as simplex PCR by replacing primers and probe by an equivalent volume of DNase/RNase-free water.
14. This qPCR can be also used for DNA quantification, using a standard curve with a serial dilution of a plasmid containing sequences corresponding to the VP72 and β-actin amplicons, as suggested in Fig. 3a.
15. Step 9 of the RNA extraction protocol is very important for the complete removal of DNA contamination (*see* Fig. 4). To test this in your own laboratory, remove an aliquot of RNA extract prior to the addition of DNAse.
16. This qPCR can be also used for DNA quantification, using a standard curve with a serial dilution of a plasmid containing sequences corresponding to the VP72 and β-actin amplicons, as suggested in Fig. 3b.

A

Standard for DNA quantification

ATGTACC**CCGGTATTGCCGACCGTATGC**AGAAGGAAATCACTGCCCTGGCCCCATCCACGATG
AAGATCAAGATCATCGCTCCCCC**CGAGAGGAAGTACTCCGTCTGG**ATCGGTGGTTCCATCCTG
GC**CTCCCTGTCCACCTTCCAGC**AGATGTAAAAATGATCCGTTTAGGTTGATGGCTTTGGTCA
TCGTGTATATAAAAACTACGATCCCCGTGCCGCAGTACTTAAAGAAACCTGTAAAGAAGTATT
AAAGGAACTCGGACAGCTAGAAAACAACCCACTCCTGCAAATAGCAATAGAATAAC**TGCTCAT**
GGTATCAATCTTATCGATAAGT**TTCCATCAAAGTTCTGCAGCTCTT**ACATACCCTTCCACTAC
GGAGGCAATGCAATTAAAACCCCCGATGATCCGGGTGCGATGATGATTACCTTTGCTTTGAAG
CCACGG**GAGGAATACCAACCCAGTGG**TCATATT

B

Standard for RNA quantification

ATGTACC**CCGGTATTGCCGACCGTATGC**AGAAGGAAATCACTGCCCTGGCCCCATCCACGATG
AAGATCAAGATCATCGCTCCCCC**CGAGAGGAAGTACTCCGTCTGG**ATCGGTGGTTCCATCCTG
GC**CTCCCTGTCCACCTTCCAGC**AGATGTTAAC**TGCTCATGGTATCAATCTTATCG**ATAAGT**TT**
CCATCAAAGTTCTGCAGCTCTTACATACCCTTCCACTACGGAGGCAATGCAATTAAAACCCCC
GATGATCCGGGTGCGATGATGATTACCTTTGCTTTGAAGCCACGG**GAGGAATACCAACCCAGT**
GGTCATATTTTACGCGATGTATCGTATGCCCCCTTTTTACCCGGACCCGAGTCTGGTCACCTA
TAGCCCGCTGGTCCCAGTACGCACCCCCTTGAGAACTCTCACTCCGGGCACTGTCATTGAACT
GCACGGACGCATACACAATACCAAAAGGTTTGCCATCAACTTGGAAACTAAGGATGGTGATAT
AGCAGATGTTGGGCAAAAGCACAGTCTTGCTTGTGGCCACAATTTTTGCGGTCGGATGTGTCG
TCGAAGGGAAGGTTTACGACAGATGTTCTCTCGCCAGTGAGCTACGCTGGAAGTACAATCTAC
CCAAGGACCAAATTGCTGATTGGGTGTGCATCGCA

Fig. 3 DNA sequences used for generating standard curves for DNA quantification (**a**) or RNA quantification (**b**) by qPCR. Beta-actin primers are in blue and the beta-actin probe in yellow. The ASFV-VP72 gene primers are in green and the ASFV-VP72 probe is in red

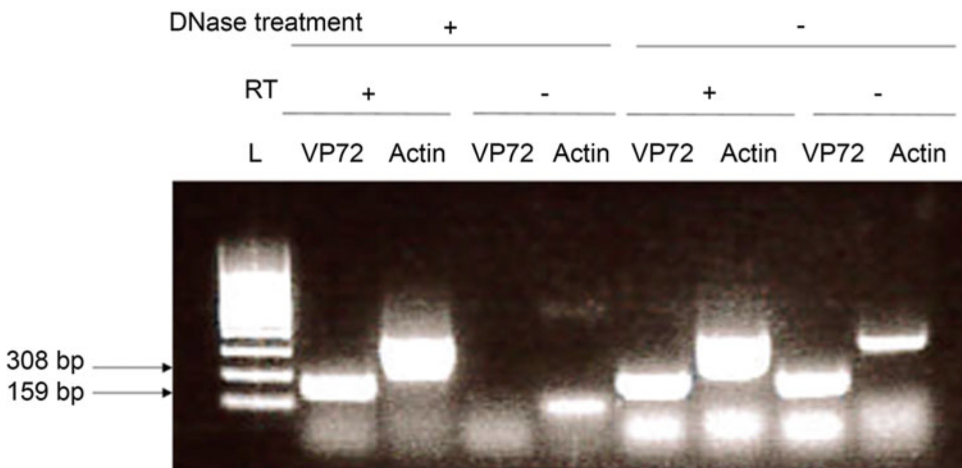

Fig. 4 Validation of the RNA extraction protocol by conventional RT-PCR. VP72 and β-actin RNA were amplified by RT-PCR. Amplification product was migrated on a 1% agarose gel. RT: reverse transcription. L ladder, bp base paired

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Chapter 8

Genotyping of African Swine Fever Virus

Paulina Rajko-Nenow and Carrie Batten

Abstract

Molecular methods are routinely used for the differential diagnosis and genetic characterization of viral diseases of livestock. Real-time PCR (qPCR) is known as the gold standard diagnostic method for most diseases and is also used for the detection of African swine fever virus (ASFV) DNA in clinical specimens. To determine the ASFV genotype or identify additional genome markers, endpoint PCR is usually performed on ASFV-positive specimens, followed by Sanger sequencing and data analysis. Here, we describe the ASFV genotyping method used at the OIE Reference Laboratory for ASF at the Pirbright Institute, United Kingdom.

Key words Gel electrophoresis, Polymerase chain reaction, Sequencing, Genotyping

1 Introduction

The African swine fever virus (ASFV) genome size varies from 170 to 193 kbp and resembles the genomic organization of other nucleocytoplasmic large DNA viruses [1]. Most regions of the ASFV genome can be amplified using gel-based PCR with a specific pair of primers and subsequently Sanger sequenced. The ASFV major capsid protein p72 (B646L gene) [2] is one of the most commonly used regions for sequencing to discriminate between the currently recognized XXIV ASFV genotypes. In addition, sequencing of p54 (E183L gene) has also been successfully used for some ASFV genotypes (e.g., genotype I) as it discriminates between additional subgroups and provides better resolution of ASFV strains [3]. Sequencing of both genetic regions p72 and p54 is often performed for ASFV initial classification to support epidemiological investigation in the event of an ASF introduction into new territories. Further discrimination between different ASFV strains can be achieved by sequencing other regions of the ASFV genome, known as genetic markers. A number of genetic markers such as the Central Variable Region (CVR) of the B602L gene [3, 4], the intergenic region between I73R and I329L genes (characterized

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by the presence of tandem repeat sequences (TRS)) [5, 6], and the CD2v lectin-like protein (EP402R gene) [7] have been widely used. However, the use of genetic markers to trace virus spread and/or identify the origin of virus in a new territory still poses a challenge [8] mainly due to the low mutation rate of ASFV and the scarcity of high-quality whole genome sequencing data [9, 10].

During endpoint PCR, two oligonucleotide primers bind to a target region of the ASFV genome and are extended by *Taq* polymerase through a series of 30–40 repeated temperature cycles. Gel electrophoresis is performed to confirm the presence of the amplified PCR products by comparing the DNA bands to a molecular-weight size standard (ladder) under UV light (Fig. 1). Once a correct size band is identified, then it must be purified to remove the excess primers, salts, and nucleotides that can interfere with the downstream sequencing reaction. Poor quality template is most common cause of sequencing errors. When well-resolved and evenly spaced peaks are visible on an electropherogram generated by an automated DNA sequencer, then data analysis can begin by assembling reverse and forward sequences obtained from the same specimen. To determine the ASFV genotype present in the specimen, sequencing data needs to be compared with a number of ASFV reference sequences by generating a multiple sequence alignment (Fig. 2) and a phylogenetic tree (Fig. 3).

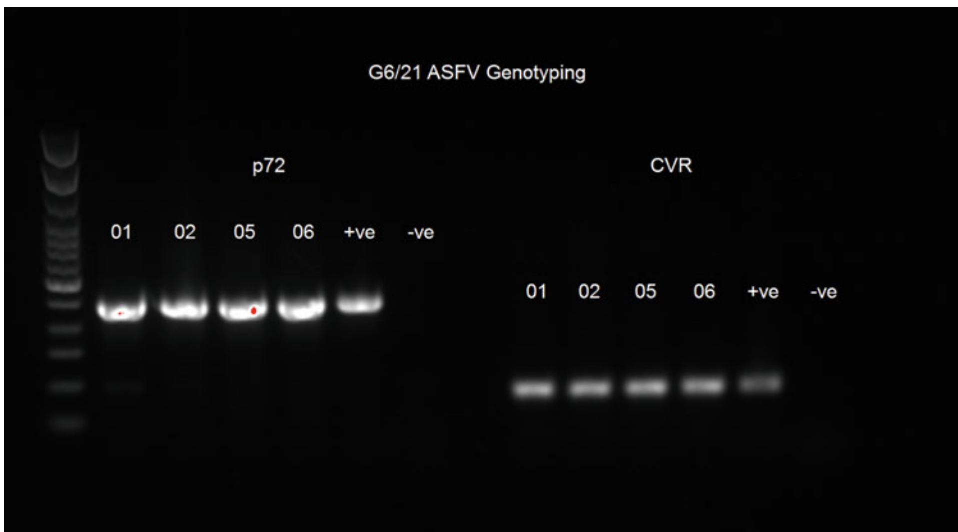

Fig. 1 Agarose gel electrophoresis of two PCR products: p72 (~478 bp) and CVR (~200 bp). First line on the left represents a 100 bp ladder; ASFV-positive samples are labelled as 01, 02, 05, and 06; positive control and negative control are labelled as +ve and −ve, respectively

A

B

Fig. 2 Multiple sequence alignment of the p72 (**a**) and the TRS regions (**b**) performed by MUSCLE algorithm in MEGA7 program. The multiple sequence alignment for p72 region was carried out on amino acid level and then translated to the nucleotide sequences prior to phylogenetic analysis. Partial nucleotide sequence alignment of the intergenic region between I73R and I329L from representative ASFV strains revealed that the ASFV/Belgium/Etalle/2018 (MH998379) strain has a 10-nucleotide insertion in comparison to other ASFV genotype II strains, e.g., Georgia/2007/1 (FR682468)

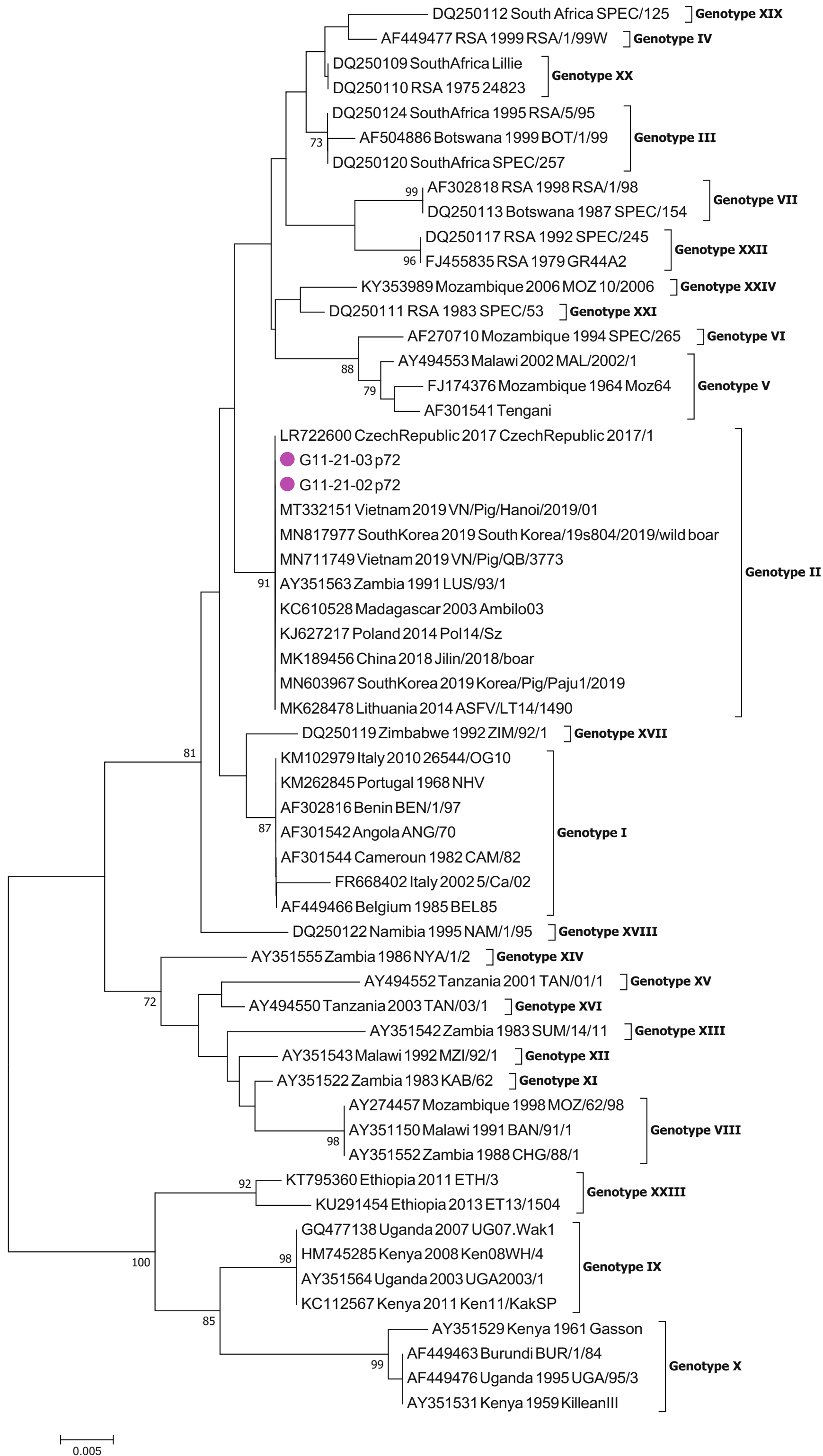

Fig. 3 Phylogenetic relationship of ASFV selected strains based on the partial p72 gene sequences. The neighbor-joining tree was constructed using MEGA7, and the evolutionary distances were computed using the

2 Materials

1. Separate workspaces and personal protective equipment (lab coats and gloves) for PCR setup and post-amplification processing, preferably with PCR cabinets.
2. Pipettes and aerosol-resistant tips.
3. PCR machine with preheated lid.
4. Molecular biology grade nuclease-free water.
5. Genotyping PCR and sequencing primers (*see* **Note 1** and Table 1).
6. DNA/RNAse-free 0.2 mL tubes, strips, or plates and 2 mL tubes.
7. PCR master mix (*see* **Note 2**).
8. DNA extractions from test samples, positive and negative controls.
9. Vortex.
10. Deionized water.

Table 1
Names and sequences of genotyping primers, as well as annealing temperatures for PCR cycling and length of predicted PCR product

Region	Product size	Annealing temperature (°C)	Primer name	Sequence (5′-3′)
p72 (B646L)	478 bp	50	P72-U P72-D	GGCACAAGTTCGGACATGT GTACTGTAACGCAGCACAG
p54 (E183L)	683 bp	50	PPA722 PPA89	CGAAGTGCATGTAATAAACGTC TGTAATTTCATTGCGCCACAAC
TRS (I73R and I329L)	356 bp	54	ECO1A ECO1B	CCATTTATCCCCCGCTTTGG TCGTCATCCTGAGACAGCAG
CVR (pB602L)	Variable	54	ORF9L-F ORF9L-R	AATGCGCTCAGGATCTGTTAAATCGG TCTTCATGCTCAAAGTGCGTATACCT

Fig. 3 (continued) maximum composite likelihood method. Scale bar indicates nucleotide substitutions per site. Bootstrap analysis was performed for 1000 replicates of the dataset, and values of >70% are displayed. Corresponding ASFV genotypes are labelled I–XXIV. Query sequences G11–21 were represented by pink points (or pink circles)

11. 0.5 M EDTA: 0.5 M ethylenediaminetetraacetic acid (EDTA) in water, pH 8.0.
12. 50× TAE buffer: Weigh 242 g of Tris-base and dissolve in approximately 700 mL of deionized water. Carefully add 57.1 mL of 100% glacial acid (or acetic acid) and 100 mL of 0.5 EDTA solution (pH 8.0). Adjust the solution to a final volume of 1 liter with deionized water, and store at room temperature (*see* **Note 3**).
13. 1× TAE buffer: Aliquot 20 mL of 50× TAE buffer into a 1 liter graduated cylinder. Make up to 1 liter with distilled water and mix throughout. For each electrophoresis, fresh 1× TAE buffer (composition 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) should be used.
14. Nucleic acid-binding dye visible under UV light.
15. Electrophoresis grade agarose.
16. Glass conical flask.
17. Heat-proof gloves and appropriate eye protection.
18. Microwave.
19. Apparatus suitable for agarose DNA electrophoresis, including caster or adhesive tape.
20. DNA loading dye.
21. DNA ladder (*see* **Note 4**).
22. UV lightbox with appropriate eye protection.
23. Gel extraction kit for PCR products (*see* **Note 5**).
24. Spectrophotometer.
25. Sanger sequencing kit (*see* **Note 6**).
26. Electropherogram analysis software (*see* **Note 7**).
27. MEGA-X installation (*see* **Note 8**).

3 Methods

To prevent contamination in the PCR laboratory, a sample processing workflow should be set according to well-established guidelines [11]. It is very important that pre-PCR and post-PCR activities are separated; each area should have dedicated set of equipment, including pipettes, reagents, pipette tips, racks, lab coats, etc. PCR good laboratory practice should always be followed by using aerosol-resistant pipette tips and wearing clean gloves and lab coats (not previously contaminated with PCR products). Gloves should be changed on regular basis whenever contamination is suspected. The workflow must be unidirectional from “clean” area (pre-PCR) to “dirty” (post-PCR), and amplified PCR

products should never be brought into the PCR setup area. Routine cleaning of lab benches and equipment should be performed with an approved cleaning product (e.g., 10% bleach solution) to prevent contamination.

3.1 Primer Preparation

1. Once the primers arrive at the laboratory, they must be taken directly to a PCR clean room.
2. To prepare 100 μM primer concentration, add the appropriate volume of nuclease-free water to the lyophilized primer, e.g., add 189 μl of nuclease-free water to 18.9 nmol of the lyophilized primer.
3. Vortex thoroughly for 30 s.
4. Prepare a 1 in 10 dilution of 100 μM primer to achieve a 10 μM working stock primer concentration, e.g., add 900 μl of nuclease-free water to 100 μl of 100 μM of primer.
5. Vortex thoroughly for 30 s.
6. Label the primer: name, date preparation, and concentration on the tube.

3.2 PCR Setup

1. Calculate a sufficient volume of PCR reagents needed for testing the required number of samples and controls. Include extra (approximately one extra reaction for every ten reactions) to allow for reagents lost during pipetting. A single PCR requires 0.75 μl of 10 μM of forward primer, 0.75 μl of 10 μM of reverse primer, 9 μl of molecular biology grade water, and 12.5 μl of master mix [*see* **Note 2**].
2. In the PCR clean room, combine all PCR reagents into a suitable plastic container such as a 2 mL microfuge tube, and vortex thoroughly.
3. Move the PCR mix to the PCR assembly room, avoid prolonged exposure of the PCR mix to direct light, and use immediately after preparation.
4. In a HEPA/UV PCR cabinet, pipette 23 μl of the PCR mix into 0.2 mL tubes, 96-well PCR plate, or the PCR tube strips as required.
5. Add 2 μl of sample nucleic acid [*see* **Note 9**] into the 96-well PCR plate or the PCR tube strips, and label accordingly.
6. Add 2 μl of negative control [*see* **Note 10**] and 2 μl of positive control [*see* **Note 11**] into the designated wells or tubes, and label accordingly. Great care should be taken to avoid the creation of aerosols and other potential routes of contamination.
7. Transfer the 96-well PCR plate or the PCR tube strips into the PCR room, and place on the thermal cycler.

8. Save the following ASF-GEN program on the thermal cycler using the appropriate annealing temperature for the specific target (see Table 1). Choose the preheated lid option and set to 100 °C, and set the reaction volume to 25 µl. Heat for 10 min at 95 °C for Taq activation, then 40 cycles of PCR, with each consisting of denaturation (95 °C for 30 sec), annealing (50 °C for 30 sec), and extension (72 °C for 45 sec), followed by final extension for 7 min 72 °C, and hold at 4 °C [*see* **Note 12**].
9. Once the PCR is completed, then the PCR products can be stored at +4 °C for 2 days or at −20 °C for up to 1 month in the post-PCR room.

3.3 Agarose Gel Preparation

1. Prepare a volume of agarose solution appropriate for the size of the gel tank to be used (e.g., 50 mL for a small tank; 100 mL for a medium tank). Weigh out 1 g of electrophoresis grade agarose on a weighing scale, and add 100 mL of 1× TAE buffer in a conical flask.
2. Wearing protective eye goggles and heat-resistant gloves, place the flask in the microwave and heat until the agarose has dissolved completely.
3. Clean an electrophoresis casting tray with distilled water, and secure the ends with a standard laboratory tape or the gel casting gates.
4. When the agarose has dissolved, allow it to cool to hand-hot before adding 10 µl of fluorescent nucleic acid dye per 100 mL agarose solution, and mix thoroughly [*see* **Note 13**].
5. Pour the gel into the tray, and place an appropriate comb into the gel and allow it to set (~40 min).
6. After the gel has set, remove the tape or the casting gates from the tray. Carefully remove the comb to keep the wells intact, and place the gel in the gel tank. Fill the gel tank with 1× TAE buffer solution to fully submerge the gel.

3.4 Agarose Gel Electrophoresis

1. Spin down PCR plate or tubes, and transfer 5 µl of the PCR product into a new tube followed by 1 µl of loading dye and mix well by pipetting.
2. Load 5 µl of molecular weight standard (choose a DNA ladder most appropriate for the expected product size) to the first gel well [*see* **Note 14**].
3. Using a pipette, load 6 µl of the first sample into the second gel well, and continue with the remaining samples. Try to avoid air bubbles as you load the samples, and record the sample order for reference.

4. Place the lid on the electrophoresis tank, and connect the electrodes to the power supply (black-to-black and red-to-red).
5. Turn on the power, and set a constant voltage of 100 V. Make sure that the dye front can be seen to migrate in the correct direction which is negative to positive.
6. Pay attention to the gel as it migrates. Turn off the power when the dye is ~2 cm from the end of the gel (30–40 min). Do not allow the dye to run off the gel.
7. Turn off the power and disconnect the leads from the tank. Remove the gel from the tank, and carry carefully on a tray for visualization.
8. Examine the electrophoresis gel under UV light to visualize PCR products and the DNA ladder. The size of the PCR fragments can be determined by comparison with the DNA ladder.
9. The PCR fragment should be visible as a single band of the correct size target without any unspecific amplification products (Fig. 1). No bands should be present in the negative control well, and the positive control should be represented by the correct size band [*see* **Note 15**].

3.5 PCR Clean-Up and Sanger Sequencing

1. To prepare template for Sanger sequencing, purify the PCR product with your method of choice (e.g., column purification, magnetic gel purification, enzymatic clean-up, or ethanol precipitation), and measure quality and the DNA concentration in each sample using a spectrophotometer (NanoDrop) [*see* **Note 16**].
2. Follow protocols for Sanger sequencing, or submit the prepared templates [*see* **Note 16**] to an external service provider.

3.6 Quality Check of Sequenced Data

1. Open .ab files provided by service provider, and check if electropherogram contains well-resolved and evenly space peaks, each with a single color. Only a little baseline noise should be visible, and peak should be assigned a high-quality value per base.
2. Combine reverse and forward sequences into one consensus sequence, check for any mismatches between them, remove primer sequences, save as a single fasta file for each sample, and label accordingly.

3.7 Acquiring the Reference Sequences

1. Download reference sequences representing all existing ASFV genotypes (Table 2) [*see* **Note 17**], and/or search for homologous sequences of interests (e.g., originating from neighborhood countries and/or emerging in the recent years) either directly from Genbank or a specialized database such as the

Table 2
Representative sequences of ASFV p72 genotypes I through XXIV

Name	Accession#	Genotype
Cameroun 1982 CAM/82_AF301544 Belgium_1985_BEL85_AF449466 Italy_2002_5/Ca/02_FR668402	AF301544 AF449466 FR668402	I
Zambia_1991_LUS/93/1_AY351563 Madagascar_2003_Ambilo03_KC610528 Poland_2014_Pol14/Sz_KJ627217 China_2018_Jilin/2018/boar_MK189456	AY351563 KC610528 KJ627217 MK189456	II
Botswana_1999_BOT/1/99_AF504886	AF504886	III
RSA_1999_RSA/1/99W_AF449477	AF449477	IV
Malawi_2002_MAL/2002/1_AY494553 Mozambique_1964_Moz64_FJ174376	AY494553 FJ174376	V
Mozambique_1994_SPEC/265_AF270710	AF270710	VI
RSA_1998_RSA/1/98_AF302818 Botswana_1987_SPEC/154_DQ250113	AF302818 DQ250113	VII
Mozambique_1998_MOZ/62/98_AY274457 Malawi_1991_BAN/91/1_AY351501 Zambia_1988_CHG/88/1_AY351552	AY274457 AY351501 AY351552	VIII
Uganda_2003_UGA2003/1_AY351564 Uganda_2007_UG07.Wak1_GQ477138 Kenya_2008_Ken08WH/4_HM745285	AY351564 GQ477138 HM745285	IX
Uganda_1995_UGA/95/3_AF449476 Kenya_1961_Gasson_AY351529 Kenya_1959_KilleanIII_AY351531	AF449476 AY351529 AY351531	X
Zambia_1983_KAB/62_AY351522	AY351522	XI
Malawi_1992_MZI/92/1_AY351543	AY351543	XII
Zambia_1983_SUM/14/11_AY351542	AY351542	XIII
Zambia_1986_NYA/1/2_AY351555	AY351555	XIV
Tanzania_2001_TAN/01/1_AY494552	AY494552	XV
Tanzania_2003_TAN/03/1_AY494550	AY494550	XVI
Zimbabwe_1992_ZIM/92/1_DQ250119	DQ250119	XVII
Namibia_1995_NAM/1/95_DQ250122	DQ250122	XVIII
South_Africa_SPEC/125_DQ250112	DQ250112	XIX
RSA_1975_24823_DQ250110	DQ250110	XX
RSA_1983_SPEC/53_DQ250111	DQ250111	XXI
RSA_1979_GR44A2_FJ455835 RSA_1992_SPEC/245_DQ250117	FJ455835 DQ250117	XXII

(continued)

Table 2
(continued)

Name	Accession#	Genotype
Ethiopia_2011_ETH/3_KT795360 Ethiopia_2013_ET13/1504_KU291454	KT795360 KU291454	XXIII
Mozambique_2006_MOZ_10/2006_KY353989	KY353989	XXIV

African Swine Fever Viruses Database (http://asfvdb.popgenetics.net/index).

2. Save all reference sequences into a single fasta file, ensuring that all strands are in the same orientation, and in the file name, include the ASF genome region, e.g., ASFV_p72_B646L.

3.8 Multiple Sequence Alignment

1. Familiarize yourself with MEGA interface and software manual [12, 13].
2. Copy and paste the sample sequences into the fasta file containing all reference strains for the target region, and save accordingly.
3. Before performing multiple sequence alignment, ensure that all downloaded sequences and the subject strand are in the same orientation. If required, use the reverse complement option to change plus strand to minus or vice versa.
4. For DNA coding sequences, multiple sequence alignment should be performed on the amino acid level to avoid placing gaps into incorrect positions that could cause frame shift in the coding sequences. Click the Translated Protein Sequences to see the corresponding protein sequence [*see* **Note 18**].
5. Perform multiple sequence alignment by choosing either ClustalW or MUSCLE method.
6. When the alignment is completed, examine visually, and possibly manually adjust if necessary, e.g., trim excess sequence or eliminate duplicate sequences (Fig. 2).
7. Save the alignment as a .mas file as well as .meg file into the designated folder.

3.9 Building the Phylogenetic Tree

1. Construct the phylogenetic tree using the neighbor-joining (NJ) method by selecting construct/test neighbor-joining tree option from the phylogeny menu [*see* **Note 19**].
2. Select the Maximum Composite Likelihood in model/method option in the analysis preferences window.
3. To estimate the reliability of each node, change test of phylogeny from none to bootstrap method and no. of bootstrap replications set to 1000, and then click calculate.

4. Once the tree is calculated, the bootstrap value or the bootstrap percentage is displayed next to each node.
5. Hide the bootstrap values lower than 70% by changing the cutoff value from 50% to 70% in the tree options settings [*see* **Note 20**].
6. Change the appearance of a tree to suit your needs, e.g., display color taxon markers next to sequences being analyzed or create a subtree to highlight sequences belonging to genotype of interest (Fig. 3).
7. Select save as option from the Image menu of the tree explorer window to save the tree image as pdf, TIFF, EMF, or PNG file.

4 Notes

1. Upon synthesis by manufacturer, primers were purified through normal phase chromatography columns to remove salts and were delivered to the laboratory in a lyophilized form. The primers in Table 1 can be used for both the PCR and sequencing reactions.
2. The AmpliTaq Gold 360 Master mix (Thermo Fisher Scientific) is used in this protocol. If different PCR reagents are preferred, then reagent volumes and primer concentrations should be adjusted according to manufacturer recommendations.
3. TBE buffer (Tris-borate-EDTA) can also be used if preferred.
4. The DNA ladder usually needs to be mixed with the loading dye before use. Use the same loading dye for the preparation of DNA ladder and the dilution of PCR products.
5. Many different kits are available and most use very similar technology; we have successfully used those from Qiagen and Cytiva, but others are just as likely to be effective.
6. We use Big Dye Terminator (Thermo Fisher Scientific) in combination with an ABI-3730 automatic sequencer in house. However, DNA templates may also be sent to commercial providers for Sanger sequencing if the samples can be demonstrated to be free of ASFV.
7. You can use specialized software to check the quality of sequencing data, e.g., Sequencing Analysis Software v5.4 from Applied Biosystems, BioEdit, or Chromas.
8. To download MEGA software, go to https://megasoftware.net/ website, and select version compatible with your operating system (e.g., Microsoft Windows 7, macOS 10.13 (High Sierra), and Linux), and follow installation guidelines. The minimum computer requirement for the version at the

time of writing (MEGA X) is at least 2048 MB of RAM and 500 MB of hard disk space. A 64-bit operating system is strongly recommended.

9. You can use nucleic acid previously extracted for qPCR assays (see Chapter 6) if it has been stored at −20 °C.
10. Use either nuclease-free water or elution buffer as a negative control.
11. The positive control can be the same as that used for the ASF real-time qPCR assay (Chapter 6). However, to avoid cross contamination, the virus load in the positive control should not be much higher than that seen in clinical specimens.
12. PCR conditions were optimized for the AmpliTaq Gold 360 Master mix and T100TM Thermal cycler (Bio-Rad). Annealing temperature for p72 and CVR regions was 50 °C, but for p54 and TRS regions, higher annealing temperature (54 °C) gave better specificity. If different PCR reagents are used, then amplification conditions should be adjusted according to manufacturer recommendations.
13. GelRed nucleic acid gel was used for staining dsDNA in this protocol to avoid handling of ethidium bromide due to its high toxicity.
14. If the correct size band is accompanied by other bands, then the condition of PCR needs to be optimized, e.g., by increasing the annealing temperature of PCR cycle.
15. The quantity of template (size fragment 200 bp–1000 bp) used in a cycle sequencing reaction should be between 3 ng and 20 ng. The purity of DNA can be determined by the ratio of absorbance at 260 and 280 nm and should be ~1.8.
16. Poor template quality or insufficient template concentration is the most common cause of sequencing problems.
17. Partial sequences of the p72 gene from ASF strains representing the 24 ASF genotypes shown in Table 2 can be downloaded from Genbank by typing the accession numbers in the search menu (https://www.ncbi.nlm.nih.gov/genbank).
18. The final character at the C-termini of the protein (stop codon) is always an asterisk (*), and it corresponds to the chain termination codon. No asterisks should appear in the middle of coding sequences. If asterisks occur in the alignment, check if sequences are in the correct reading frame.
19. Determine the suitability of the data for a NJ tree by calculating the overall mean Jukes-Cantor distance. If distance is >1.0, then data are not suitable for making NJ trees, and another method should be considered.
20. Only nodes with >70% are considered reliable.

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Chapter 9

African Swine Fever Virus (ASFV) Indirect ELISA Test Based on the Use of the Soluble Cytoplasmic Semi- purified Antigen (ASFV CP-Ag)

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Abstract

The present chapter describes a simple and economic indirect enzyme immunoassay (ELISA) for African swine fever virus (ASFV) antibody detection based on the use of the soluble cytoplasmic fraction of ASFV-infected monkey stable cells (MS). The soluble antigen proteins of ASFV-infected cells are separated by sucrose precipitation centrifugation, and the supernatant above the sucrose layer is used as an ELISA antigen. The test serum sample reacts with the cytoplasmic soluble fraction, and antibodies are detected using a protein A-peroxidase conjugate. This crude antigen is currently recommended as a test reagent in screening and diagnostic tests by the World Organization for Animal Health (OIE).

Key words African swine fever virus (ASFV), ASFV antibodies, ELISA, Soluble cytoplasmic antigen

1 Introduction

Serological assays are the most widely used diagnostic tests due to their simplicity, relatively low cost, and the fact that they require little specialized equipment or facilities. This is particularly relevant in the diagnosis of African swine fever (ASFV), since anti-ASFV antibodies appear shortly after infection (from day 7) and persist for several months [1]. In endemic areas, where low virulence and attenuated virus isolates circulate alongside virulent isolates, serological detection is crucial to identify survivors and asymptomatic recovered infected animals. It should be noted that when pigs have been infected with avirulent or low virulence strains, serological tests may be the only way to detect infected animals due to the absence of specific clinical signs and, in some animals, the absence of viremia [2, 3]. The use of antibody detection assays was crucial for successful eradication programs in the past in the Iberian Peninsula [4, 5].

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ELISA (also known as enzyme immunoassay) is the most commonly used method for large-scale ASF serological tests to detect antibodies in serum or plasma. There are several ELISA formats for the detection of antibodies against ASFV. They are based on a competitive or indirect format, which are validated for their use in different epidemiological situations [1, 6]. The commercial ELISA tests might be costly and not easily available for all geographic locations. In the last years, several new tests have been developed, and it is important to get information about its full validation for the specific purpose.

The method described here, originally described by Sanchez-Vizcaíno et al., in 1979 [7], is an indirect ELISA based on the use of the cytoplasmic soluble antigen (CP-Ag) obtained from MS cells (monkey kidney cell line), grown in the presence of pig serum infected with an ASFV isolate passaged 48 times on MS. The use of pig serum in cell cultures instead of bovine serum evades antigen contamination with albumin from the latter, which is a key factor responsible for false-positive reactions in ELISA [8]. The soluble protein fraction from infected cells is prepared by cell disruption, elimination of nuclei, and sedimentation of cellular debris by a 20% (w/w) sucrose cushion (Fig. 1). The supernatant above the sucrose layer is used as the ELISA antigen. The antigen is adsorbed onto a well of an ELISA plate, and then serum or plasma is added. If antibodies are present, they will bind to the antigen. Second, an enzyme-conjugated secondary antibody is applied that is directed against the primary antibody present in the clinical specimen. After washing to remove any unbound antibody, a colorless substrate (chromogen) is added. The presence of the enzyme converts the substrate into a colored end product (Fig. 1).

The method used to obtain the soluble antigen is based on a NaCl-sucrose purification procedure that allows the integrity and high purity of the intracellular virus obtained. The CP-Ag contains the immediate early, early, and the late proteins generated during ASFV infection and thus the most antigenic ASFV-induced proteins [9, 10]. Although correlations between ASFV proteins recognized by a serum and the stage of infection are difficult to assess, in general terms, sera from infected pigs react with most of the early and late antibody-inducing proteins but at different phases. In time-course experiments to detect appearance of ASFV antibodies, antibodies can be detected as earliest on day 7 post-infection [1, 2, 4]. At that time, only the early polypeptides IP25, IP25.5, IP30, and IP31 are recognized, whereas IP73 (IP72) and IP12 are usually detected 3 and 4 days later, respectively. Proteins such as p23.5 and p15 induced antibodies up to 12 days after infection, whereas the p34 corresponds to animals in late stages of infection [11, 12]. This characteristic makes the use of CP-Ag advantageous for the early serological detection of ASFV-infected pigs, as well as in the detection of asymptomatic surviving animals, compared to use-based ELISA tests of individual proteins [6, 12, 13].

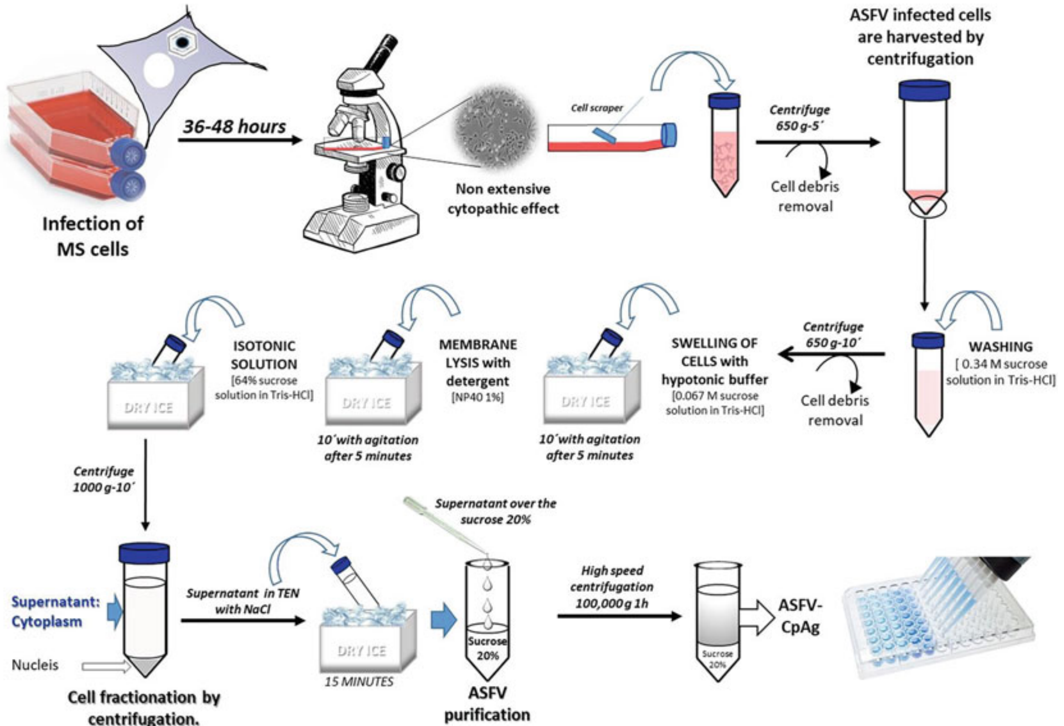

Fig. 1 Scientific diagram | Purification of ASFV obtained from the cytoplasm of monkey stable cells infected with adapted ASFV by sucrose cushion centrifugation

The CP-Ag indirect ELISA method described in this chapter is included in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual) as prescribed serological test for antibody screening [2]. It is fast, easy to perform, and economical. The main disadvantage is that preparation of the CP-Ag requires BSL-3 biocontainment facilities capable of handling live ASFV. Furthermore, despite having higher specificity than other competition format-based ELISA tests, positive ELISA results should always be confirmed by alternative methods such as the indirect immunoperoxidase test (IPT) or the indirect immunofluorescence test (IFAT), as recommended by the OIE [1, 2, 14].

2 Materials

Use deionized, distilled water in all recipes and protocol steps. Diligently follow all waste disposal regulations when disposing waste materials. The ASFVs should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with the Chapter 1.1.4 Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities included in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual) [15].

2.1 ASFV Cytoplasmic Soluble Antigen Isolation

The necessary equipment and material is that required for all cell culture laboratories that have the common requirement of being free from pathogenic microorganisms (i.e., asepsis) and share some of the same basic equipment that is essential for culturing cells. All reagents that are used with cells must be sterile. Always use proper sterile technique, and work in a laminar flow hood (class II for ASFV).

1. Class II biosafety cabinet.
2. CO_2 cell incubator.
3. Water bath.
4. Centrifuge.
5. Refrigerator (4 °C).
6. Freezer (−20 °C).
7. Inverted microscope.
8. Liquid nitrogen (N_2) freezer or cryostorage container.
9. Sterilizer (i.e., autoclave).
10. Aspiration pump (peristaltic or vacuum).
11. Analytical balance.
12. pH meter.
13. Plate-shaker (with and without incubator).
14. Low-speed centrifuge rotor with a capacity ≥1 L and maximum speed of 15,000 rpm/34,155 × g.
15. High-speed centrifuge rotor with a total capacity ≥1 L and maximum speed of 100,000 rpm/803,000 × g.
16. 0.2 M ethylene-diamine-tetra-acetic acid (EDTA) solution: Weigh 0.90 g of EDTA in 10 mL of distilled water. Store at 4 ± 3 °C.
17. Filtered porcine sera (*see* **Note 1**): Porcine sera is filtered through a 0.45 micrometer filter, and store in aliquots at <−10 °C.
18. Monkey stable cells (MS) [ATCC/ECACC 91070510]. Keep in liquid nitrogen <−180 °C.
19. Monkey stable cells (MS) culture medium: Eagle's Minimum Essential Medium (EMEM) supplemented with 10% of sterile-filtered porcine sera, 1% non-essential amino acid solution 100×, 1% glutamine [4 mM], 50 µg/mL of gentamicin sulfate [50 mg/mL]. Store at 4 ± 3 °C.
20. Tissue culture flasks and plates.
21. 10% Nonidet P-40 (NP40) solution in distilled water: Mix 20 mL of NP40 with 180 mL of distilled water. Store at room temperature.

22. Phosphate buffered saline (PBS 1×) pH 7.2 $_{(\pm 0.2\ UpH)}$: Add about 100 mL water to a 1 L graduated cylinder or a glass beaker. Weigh 8.0 g of sodium chloride (ClNa), 0.2 g of potassium chloride (ClK), 0.2 g of potassium di-hydrogen phosphate (PO_4H_2K), and 1.15 g of di-sodium hydrogen phosphate (PO_4HNa_2), and transfer to the cylinder. Add water to a volume of 900 mL. Mix and adjust pH with HCl (*see* **Note 2**). Store at room temperature.
23. 5 M sodium chloride (NaCl) solution: Dissolve 7.30 g of NaCl in 25 mL of distilled water, and gently warm the solution to completely dissolve it. Store at room temperature.
24. 0.34 M sucrose (*see* **Note 3**) in Tris-HCl 5 mM pH 8 $_{(\pm 0.2UpH)}$: Weight 34.9 g of sucrose for biochemistry in 1.5 mL of Tris-HCl 1 M, and complete until 300 mL of distilled water. Store at <−10 °C.
25. 0.067 M sucrose in Tris-HCl 5 mM pH 8 $_{(\pm 0.2UpH)}$: Weigh 6.88 g of sucrose for biochemistry in 1.5 mL of Tris-HCl 1 M until 300 mL of distilled water. Store at <−10 °C.
26. Sucrose 64% (w/v) solution in Tris-HCl 0.4 M pH 8 $_{(\pm 0.2UpH)}$: Add 128 g of sucrose for biochemistry in 80 mL of Tris-HCl 1 M, and complete with distilled water until 200 mL. Store at <−10 °C.
27. Sucrose 20% (w/w) solution in Tris-HCl 50 mM pH 8 $_{(\pm 0.2UpH)}$: Add 60 g of sucrose density gradient in 20 mL of Tris-HCl 1 M, and complete with distilled water until the total mass equals 300 g (w/w). Store at <−10 °C.
28. TEN solution: Mix 175 μl of Tris-HCl 1 M with 700 μl of EDTA 0.2 M and 250 μl β-mercaptoethanol (*see* **Note 4**), and complete with 2.37 mL of distilled water. Prepare immediately before use.
29. 1 M tris (hydroxymethyl)aminomethane)-HCl pH 8 $_{(\pm 0.2UpH)}$: Weigh 60.57 g of Tris in 300 mL of distilled water. Adjust pH to 8$_{(\pm 0.2UpH)}$ with HCl, and complete the volume up to 500 mL with distilled water. Store at room temperature.
30. Trypsin-EDTA 1× solution containing 170,000 U/L trypsin 1:250 and 0.2 g/L EDTA manufactured with irradiated porcine trypsin, tested for porcine parvovirus and mycoplasma. Store at 4 ± 3 °C.
31. Thimerosal (*see* **Note 5**).

2.2 Indirect ELISA for ASFV Cytoplasmic Antigen

1. Plate-shaker incubators.
2. Single- and multi-channel pipettes.
3. Spectrophotometer UV/VIS with filter 620 nm.

4. pH meter.
5. Ventilated fume hood.
6. ASFV-CpAg coating solution (carbonate/bicarbonate buffer 0.05 M pH 9.6): Dissolve 2.93 g of sodium bicarbonate ($NaHCO_3$) pH 8.3 in 500 mL of distilled water (sodium bicarbonate buffer). Dissolve 1.59gr of sodium carbonate (Na_2CO_3) in 500 mL of distilled water (sodium carbonate solution). Store at room temperature. Immediately before using mix the carbonate/bicarbonate sodium buffers until to achieve a pH of 9.6 $_{(\pm 0.2\ \mathrm{UpH})}$ (*see* **Note 6**).
7. DMAB solution (3-dimethylaminobenzoic acid): Dissolve 13.3 g of DMAB in 900 mL of phosphate buffer 0.1 M pH 7 $_{(\pm 0.2\ \mathrm{UpH})}$. Mix during 1 h at room temperature, and adjust the pH to 7 $_{(\pm 0.2\ \mathrm{UpH})}$ with NaOH 5 M. Adjust the final volume to 1 L with phosphate buffer 0.1 M pH 7 $_{(\pm 0.2\ \mathrm{UpH})}$. Filter with paper filter and prepare aliquots of 10 mL, 5 mL, and 3 mL. Store at <-10 °C in darkness.
8. HRP conjugate: Protein A peroxidase 1 mg/mL. Prepare and store following manufacture instructions.
9. Hydrogen peroxidase 30% (H_2O_2). Store at 4 ± 3 °C.
10. MBTH solution: Dissolve 0.36gr of 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) in 900 mL of phosphate buffer 0.1 M pH 7 $_{(\pm 0.2\ \mathrm{UpH})}$. Mix during 1 h at room temperature, and adjust the pH to 6.25 $_{(\pm 0.2\ \mathrm{UpH})}$ with concentrate HCl. Adjust the final volume to 1 L with phosphate buffer 0.1 M pH 7 $_{(\pm 0.2\ \mathrm{UpH})}$. Filter with paper filter and prepare aliquots of 10 mL, 5 mL, and 3 mL. Store at <-10 °C in darkness.
11. 0.1 M phosphate solution pH 7 $_{(\pm 0.2\ \mathrm{UpH})}$: Weigh 5.3 g of potassium phosphate (PO_4H_2K) and 8.65 g of sodium phosphate (PO_4HNa_2) in 1 L of distilled water. Store at room temperature.
12. Stop solution (sulfuric acid 3 N): Mix 16.1 mL of sulfuric acid 95–97% in 183.9 mL of distilled water (*see* **Note 7**). Store at room temperature.
13. Reference standard swine sera including hyperimmune positive control serum (ASF-PC), and limit control sera (ASF-LC) and negative control serum (ASF-NC) (*see* **Note 8**)
14. Washing solution: PBS 1× (see receipt point 2.1) with 0.05% Tween™ 20 (PBS-Tw): Dissolve 0.5 mL of Tween-20 in 1 L of PBS 1× pH 7.2 $_{(\pm 0.2\ \mathrm{UpH})}$. Store at room temperature. Check the pH before using.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Procedure to Obtain the Semi-purified ASFV Cytoplasmic Soluble Antigen (ASFV-CpAg) in African Green Monkey Cell Lines

The monkey stable (MS) cell line used here from African green monkey species is cultured according to standard protocols in a 37 °C, 5% CO_2 humidified incubator. The established cell line is grown in plastic tissue culture flasks and subcultured by trypsinization according to standardized protocols. All of these protocols can be applied, with minor modifications, to other ASFVs and cell models.

3.1.1 Growing and Infection of MS Cells with the Spanish MS-Adapted E70 ASFV Isolate

1. Bring the trypsin-EDTA solution balanced and complete growth medium to the appropriate temperature of 37 °C for the MS cell line (*see* **Note 9**).
2. From 90% confluent cells grown in T150 flasks [growth area 150 cm^2/~1.5×10^7 cells], remove and discard the cell culture medium.
3. Wash the cells with ~13–15 mL of trypsin-EDTA solution (approximately 1 mL per 10 cm^2 culture surface area), spread the liquid onto the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer, and rock the vessel back and forth several times. The wash step removes any traces of serum, calcium, and magnesium that would inhibit the action of the trypsin-EDTA solution (dissociation reagent).
4. Remove and discard the wash solution.
5. Add ~7–8 mL of trypsin-EDTA to cover the entire surface (approximately 0.5 mL per 10 cm^2 culture surface area), and incubate 2–3 min at 37 °C in 5% CO_2 humidified incubator until the cells appear to be detached (they will appear rounded and retractile under the microscope). Check the progress of cell dissociation by microscopy. To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for them to detach.
6. Once the cells appear to be detached, add the corresponding volume of pre-warmed complete growth medium supplemented with 10% of FSBi to the cell suspension to inactivate the trypsin.
7. Disaggregate the cells by pipetting up and down to disaggregate cell clumps. Check the cells with the microscope to be sure that most (>95%) are single cells. If cell clusters are apparent, continue to disperse the cells with gentle pipetting.

8. Using this cell suspension, pipette required volume of cells into the required number of T150 flasks to be prepared (50 mL per flask) (*see* **Note 10**).
9. Tighten caps and incubate 48 h at 37 ± 3 °C in 5% CO_2 humidified incubator to get 80% confluent flasks [growth area/well 150 cm^2, average cell yield ~1.2×10^7].
10. After the incubation time, carefully decant the medium of the cell cultures grown in the T150 flasks.
11. In a separate glass sterile bottle, prepare the appropriate dilution (in culture medium without serum) of the MS cell-adapted ASFV (*see* **Note 11**) to infect at a multiplicity of infection (MOI) of 10. For figuring out the amount of virus you need to add for a certain MOI, use the formula: number of cells x desired MOI = total PFU (or Plaque Forming Units) needed (*see* **Note 12**). Then use the formula: (total PFU needed) / (PFU/mL) = total mL of virus needed to reach the desired MOI.
12. Tighten caps and incubate for 2 h at 37 ± 2 °C shaking gently by rocking (adsorption of the virus).
13. After 2 h complete the corresponding volume of the 150 cm^2 flasks (recommended volume of 50 mL) with medium containing 2% porcine serum.
14. Tighten caps and incubate for 36–48 h at 37 ± 3 °C in 5% CO_2 humidified incubator.
15. After 36–48 h post-infection, detach the infected cells with a cell scraper or, if possible, by shaking. The cells are harvested before extensive cytopathic effect (CPE) is observed to recover the intracellular virus (*see* **Note 13**).

3.1.2 Generation of Semi-purified ASFV Cytoplasmic Soluble Antigen

1. Centrifuge the harvested cells at 650 g for 5 min.
2. Wash the cell pellet with 20 mL of 0.34 M sucrose solution prepared in the Tris-HCl buffer, and centrifuge at 650 g for 10 min to pellet cells.
3. Resuspend the cell pellet in 0.067 M sucrose solution prepared in the Tris-HCl buffer (1.8 mL per 150 cm^2 flask), and leave for 10 min on dry ice with agitation after 5 min allowing cells to swell.
4. Add non-ionic detergent Nonidet P-40 to a final concentration of 1% (w/v), and leave for 10 min on dry ice (with agitation after 5 min) to lyse the cells.
5. Add 1/7 of the total volume of the 64% sucrose solution prepared in Tris-HCl buffer (*see* **Note 14**), and centrifuge at $1000 \times g$ for 10 min to pellet nuclei.

6. Collect the supernatant and add 1/19 of the total volume of TEN buffer and 1/10 of the total volume of NaCl 5 M to achieve a final concentration of 0.5 M. Incubate 15 min on ice (*see* **Note 15**).
7. Centrifuge at 100,000 × *g* for 1 h at 4 ± 1 °C over a layer of 20% (w/w) sucrose solution.
8. Remove the band immediately above the sucrose layer that will contain the soluble cytoplasmic antigen *(ASFV-CpAg)*, and add thimerosal for a final concentration of 0.01% (*see* **Note 16**).

3.2 Indirect ELISA Test Procedure for ASFV Antibody Detection

1. Dilute the soluble antigen in carbonate/bicarbonate buffer pH 9.6 (±0.2 UpH) at the recommended working dilution. Add 100 μl per well of a polypropylene-polysorp microtiter plates (*see* **Note 17**). Incubate at 4 ± 3 °C for 16–18 h (overnight).
2. Wash the plates four times with washing buffer. Plates are flicked free of antigen and then blotted onto paper towels. The sensitized and dry plates can be used directly or stored at <−10 °C for 18 months.
3. Dilute the test sera and the reference sera (positive, limit, and negative) 1/30 in washing solution, and add 100 μl/per well. Include blank control well (100 μl of washing solution). Test the reference sera by duplicate.
4. Incubate 1 h at 37 ± 2 °C on a plate incubator shaker.
5. Wash as step 2.
6. Addition of HRP conjugate: To each well add 100 μl of protein-A/horseradish-peroxidase conjugate at the recommended or pretitrated dilution.
7. Incubate 1 h at 37 ± 2 °C on a plate incubator shaker.
8. Wash as step 2.
9. Add 200 μl of substrate DMAB/MBTH (*see* **Note 18**) to each well prepared as follows: The volume required per plate is 10 mL of DMAB solution +10 mL of MBTH solution +5 μl H_2O_2 30%. Incubate at room temperature for approximately 6–10 min (before the negative control begins to be colored) (*see* **Note 19**).
10. Stop the reaction by adding 100 μl of stopping solution to each well.
11. The results can be obtained using a spectrophotometer UV/VIS to read microtiter plates at 620 nm wavelengths.

3.3 Test Validation

The test is validated when the optical density (OD) of the PC is, at least, four times greater than the OD of the NC if the following criterions are accomplished:

- MEAN $OD_{PC} \geq 1.0$ (the tolerance interval for the OD values of duplicate PC must be ± 0.3)
- MEAN $OD_{NC} \leq 0.250$ (the tolerance interval for the OD values of duplicate NC must be ± 0.1)
- MEAN $OD_{LC} \geq CO - 0.1$
- MEAN $OD_{BLANK} \leq 0.1$

3.4 Interpretation of the Results

To achieve a correct interpretation of the results, it is necessary to calculate the CUT OFF (CO) which will define negative, ambiguous, and positive sera. The CO is calculated by a simple equation: CO = MEAN OD_{NC} + [MEAN $OD_{PC} \times 0.2$].

- Sera with OD *lower* than the *CUT OFF − 0.1* are considered as *negative sera*.
- Sera with OD *greater* than the *CUT OFF + 0.1* are considered as *positive sera*.
- Sera with OD *within the range* $CO - 0.1 \leq OD\ sample \leq CO + 0.1$ are considered as *ambiguous sera*, and they have to be confirmed by alternative ASF confirmatory antibody detection technique such as the indirect immunoperoxidase test (IPT) (Chapter 10).

4 Notes

1. Sera from domestic pigs produce false-positive reactions in the serodiagnostic techniques when bovine serum albumin is present in the soluble cytoplasmic antigen. It occurs when the infected cells are cultured in the presence of bovine fetal serum. Instead, it is important the soluble cytoplasmic antigen be obtained from cell cultures infected with ASFV in the presence of porcine serum. The porcine serum will avoid the false-positive reactions.
2. Concentrated HCl (12 N) can be used at first to narrow the gap from the starting pH to the required pH. From then on it would be better to use a series of HCl (e.g., 6 and 1 N) with lower ionic strengths to avoid a sudden drop in pH below the required pH.
3. The sucrose solutions must be used within 1 month after the preparation.
4. 2-Mercaptoethanol is extremely toxic if swallowed or inhaled and can be fatal if absorbed through the skin. It is corrosive and can cause severe burns to the skin and eyes. It is also a combustible liquid. This compound is extremely toxic to aquatic life and has long-lasting effects in the environment. Additionally, it

has an extremely unpleasant odor that can be detected at very low concentrations (stench chemical). Handling, storage, and waste disposal must achieve all of the biosafety requirements.

5. Thimerosal is a mercury-containing organic compound (an organomercurial) that has been used since the 1930s as a preservative in a number of biological and drug products, including many vaccines, to help prevent potentially life-threatening contamination with harmful microbes. It is approximately 50% mercury by weight, and it is metabolized or degraded to ethylmercury and thiosalicylate. At concentration used in this protocol, thimerosal meets the requirements for a preservative as set forth by the Internationals Standards that is of 0.001% (1 part in 100,000) to 0.01% (1 part in 10,000). Handling, storage, and waste disposal must achieve all of the biosafety requirements.
6. To achieve a pH of 9.6, adjust with carbonate (to decrease the pH) or bicarbonate (to increase the pH), but do not add HCl nor NaCl.
7. Concentrated sulfuric acid cannot be handled out of the ventilated fume hood and has to be used on a cleared space away from any strong base, reducing agent, and metals. Due to the exothermic nature of the reaction, dilution of sulfuric acid has to be done by slowly adding the acid to water to limit the risk of splashing concentrated acid out. Concentrated sulfuric acid is also a strong oxidizing agent incompatible with flammable organic chemicals. Diluted solutions of sulfuric acid can be handled out of the ventilated fume hood. Sulfuric acid has to be disposed as a hazardous waste in the appropriate acidic waste container and kept closed at all times.
8. The reference standard sera can be obtained at the European Union (EU) Reference laboratory for ASF (https://asf-referencelab.info/asf/en/).
9. After recovery from frozen stock, MS cells usually take two to three passages to reach their regular growth rate, and this should be taken into account if planning to use the cells for infections. It is important to note that cell cultures have the potential to change with time due to selective pressures in the culture environment and, particularly with continuous passages, due to genetic instability. Cells may continue to proliferate, but as the passage number increases, changes such as loss of differentiated properties or changed susceptibility to the viral infection over time can occur. The maximum passage level recommended is 150.
10. The minimum number of T150 flasks recommended is 15 to render 5–9 μg/mL of the antigen considered as optimal for the indirect ELISA test procedure for ASFV antibody detection.

11. This procedure describes the cell infection using the ASFV E70 adapted to MS cells, but any ASFV adapted to these cell lines can be used with the appropriated standardization.
12. Tissue culture infection doses which will infect 50% of the cell monolayers challenged with the defined inoculum (TCID50) are related to PFU by the following formula PFU/mL = 0.7 * TCID50.
13. Store 1 T150 flask with cells infected with the virus for virus titration to serve as inoculum. The cells are infected with sonicated virus. After 36–48 h, the infected cells from one of the T150 flasks are harvested and lysed during repeated freeze-thaw cycles. The virus stock is then sonicated, titrated in MS cells, aliquoted, and stored at −80 °C.
14. The sucrose solution neutralizes the detergent action preserving the cell membrane.
15. This step disaggregates the mixed virus and capsid clusters throughout the action of the TEN buffer. The tonicity is recovered by the action of sodium chloride.
16. The ASFV-Ag batches should comply with appropriated quality control tests and tested against the reference positive, limit, and negative standard sera following the international EU reference laboratory guidelines.
17. One of the major components of the ELISA is the surface to which antigens and antibodies are immobilized. The surface is as crucial as the antigen-antibody complex with which it interacts. The attachment phenomenon is controlled by the chemical properties of the surface, but can be influenced by (i) the physical properties of the containment vessel (96-well microplate) and (ii) other factors such as pH and temperature. In this ELISA test, hydrophobic PolySorp polystyrene plates are best suited for ASFV-Ag binding using the recommended coating buffer. Plates made with hydrophilic surfaces like MaxiSorp induce lower levels of ASFV-Ag adsorption.
18. This indirect ELISA procedure could be improved to have higher sensitivity to detect antibodies in serum samples collected at earlier stages of infection, by using ABTS [2,2′-azino-di(3-ethyl-benzothiazoline)-6-sulfonic acid]-di-ammonium salt) as substrate. In this case the incubation time, incubation temperatures, buffers, concentrations of the antigen and samples, and the type and concentration of the conjugate should be modified and used as is described in the OIE [2]. It should be noted that although sensitivity is improved, the specificity is lower when compared to the procedure described in this chapter. This test is useful in case of regions where ASF is present.
19. The time necessary for the color to develop will depend on both the temperature of the substrate when added to the wells and the room temperature.

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Chapter 10

Indirect Immunoperoxidase Test (IPT) for Detection of Antibodies Against African Swine Fever Virus (ASFV) on African Green Monkey Cell Lines (Vero, MS)

Gallardo Carmina, R. Nieto, and M. Arias

Abstract

The immunoperoxidase technique (IPT) is an immunocytochemistry technique on fixed infected cells to determine the antibody-antigen complex formation through the action of the peroxidase enzyme. In this procedure, African green monkey established cell lines, such as Vero or MS, are infected with African swine fever virus (ASFV)-adapted isolates to these cell cultures. The infected cells are fixed and then used as antigens to determine the presence of the specific antibodies against ASFV in samples of swine origin. IPT has many advantages for the serological diagnosis of ASF. Firstly, IPT has a higher sensitivity than ELISA or immunoblot assay (IB) for the detection of antibodies against ASFV. Secondly, it has greater versatility to analyze any type of clinical sample of porcine origin and to conduct research studies on the epidemiological situation, through the presence of antibody titers in exudates from organs and tissues. Thirdly, the preparation of IPT plates with cells infected with ASFV is simple, although laborious, that can be stored for a long time and allow the analysis of a large number of samples.

Key words African swine fever virus (ASFV), ASFV antibodies, Immunochemistry peroxidase staining

1 Introduction

Immunocytochemistry (ICC) is a standard virological technique to identify the presence of antibodies by their specific ability to react with viral antigens expressed in infected cells. The antibody is directly or indirectly linked to a reporter such as a fluorophore or enzyme that gives rise to a fluorescence or to a color from an enzymatic reaction, which can be detected using fluorescence or light microscopy, respectively. In ICC, the staining technique is applied on cultured cells. This is in contrast to immunohistochemistry (IHC), where cells are analyzed within intact tissue sections. As with the enzyme-linked immunosorbent assay (ELISA), ICC methods may be direct, in which a labeled primary antibody binds

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an antigen, or indirect, in which secondary antibody binds antibodies present in a sample that react to a prepared antigen fixed on microplate wells [1]. These methods rely on antigen–antibody interactions, and they can be adapted to allow direct detection of the virus (antigen detection) or to identify the host's immune response to the virus infection (antibody detection).

The indirect immunofluorescence antibody technique (IFAT) was widely and successfully employed in the past for African swine fever (ASF) antibody detection in affected countries in combination with the direct method (FAT) for the antigen detection. Both together allowed the diagnostic of 85–95% of all cases of ASF (acute, subacute, and chronic) during the Spanish eradication program that was carried out from 1985 up to 1995 [2–4]. But although these assays played a key role in ASF surveillance and eradication programs in affected countries [5–8], they were laborious and not readily adaptable to large-scale surveys. Currently, IFAT is only used as a confirmatory test in the serological diagnosis of ASFV in laboratories with extensive experience with the test. Disadvantages of IFAT include the need for fluorescent microscopes and the difficulty in interpreting some clinical samples that have a high level of nonspecific fluorescence.

An alternative ICC method based on an enzyme reaction is the indirect immunoperoxidase test (IPT), also called the indirect immunoperoxidase monolayer assay (IPMA). The IPT as a diagnostic serological test for ASFV antibody detection was initially described by Pan et al., in 1982 [8] with percentages of sensitivity and specificity comparable to those of the IFAT. In 2013 Gallardo et al. [9] described the use of the IPT as a valuable confirmatory serological test after a comparative study carried out to evaluate the performance of the World Organization for Animal Heath (OIE)-prescribed antibody detection tests. Within this study, new antigen-specific ELISAs and new IPTs were developed based on the use of COS-1 cells infected with ASFVs of different genotypes. The assays developed were compared with those recommended by the OIE by analyzing more than 1000 field and experimental sera from Africa and Europe. The results showed strong agreement with OIE tests and confirmed the suitability of the IPT assay as a valuable diagnostic tool to be used as a confirmatory test in the serological diagnosis of ASFV. In 2015, an evaluation study of ASF diagnostic techniques, which included the analysis of more than 700 sera collected from the European Union (EU), showed the IPT, together with PCR, as the most reliable methods to detect the disease during the outbreaks of ASF of the European Union since 2014 [10].

Currently, the ELISA test remains as the most useful method for large-scale serological studies; nevertheless, it is usually necessary to strike a balance between sensitivity and specificity that will enable the user to detect specific antibodies yet yield a minimum of

false-positive readings. At best, ELISA tests are suitable for screening, and a more critical test must be used to confirm the positive and doubtful reactions. The IPT method is included in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual) as a confirmatory test for positive sera from areas that are free from ASF and are positive in the ELISA and for sera from endemic areas that give an inconclusive result in the ELISA. Given its superior sensitivity and its performance, this is defined also as the test of choice to analyze blood, fluids, or tissue's exudate samples [10–13]. The IPT in coordination with PCR results in blood and tissue samples allows to carry out an accurate epidemiological investigation in whatever the scenario. By means of IPT titration of the antibody presence in exudate tissue samples, it is possible to perform a precise assessment of the infection status of the animal analyzed.

A major shortcoming of the IPT confirmatory serological tests is that fixed microplates with infected cultures are not commercially available yet, and laboratories with the appropriated biosafety facilities are required.

This chapter describes the OIE-IPT "in house" method for the detection of ASFV antibodies [13]. The technique is performed in two steps. First, African green monkey kidney cells, Vero or MS, are seeded on a solid support, which is usually a flat bottom multiwell plate. Since adherent cells are used, the cells will attach to the solid support surface during the incubation, which varies from half an hour to 24 h for the different cell types. Then cells are infected with the ASFV isolates adapted to these established cell lines and fixed [12–14]. In the second step, the samples to be analyzed are incubated with the fixed infected cells, and if the sample is positive, specific antibodies in the sample will attach to the antigens coupled to the solid phase. Then, the enzyme horseradish peroxidase (HRP), which is specific to Fc fragment of the IgG molecule of the primary antibody, is applied, and after the addition of a substrate, the enzyme catalyzes a reaction that generates a red-brown precipitate at the site where the enzyme-coupled antibody is bound in the cells (Fig. 1).

In conclusion, the IPT is versatile, as it can test any kind of porcine material, including exudates from any organ or tissue including bone marrow, very useful in case of wild suids. It is highly sensitive and specific and well suited for confirmatory testing or for small to medium sample sets especially early after infection to perform epidemiological investigation. As to the efficiency of the IPT, a trained technician is able to process up to 400 samples day in case it could be required. The completed test plates may be stored as permanent records.

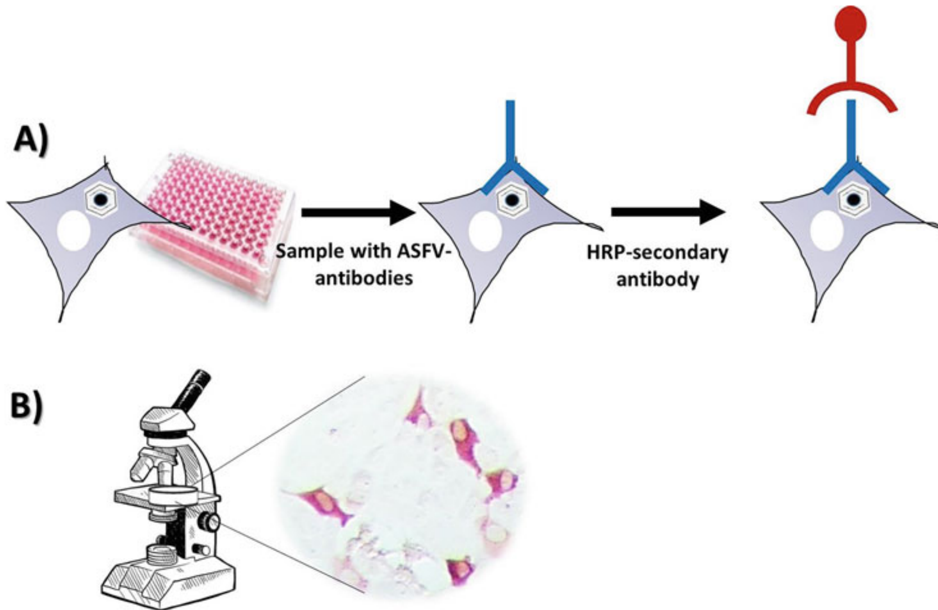

Fig. 1 Indirect immunoperoxidase test (IPT). (**a**) A diagram illustrating the procedure involved in IPT. After cells grown on multiwell plate are fixed and infected with ASFV-adapted isolates, the sample with antibodies against ASFV is added, and the secondary antibody conjugated to peroxidase protein (HRP) is sequentially added to bind to the primary antibody. Its presence must be made visible using the 3-amino-9-ethylcarbazole substrate that, when oxidized by HRP using hydrogen peroxide as the oxidizing agent, yields a characteristic red-brown color change that is detectable in the microscope. (**b**) Microscope image obtained in Vero cells infected with ASFV showing the intensive red color in the cytoplasm

2 Materials

Use deionized, distilled water in all recipes and protocol steps. Diligently follow all waste disposal regulations when disposing waste materials. The ASFVs should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4 Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities included in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual) [15].

2.1 Preparation of ASFV-Infected Cell Culture Plates

The necessary equipment and material are that required for all cell culture laboratories that have the common requirement of being free from pathogenic microorganisms (i.e., asepsis) and share some of the same basic equipment that is essential for culturing cells. All reagents that are used with cells must be sterile. Always use proper sterile technique, and work in a laminar flow hood (class II for ASFV).

1. Class II biosafety cabinet.
2. CO_2 cell incubator.
3. Water bath.

4. Centrifuge.
5. Refrigerator (4 °C).
6. Freezer (−20 °C).
7. Inverted microscope.
8. Liquid nitrogen (N_2) freezer or cryostorage container.
9. Sterilizer (i.e., autoclave).
10. Aspiration pump (peristaltic or vacuum).
11. Analytical balance.
12. pH meter.
13. Plate -shaker (with and without incubator).
14. Heat-inactivated filtered fetal bovine serum (FBSi) (*see* **Note 1**): FBS is heated to 56 ± 2 °C for 30 ± 2 min in agitating water bath to inactivate complement. Then it is filtered through a 0.20 μm filter and stored in aliquots at < −10 °C.
15. Methanol/acetone fixative solution: Prepare a mix solution containing 30% of acetone and 70% of methanol. Store at < −10 °C.
16. Monkey stable cells (MS) [ATCC/ECACC 91070510].
17. Monkey stable cell (MS) culture medium: Eagle's Minimum Essential Medium (EMEM) supplemented with 10% of FBSi, 1% non-essential amino acid solution 100×, 1% glutamine [4 mM], and 50 μg/mL of gentamicin sulfate [50 mg/mL]. Store at 4 ± 3 °C.
18. Tissue culture flasks and flat-bottomed 96-well plates.
19. Phosphate-buffered saline (PBS 1×) pH 7.2 (±0.2 UpH): Add about 100 mL water to a 1 L graduated cylinder or a glass beaker. Weigh 8.0 g of sodium chloride (ClNa), 0.2 g of potassium chloride (ClK), 0.2 g of potassium di-hydrogen phosphate (PO_4H_2K), and 1.15 g of di-sodium hydrogen phosphate (PO_4HNa_2), and transfer to the cylinder. Add water to a volume of 900 mL. Mix and adjust pH with HCl (*see* **Note 2**). Store at room temperature.
20. Trypsin-EDTA 1× solution containing 170,000 U/L trypsin 1:250 and 0.2 g/L EDTA manufactured with irradiated porcine trypsin, tested for porcine parvovirus and mycoplasma. Store at 4 ± 3 °C.
21. Vero cell line (ATCC CCL-81).
22. Vero cell line culture medium: Dulbecco's modified Eagle's medium with 4.5 g/L glucose (DMEM) supplemented with 10% of FBSi, 1% of non-essential amino acid solution 100×, 1% Na pyruvate, 1% glutamine [4 mM], 50 μg/mL of gentamicin sulfate [50 mg/mL], and 100 U/mL of nystatin [10,000 U/mL]. Store at 4 ± 3 °C.

2.2 Indirect Immunoperoxidase Test (IPT)

1. Plate shaker.
2. Single- and multichannel pipettes and tips.
3. Inverted microscope.
4. Acetate buffer solution: Dissolve 1.155 mL of acetic acid (glacial) in 100 mL of water (solution A, acetic acid 0.2 N). Dissolve 2.72 g of sodium acetate trihydrate ($C_2H_9NaO_5$) in 100 mL of water (solution B, $C_2H_9NaO_5$ 0.2 M). Mix 74 mL of solution A and 176 mL of solution B. Store at room temperature.
5. Phosphate-buffered saline (PBS 1×) pH 7.2 (±0.2 UpH): Add about 100 mL water to a 1 L graduated cylinder or a glass beaker. Weigh 8.0 g of sodium chloride (ClNa), 0.2 g of potassium chloride (ClK), 0.2 g of potassium di-hydrogen phosphate (PO_4H_2K), and 1.15 g of di-sodium hydrogen phosphate (PO_4HNa_2), and transfer to the cylinder. Add water to a volume of 900 mL. Mix and adjust pH with HCl (*see* **Note 2**). Store at room temperature.
6. Blocking solution: PBS 1× with 0.05% Tween™ 20 (PBS-Tw) and 5% of powdered skimmed milk. Prepare blocking solution immediately before use (*see* **Note 3**).
7. HRP conjugate: Protein A peroxidase 1 mg/mL. Prepare and store following manufacture instructions.
8. Hydrogen peroxidase 30% (H_2O_2). Store at 4 ± 3 °C.
9. Preincubation sample solution: PBS 1× with 0.05% Tween™ 20 (PBS-Tw), 5% of powered skim milk, and 2% of FSBi. Prepare preincubation solution immediately before use.
10. Reference standard swine sera including hyperimmune positive control serum (ASF-PC), limit control sera (ASF-LC), and negative control serum (ASF-NC) (*see* **Note 4**).
11. Substrate stock solution: Dissolve 20 mg (one tablet) of 3-amino 9-ethylcarbazole (AEC) in 2.5 mL of N, N-dimethylformamide. Store at 4 ± 3 °C in a bottle wrapped with aluminum foil. Do not equilibrate at room temperature before use.
12. Substrate solution: Mix 300 μl of substrate stock solution in 5 mL of acetate buffer and 5 μl of H_2O_2 (this volume is recommended for one 96-well plate). Prepare substrate solution immediately before use.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Preparation of the 96-Well Cell Culture ASFV-Infected Plates to Be Used as an Antigen in the IPT Assay for ASFV-Specific Antibody Detection

All of the established cell lines used here from African green monkey species (Vero and MS) are cultured according to standard protocols in a 37 °C, 5% CO_2 humidified incubator. Established cell lines are grown in plastic tissue culture flasks and/or plates and subcultured by trypsinization according to standardized protocols. All of these protocols can be applied, with minor modifications, to other ASFVs and cell models.

1. Bring the trypsin-EDTA solution and complete growth medium to the appropriate temperature for the cell line. For Vero and MS cell lines, 37 °C is the temperature used to grow the cells (*see* **Note 5**).
2. From 90% confluent cells grown in a T150 flask [growth area 150 cm^2/~1.5×10^7 cells], remove and discard the cell culture medium.
3. Wash the cells with ~13–15 mL of trypsin-EDTA solution (approximately 1 mL per 10 cm^2 culture surface area), spread the liquid onto the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer, and rock the vessel back and forth several times. The wash step removes any traces of serum, calcium, and magnesium that would inhibit the action of the trypsin-EDTA solution (dissociation reagent).
4. Remove and discard the wash solution.
5. Add ~7–8 mL of trypsin-EDTA to cover the entire surface (approximately 0.5 mL per 10 cm^2 culture surface area), and incubate 2–7 min (*see* **Note 6**) at 37 °C in 5% CO_2 humidified incubator until the cells appear to be detached (they will appear rounded and retractile under the microscope). Check the progress of cell dissociation by microscopy. To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for them to detach.
6. Once the cells appear to be detached, add the corresponding volume of pre-warmed complete growth medium supplemented with 10% of FSBi to the cell suspension to inactivate the trypsin.
7. Disaggregate the cells by pipetting up and down to disaggregate cell clumps. Check the cells with the microscope to be sure that most (>95%) are single cells. If cell clusters are apparent, continue to disperse the cells with gentle pipetting.
8. Using this cell suspension, pipette required volume of cells into the required number of 96-well cell culture plates (*see* **Note 7**) at 1:2 split ratio (*see* **Note 8**).
9. Incubate 24 (Vero) or 48 (MS) hours at 37 ± 3 °C in 5% CO_2 humidified incubator to get an 80–90% confluent plate [growth area/well 0.32 cm^2, average cell yield 3.2×10^4].

10. After the incubation time, carefully decant the medium of the cell cultures grown in the 96-microwell plates.
11. In a separate glass sterile bottle, prepare the appropriate dilution (in culture medium without FBSi) of the cell-adapted ASFV (*see* **Note 9**) to infect at a multiplicity of infection (MOI) between 0.025 and 0.05. For figuring out the amount of virus you need to add for a certain MOI, use the formula: number of cells $\times$ desired MOI $=$ total PFU (or plaque-forming units) needed (*see* **Note 10**). Then use the formula: (total PFU needed)/(PFU/mL) = total mL of virus needed to reach the desired MOI.
12. Add 100 μl/well of the inoculum, and incubate at 37 $\pm$ 3 °C in 5% CO_2 humidified incubator for 2 h. Then complete the volume up to 200 μl per well with cell medium supplemented with FBSi to have a final concentration of 2%.
13. Incubate at 37 $\pm$ 3 °C in 5% CO_2 humidified incubator 18 $\pm$ 1 h in case of the ASFV-infected Vero cells and 24 $\pm$ 1 h in case of the ASFV-infected MS cells.
14. Remove the viral inoculum by vacuum suction or by inverting the plate to throw the liquid and tap on absorbent paper to dry. Fix the cell sheets by adding 100 μl/well of the acetone/methanol cold fixative solution. Keep the plates 8 $\pm$ 2 min at room temperature, and then wash the plates with PBS1x three times for 10 min each, and gently shake on a plate shaker.
15. Dry the plates at room temperature for 30 min. The fixed and dry IPT plates can be used directly for the detection of antibodies against ASFV following the IPT procedure (Subheading 3.2) or stored at < -10 °C (*see* **Note 11**).

3.2 Indirect Immunoperoxidase Test (IPT) Procedure for ASFV Antibody Detection

1. Keep the ASFV-IPT plates at room temperature (18–25 °C) for 30 min after defrosting.
2. Blocking step: Block the plates by adding 100 μl per well of blocking solution. Incubate 1 h at 37 $\pm$ 2 °C on a plate incubator shaker.
3. Sample preincubation (*see* **Note 12**): During the blocking step (step 2), dilute the samples and the reference standard sera in separate 96-well microtiter plates in preincubation solution. Add 100 μl per well and incubate 1 h at 37 $\pm$ 2 °C on a plate incubator shaker. Samples should be analyzed at the recommended desired dilution to fit the purposes of the analysis (*see* **Note 13**).
4. Sample incubation: After 1 h discard the blocking solution from the ASFV-IPT-plates, and add 100 μl per well of the pre-incubated samples and reference standard sera. Incubate 45 min at 37 $\pm$ 2 °C on a plate incubator shaker.

5. Wash three times by adding 100 μl per well of PBS 1× for 5 min each time at 37 ± 2 °C on a plate incubator shaker.
6. Add 100 μl per well of the HRP-protein A conjugate (1:5000 dilution diluted in blocking solution), and incubate for 45 min at 37 ± 2 °C on a plate incubator shaker.
7. Wash as step 5.
8. Add 50 μl/well of substrate solution, and incubate for 10 min at room temperature (18–25 °C).
9. Add 100 μl per well of PBS 1× to stop the reaction (*see* **Note 14**).
10. Results are visualized using an inverted microscope (Figs. 2 and 3) (*see* **Note 15**).

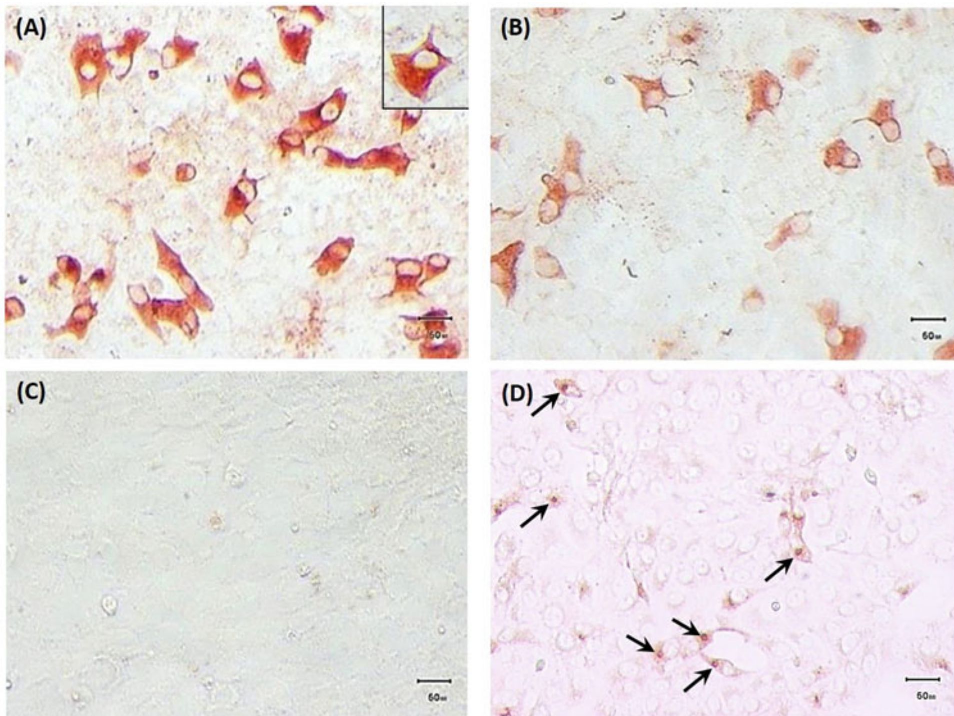

Fig. 2 IPT result in Vero cells infected with the Spanish Ba71V –Vero-adapted ASFV incubated with: (**a**) Antibody positive serum sample (titer of 5.21 Log10). An intensive red staining is observed in the cytoplasm of the ASFV-infected Vero cells. (**b**) Weak antibody positive serum (titer 3.1 Log10) showing a weak red staining in the cytoplasm of the ASFV-infected Vero cells. (**c**) Negative serum sample. (**d**) Limit antibody-positive serum sample with a titer of 1.6 Log10. Arrows show cytoplasmic inclusion bodies (IB) in an area close to the nucleus observed in samples collected from animals at early times after the infection

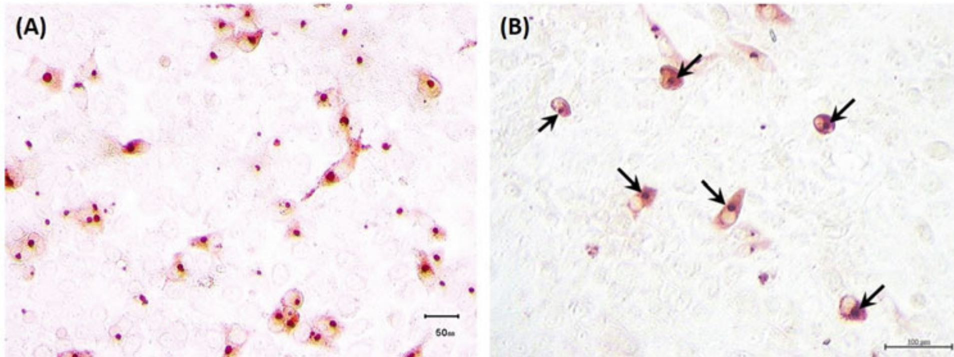

Fig. 3 Monkey stable (MS) cells infected with the Spanish E70 –MS-adapted ASFV incubated with (**a**) antibody-positive lung's exudate (titer 4.01 Log10) and (**b**) antibody-positive serum (titer 2.8 Log10). Arrows show cytoplasmic inclusion bodies (IB) in an area close to the nucleus observed in samples collected from animals at early times after the infection. Cytoplasm staining is also observed

4 Notes

1. Cell culture and infection steps can be affected by the batch-to-batch variation of the fetal bovine serum (FBS). It is recommended to perform quality tests on the new batch.
2. Concentrated HCl (12 N) can be used at first to narrow the gap from the starting pH to the required pH. From then on it would be better to use a series of HCl (e.g., 6 and 1 N) with lower ionic strengths to avoid a sudden drop in pH below the required pH.
3. We find that it is best to prepare this fresh each time.
4. The reference standard sera can be obtained at the European Union (EU) and FAO Reference laboratory for ASF (https://asf-referencelab.info/asf/en/).
5. After recovery from frozen stock, Vero and MS cells usually take two to three passages to reach their regular growth rate, and this should be taken into account if planning to use the cells for infections. It is important to note that cell cultures have the potential to change with time due to selective pressures in the culture environment and, particularly with continuous cell lines, due to genetic instability. Cells may continue to proliferate, but as the passage number increases, changes such as loss of differentiated properties or changed susceptibility to the viral infection over time can occur. The maximum passage level recommended is 150.

6. Incubation time varies with the cell line used. Usually MS takes 2–3 min, whereas Vero cells need longer incubation time up to 5–7 min.
7. 96-well culture single-break strip plates can be used since they can be broken off separately and number of tests performed can be adjusted to number of samples.
8. One T150 flask [150 cm^2/~ 1.5 × 10^7 cells] is equivalent in 1: 1 ratio to (passage 1:1) to five 96-culture microwell plates [32 cm^2 plate/ ~ 3. 2 × 10^6 cells/plate]. Therefore to passage one T150 flask in a 1:2 ratio, we have to add 1 mL of cell suspension to 9 mL of cell culture medium supplemented with 10% of FSBi for one plate.
9. This procedure describes cell infection using ASFV E70 adapted to MS cells and Ba71VR adapted to Vero cells, but any ASFV adapted to these cell lines can be used for infection.
10. Tissue culture infectious dose which will infect 50% of the cells in monolayer (TCID50) is related with PFU by the following formula PFU/ml = 0.7 × TCID50.
11. The IPT plate batches should comply with appropriated quality control tests and be tested against the reference positive, limit, and negative standard sera following the international EU reference laboratory guidelines.
12. To reduce the IPT background observed mainly with sera collected from domestic pigs vaccinated with vaccines produce in monkey established cell lines.
13. The working dilution for routine diagnosis recommended by the international EU and OIE reference laboratories is 1:40 in either in serum, blood, tissue exudates, or fluids and 1:5 for tissue's homogenate (https://asf-referencelab.info/asf/en/).
14. It is recommended to perform this step three times to avoid background. Leave the plate in the last PBS wash step.
15. The test is validated when there are an intensive red cytoplasmic coloration in positive control wells and an absence in case of negative controls. In samples positives for antibody detection against ASFV, a red cytoplasmic staining will be observed in the infected cells. In some specific situations related to samples collected from vaccinated animals against other diseases, some slight background can be observed with a nonspecific red coloration in the wells. Positive- and false-positive reactions are easily distinguished analyzing pig samples against infected and uninfected cell cultures.

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Chapter 11

African Swine Fever Virus Hemadsorption Inhibition Assay

Alexander Malogolovkin and Alexey Sereda

Abstract

Adsorption of red blood cells (hemadsorption) on the surface of macrophages infected with African swine fever virus (ASFV) is a unique phenomenon allowing to determine virus infectious titer in hemadsorption unit (HAU) and differentiate virus strains phenotypically. In the meantime, hemadsorption of particular ASFV strain can by inhibited by homologous anti-ASFV serum containing antibody to the serogroup-specific virus protein (CD2v). Here, we describe a hemadsorption inhibition assay (HADIA) to phenotype ASFV strains to one of the known nine serogroups using blood-derived swine macrophages. The HADIA is a powerful method in the ASFV immunopathology and vaccine research since it provides additional antigenic and phenotypic characteristics of virus strains that can't be defined by other assays.

Key words African swine fever virus, Hemadsorption inhibition, Phenotyping, Serogroup, Vaccine

1 Introduction

African swine fever virus (ASFV) is a genetically and phenotypically extremely heterogeneous virus [1]. Nevertheless, good laboratory practice has been successfully established to isolate and characterize various ASFV strains [2]. Genotyping of ASFV based on the partial p72 (B646L) gene sequencing is a reliable method for understanding of virus origin and phylogenetics [3, 4]. Based on the ASFV p72 gene sequencing, ASFV strains were classified in XXIV genotypes, but more genotypes may exist [5]. However, p72 genotyping analysis alone does not always allow to discriminate between viruses of different biological phenotype or virulence [6]. New genetic markers and recent advances of whole genome sequencing can provide comprehensive genotyping and critical data for further elucidation of ASFV genetic diversity.

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Antigenic variability of ASFV strains was initially picked up by Malmquist and Hay [7, 8]. ASFV strains demonstrated different virulence, hemadsorption (HA) phenotype (*see* supplementary video) (number of adsorbing red blood cells/inhibition by homologous serum), and antigenic specificity [9]. Among these, two key features were taken into account to differentiate ASFV: (1) ability to stimulation of type-specific (homologous) immune response and (2) induction/inhibition of a hemadsorption (HA) phenotype in infected cells using an HA inhibition assay (HADIA) to assess serological cross-reactivity with different ASFV isolates in vitro [8, 10]. HADIA-based ASFV classification was developed to differentiate virus antigenic types (serogroup). Nine ASFV serogroups (SG) were defined and subsequently thoroughly characterized at the Federal Research Center for Virology and Microbiology (Pokrov) [11, 12] (Table 1).

Table 1
Serogroup classification of African swine fever virus (ASFV) strains, isolates, and variants

Serogroup	ASFV reference strain	ASFV strains and isolates	
		Highly/moderately virulent	Low virulent attenuated/avirulent
1	Lisbon-57	Lisbon-57 (L-57), Kimakia, Katanga-78, Katanga-105, Katanga-115, Madeira-65, Diamant	LS, L-50, LF-97, Kimakia-155, Diamant-160, Katanga-139 (Kc-139), Katanga-149 (Kc-149), Katanga-160 (Kc-160), LK-111, Katanga-350
2	Congo-49	Congo-49 (K-49), Yamba-74, Le Bry-73, Sylva	NVL-1, Mfuati-79, Ndjassi-77, KK-202, KK-262/C
3	Mozambique-78	Mozambique-78 (M-78), MK-101	MK-200, MK-210
4	France-32	France-32 (F-32), Cuba-71, Brazil-80, Cuba-80, Malta-78, Sao-Tome and Principe-79 (STP-1), DNOPA-Luanda, Odessa-77	FK-32/135
5	TSP-80	TSP-80	TSP-80/300
6	TS-7	TS-7	TS-7/150 TS-7/230
7	Uganda	Uganda	UK-50, UK-80
8	Rhodesia, Stavropol 01/08	Rhodesia, Stavropol 01/08	St-CV_1/20
9	Davis	Davis	None
Heterogeneous isolates			
	Kiravira-67	Kiravira-67	None

The HADIA-based assay was used for ASFV strain differentiation and screening for development of potential live-attenuated vaccine candidates [13, 14]. Each serogroup has a virulent hemadsorbing ASFV reference strain used as a standard for HAU (Table 1). Non-hemadsorbing ASFV strains are being classified using animal challenge experiments [15, 16].

In order to facilitate ASFV serogroup classification, CD2v/C-type lectin genes-based analysis has been proposed as a fast and precise alternative. The CD2v gene sequencing, in particular, with following phylogenetic analysis could adequately predict ASFV strains serogroup and characterize its phenotype [17, 18]. Nevertheless direct evidence of ASFV phenotype and protective potential can be withdrawn from either HADIA assay or pig immunization/challenge experiments. Hemadsorption inhibition assay remains a reliable tool in defining ASFV antigenic diversity and ASF vaccine efficiency. It is worth noting that there are several HADIA modifications proposed by different authors using PBMC or bone marrow cell culture (*see* **Note 6**) and various flask/plate formats [19, 20]. Here we present the most convenient and high-throughput 96-well format HADIA using PBMC.

2 Materials

1. 70–80 mL of blood drawn from clinically healthy pigs (*see* **Notes 1, 2,** and **3**).
2. Anti-ASFV Immune sera: Hyperimmune sera against ASFV strains from different serogroups are the key component for HADIA. The ASFV-specific sera can be collected from pigs recovered from ASF or immunized with moderately virulent ASFV strains. The highly active ASFV specific serum also can be generated by inoculating pigs with virulent ASFV along with phosphonic acid (method 3.1).
3. ASFV reference strains: In order to serotype the ASFV strains using HADIA, we used the ASFV reference strains from the FRCVM collection (Table 1).
4. Tissue culture grade 96-well plates.
5. Complete macrophage growing media: 30 mL RPMI 1640 media plus 5 mL fetal bovine serum (FBS) and 15 mL swine plasma supplemented with 500 μl 100× penicillin/streptomycin solution.
6. Anticoagulant: Any one of EDTA (10% ethylenediaminetetraacetic acid tripotassium salt dihydrate in water), heparin (200 U/mL), sodium citrate (3.2% in water).
7. Histopaque-1077/1083 or Ficoll 1.077/ 1.084.
8. Water bath.

9. Serological pipettes (10 or 25 mL).
10. Aerosol-resistant filter tips.
11. Phosphate-buffered saline, divalent cation-free (PBS).
12. Distilled water.
13. Red blood cell lysis buffer: 155 mM ammonium chloride, 12 mM sodium hydrogen carbonate, 0.1 mM ethylenediaminetetraacetic acid.
14. Hemocytometer.
15. 0.4% (w/v) trypan blue.
16. Multichannel pipettes.
17. Microcentrifuge.
18. 10% RMPI: RPMI 1640 media supplemented with 10% FBS and 1× penicillin/streptomycin.

2.1 Hyperimmune Sera

1. Hemabsorbing strain of ASFV (*see* **Notes 4** and **5**).
2. 40% solution of phosphonic acid ($HOOCCH_2P(O)(OH)_2$) in sterile water.

3 Methods

3.1 Hyperimmune Anti-ASFV Sera for HADIA

1. Pigs (30–35 kg) are infected intramuscularly with a virulent hemadsorbing ASF virus with 10^2–10^5 HAU50. The virus dose depends on the ASFV strain virulence (*see* **Note 2**).
2. The clinical signs are monitored and temperature is recorded daily.
3. When the body temperature starts to arise (>40 °C) and clinical signs of the disease appear (approx. 2–3 days post-infection), administer 40% phosphonic acid solution intramuscularly (100–150 μg/kg of body weight). Twice a day for first 3 days and then once a day for the following 10 days.
4. On day 14 after the last temperature pick, the animals are exsanguinated and the sera are checked in the HADIA.
5. Treat anti-ASFV serum at 56 °C for at least 30 min in water bath to inactivate complement system.
6. The titer of antibodies in HADIA assay may reach 1:40–1:640.
7. Up to 50% of animals may reach humane endpoints from ASF depending on the virulence of the ASFV strain.

3.2 Preparing Swine PBMC

1. Add 15 mL room temperature Histopaque or Ficoll-paque to 50 mL tubes, and overlay with diluted pig blood 1:1 with PBS [21]

2. Centrifuge the overlaid tubes at 1000 × *g* for 30 min at room temperature with brake off.
3. Collect the buffy coat between the two layers using a pipette into a new 50 mL tube and top up with PBS
4. Centrifuge at 400 × *g* for 10 min at room temperature.
5. Discard supernatant and inspect the pellet for contaminating red blood cells. If there is heavy contamination, add 5 mL of red blood cell lysis buffer per tube, resuspend the pellet, and incubate at room temperature for 5 min. Add 40–45 mL. Centrifuge at 400 × *g*, for 10 min.
6. Wash cells with PBS at least two more times (three washes total). Resuspend cells in blood macrophage media. Take 100 μl of resuspended cells, and add to 800 μl PBS and 100 μl trypan blue solution. Count clear cells and determine cell concentration.

   Cell concentration = (number of cells counted × dilution factor) ÷ volume counted. For a typical hemocytometer, the volume of one of the big squares is 0.1 μl for a 0.1 mm depth chamber or 0.2 μl for a 0.2 mm depth chamber.

3.3 Preparing Washed Red Blood Cells

1. Centrifuge 100 μl of swine whole blood at 3000 rpm for 5 min.
2. Remove plasma and buffy coat layer.
3. Resuspend the red cells in phosphate buffer saline (PBS) with approximately three times the volume of the red cells, and invert the tube to mix.
4. Centrifuge for 5 min at 2000 rpm and discard the supernatant. Repeat the washing step with PBS twice for a total of three washes or until the supernatant is clear.
5. Resuspend the RBC in 1 mL of RPMI (10%), and dilute 1:10 to prepare 1% solution.

3.4 Hemadsorption Inhibition Assay

1. Day 1. Prepare 96-well plates of PBMCs processed from swine blood according to Subheading 3.2. Seed plates at 1.3×10^6 cells/well (*see* **Note 5**).
2. Day 4 (3 days post-seeding). Monocytes can be infected with the ASFV strain that will be tested for serospecificity.
3. Calculate dilution of viral stock needed for 96-well plate.
4. Dilute virus in complete RPMI macrophage medium.
5. Check status of macrophages. Wash the cells by pipetting the medium up and down using automatic multichannel pipette with 200 μl filter tips to wash away non-adherent cells. Discard the medium. Repeat the washing step two more times.
6. Infect cells with ASFV 10^3 HAU50 virus/100ul/well using multichannel pipette.

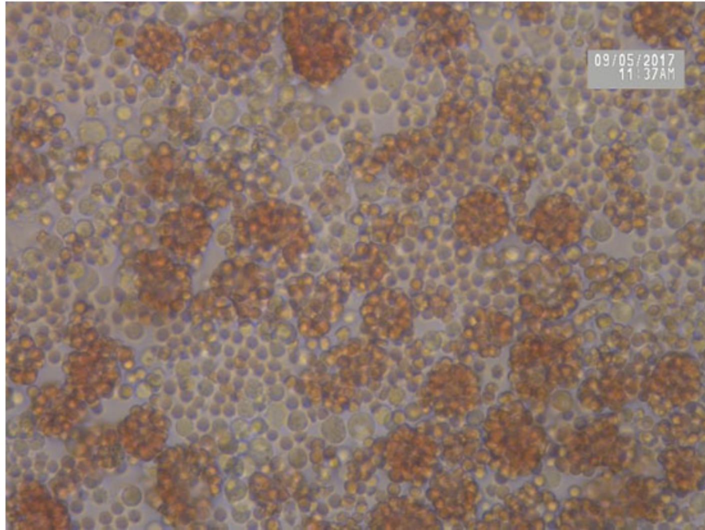

Fig. 1 Macrophages infected with African swine fever virus in the presence of red blood cells showing the characteristic hemadsorption pattern

7. Incubate infected cells overnight at 37 °C with 5% CO_2.
8. Day 5. In a separate 96-well plate, prepare two-fold serum dilutions (1:2 to 1:256) of swine anti-ASFV sera in complete macrophage medium.
9. Add 100 μl of complete macrophage medium to all wells of the new 96-well plate.
10. Add 100 μl of swine serum to each well of Row A (top row) to generate a 1:2 dilution. Run duplicates of each sample. Include appropriate positive and negative controls.
11. Using multichannel pipette, do two-fold dilutions going down the rows.
12. Add 100 μl of the serum dilutions to the infected cells.
13. Incubate plates at 37 °C for 2 h in 5% CO_2.
14. To each well, add 20 μl 1% swine red blood cells.
15. Incubate plates at 37 °C overnight in 5% CO_2.
16. Day 6. Read HADIA titers and hemadsorption using inverted microscope and 5×, 10× magnifications (Fig. 1 and *see* **Note 7**).
17. Note maximum dilution of each serum at which HADIA is complete (no rosetting cells) and write the value. Macrophages infected with ASFV with no anti-ASFV serum display representative hemadsorption pattern (Fig. 1).
18. Record the results. The HADIA result interpretation is represented in Table 2 (*see* **Note 8**).

Table 2
The results of the HADIA using ASFV reference strains and anti-ASFV sera

ASFV strains	Anti-ASFV reference serum								Serogroup
	1	2	3	4	5	6	7	8	
Lisbon-57	–	+	+	+	+	+	+	+	1
K-49	+	–	+	+	+	+	+	+	2
M-78	+	+	–	+	+	+	+	+	3
F-32	+	+	+	–	+	+	+	+	4
TSP-80	+	+	+	+	–	+	+	+	5
TC-7	+	+	+	+	+	–	+	+	6
Uganda	+	+	+	+	+	+	–	+	7
Rhodesia	+	+	+	+	+	+	+	–	8
Unknown ASFV	+	+	+	–	+	+	+	+	4

"–" Indicates that the hemadsorption of the particular ASFV was inhibited by the reference serum from certain serogroup. The serogroup can be defined

"+" Indicates that the hemadsorption was registered regardless of the reference serum dilution. Unknown ASFV isolate demonstrate HADIA with reference serum from serogroup 4 and no HADIA with sera from different serogroups. Therefore, the unknown ASFV strains belongs to serogroup 4

4 Notes

1. The number and quality of PBMC strictly depend on the physiological conditions of pigs. The blood should be withdrawn from healthy pigs preferably once a week early in the morning before feeding. Usually, 75–80 mL of blood is enough to set up an HADIA experiment with 96-well format plates.
2. We recommend to use an individual sterile vacuum collection blood tubes prefilled with anticoagulant (e.g. Vacutainer).
3. Upon withdrawal the blood should be carefully mixed with anticoagulant (e.g. heparin sulfate) and transported to the laboratories on ice. In case if the PMBC processing can't be done immediately, keep the blood samples on ice.
4. The dose of the ASFV for pig immunization to produce hyperimmune serum may vary depending on virulence of the isolate. For instance, ASFV F-32 (Genotype I, SG4) is a low-virulent isolate and can be inoculated with higher dose 10^4–10^5 HAU. However, highly virulent ASFV isolates, such as ASFV M-78 (Genotype 5, SG3) and K-49 (Genotype I, SG2), should be inoculated at a lower dose 10^2 HAU.

5. The hemadsorption inhibition assay can be only used to phenotype hemadsorbing ASFV strains only. Non-hemadsorbing ASFV can be phenotyped using immunization/challenge animal trials using reference ASFV strains.
6. Pig bone marrow cell culture also can be used for HADIA assay (*see* Chapter 4). Bone marrow cell culture has a mixture of hematopoietic, myeloid, and lymphoid progenitor cells in different proportions and differentiation states. This culture gives an incredibly high ASFV yield and is permissive for most known ASFV strains. However, the process for bone marrow cell culture preparation is laborious, time-consuming, varies between laboratories, and as a result is hard to standardize.
7. Short educational video showing hemadsorption formation in macrophages infected with ASFV K-49 strain is available in Supplementary material.
8. Highly active anti-ASFV sera can be lyophilized and then used routinely for HADIA assay. Minimal working dilution of anti-ASFV serum is 1:40, meaning that a serum inhibiting the hemadsorption of a homologous ASFV strain at dilution of 1: 40 or higher can be used for HADIA assay.

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Chapter 12

ELISpot Assay for the Detection of ASFV-Specific Interferon-Gamma (IFN-γ)-Producing Cells

Raquel Portugal

Abstract

The enzyme-linked immunospot (ELISpot) assay is a technique of unparalleled sensitivity to determine the frequency of antigen-specific immune cells secreting an immunomodulatory mediator upon recall antigen stimulation, making it a valuable tool in vaccine research. Typically done in multi-well microplate format, it also allows a high-throughput analysis of numerous immune cell samples, e.g., from different donor subjects, especially with the help of automated plate readers and specialized software that currently exist in most laboratories. IFN-γ is a hallmark cytokine secreted especially by T-cell subsets in recall response to pathogens, and consequently the IFN-γ ELISpot assay is one of the most widely used. The cellular arm of the immune response is known to be fundamental in protection against virulent ASFV, and therefore this assay is frequently employed in ASFV vaccine research to evaluate the results from immunization experiments.

The technique involves the use of plates with wells that have a membrane for base with a strong binding capacity for amino acids that thus can be densely coated with an antibody for IFN-γ. Upon adding cells and specific antigen or other control stimuli, responding cells will release IFN-γ that is captured by the antibody in close proximity and revealed using a second antibody (sandwich method) through either chromogenic or fluorescent methods, leading to the detection of a "spot" on the membrane for each positive cell. Here we detail our protocol to detect the frequency of ASFV antigen-specific IFN-γ-producing cells in immunized pig lymphocytes and give an example of a typical result using the technique.

Key words ELISpot, Immune response, Cellular immunity, IFN-γ, Antigen-specific response, Released immunomediator

1 Introduction

The enzyme-linked immunospot (ELISpot) assay is a technique of election for monitoring the response of live immune cell populations to recall antigen during vaccine development and hence assessing if an immune response is being specifically induced. It has been a fundamental tool since it was first implemented in 1983 to detect specific antibody-secreting cells [1, 2] and since then has been

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developed also for the quantification of specific T-cell responses [3, 4]. It allows the sensitive detection and quantification of antigen-specific immune cells that can exist at very low frequency, namely, in peripheral blood, secreting immunomodulatory molecules upon recall antigen stimulation. It also allows high-throughput analysis since it is typically performed in multi-well plates.

The ELISPOT assay is performed typically on 96-well plates with wells that have a membrane for base, at present mostly polyvinylidene fluoride (PVDF) since this type of membrane sustains a stronger interaction with amino acids than mixed cellulose ester ones (nitrocellulose) that were used when the technique was first developed [5]. The PVDF wells of an ELISpot plate can therefore be coated with a high density of antibody or antigen that will capture secreted immunomediators (cytokines, cytolytic mediators, antibodies) from living cells that are added to the well. However, whereas nitrocellulose membranes are hydrophilic and therefore can be coated directly, PVDF membranes are hydrophobic and frequently require a pre-wetting step with alcohol, although this may depend on the hydrophobicity of the coating antibody. Typically, the wells are coated with antibody specific for an immunomediator of choice (capture antibody) followed by addition of peripheral blood mononuclear cells (PBMCs) or other immune cell preparations such as purified lymphocyte subsets. The cells are then stimulated with a recall antigen (e.g., virus, peptides), mock stimulated as a negative control, or with activators that induce secretion of the target cytokines as a positive control. The T-cell mitogen phytohemagglutinin (PHA) is a known inducer of IFN-gamma secretion in T-cells [6], but several other activators for the production of different cytokines are reported in the literature depending on the cell type/organism (e.g., lipopolysaccharide (LPS), concanavalin A (Con A), pokeweed mitogen extract (PWM), ponomycin, phorbol myristate acetate). The time period of stimulation depends on the cell type, stimulant, and kinetics of the release of the immunomediator/s to be captured in the assay. An overnight stimulation (16 hours) is usually sufficient for antigen-specific lymphocytes producing interferon-gamma to be detected, but for other cytokines or T-cell subsets, detection may need stimulation in the range of several days [7]. The immunomediators are retained by the capture antibody in the vicinity of the secreting cells and revealed with the aid of a second antibody (detection antibody) followed by either chromogenic enzymatic or fluorescence detection. The outcome of an ELISPOT assay is the formation of a number of "spots" per well (or per number of cells), corresponding to the number of cells in the well that secreted the immunomediator. Currently the analysis in multi-well plates

can be done easily with the aid of automated readers and dedicated software (e.g., ImmunoSpot Analyzer, CTL). After applying specific settings for spot recognition, these can be used across multiple plates for similar immunomodulators and cell types, thus greatly facilitating the analysis of multiple plates and numerous samples. This is especially useful, for example, in T-cell epitope determination assays, where libraries of many overlapping peptides for different viral proteins can be tested for inducing a recall response of immunized cells [8].

With latest developments especially in low auto-fluorescing PVDF membranes, several different released immunomediators can also be detected in the same well, thanks to the use of fluorescently labelled antibodies, tuning the ELISpot assay into a multiplexed and more informative assay while simultaneously allowing to preserve limited samples [7, 9, 10].

An important strength of the ELISpot is that it reveals a biological effect since it shows cells that are actively releasing immunomodulatory molecules (e.g., cytokines, perforin, granzyme B) in response to antigen, thus having a clear effect in the environment. Since immune cells produce and store immunomediators but may not release them, this constitutes a limitation of other immune assays such as ELISA (requiring cell lysis) or intracellular cytokine staining (ICS) for flow cytometry analysis, which simply detects the production of the molecule. Another aspect of the ELISpot technique that might be relevant is the fact that the cells are kept alive and can be recovered after the incubation period and re-used for other assays, or the supernatant used for detection of further immunomediators (e.g., in ELISA).

Currently there still no effective and safe vaccine for ASFV. Immunization studies with low virulence isolates have shown that protection against virulent challenge correlates with the development of cellular immune responses, especially cytotoxic T-cell and NK activities [11–13]. A hallmark of activation of these cell types is the production of IFN-γ, and hence the IFN-γ ELISPOT became a fundamental tool for assessing whether vaccine formulations for ASFV are inducing the desired cellular arm of the immune response. Here we detail our current IFN-γ ELISPOT protocol and give an example of a typical assay and its result. It should be noted however that we sometimes observe a poor correlation between the result of the IFN-γ ELISPOT assay and protection outcome, as has been observed in other disease models [14, 15]. Therefore additional assays beyond the general release of IFN-γ by immunized cell populations will need to be developed to determine immune responses that may correlate with protection against ASFV.

2 Materials

1. Biological safety cabinet.
2. Humidified cell culture incubator.
3. Single- and multi-channel pipettes with compatible aerosol-resistant tips.
4. Plate shaker.
5. ELISpot 96-well plate: Sterile, clear 96-well filter plate with 0.45 μm pore size and hydrophobic PVDF membrane (*see* **Note 1**).
6. Coating buffer: 0.5 M carbonate-bicarbonate buffer (1.59 g/L Sodium Bicarbonate, 2.93 g/L Sodium Hydrogen Carbonate, 0.0002% Phenol Red; final pH 9.5-9.9).
7. Capture antibody: pig IFN-gamma monoclonal antibody (clone P2F6).
8. Sterile PBS.
9. Culture medium: RPMI with L-glutamine supplemented with penicillin and streptomycin, 50 μM β-mercaptoethanol (sterile filtered), and 10% fetal calf serum (v/v).
10. Lymphocyte preparation: Peripheral blood mononuclear cells (PBMCs) or other immune cell preparation (e.g., spleen cells, lymph node, sorted cell subsets).
11. Positive control: 20 μg/ml phytohemagglutinin (PHA) diluted before use in culture medium. For convenience a 1 mg/ml stock in tissue culture grade water may be stored at −20 °C.
12. Antigen: ASFV preparation for final 2×10^5 $TCID_{50}$ per well; peptide pool for final approximately 10–30 μg/ml each peptide.
13. Negative control: Mock medium (non-infected culture supernatant from the same cells used to prepare the virus stock) or other media used to dilute the antigen.
14. Ultra-pure water.
15. Detection antibody: biotinylated mouse anti-pig IFN-gamma monoclonal antibody (clone P2C11).
16. Sealing tape for 96-well plates (if working with infectious samples).
17. Streptavidin alkaline phosphatase (e.g., Invitrogen, SA1008).
18. Alkaline phosphatase conjugate substrate kit (e.g., Bio-Rad, 170-6432).
19. ELISpot plate reader and analyzer.

3 Methods

1. Dilute capture antibody in coating buffer for a final concentration of 4.0 μg/ml. Add 100 μl per well, and shake plate carefully to ensure even coating (see **Notes 1** and **2**).
2. Incubate overnight at 4 °C (see **Note 2**).
3. Wash plate four times with 200 μl/well of sterile PBS. Leave final wash on plate until just before adding cell samples to keep the wells from drying (see **Note 3**).
4. According to ELISpot plate layout, add 100 μl of PBMCs in complete culture medium at 4×10^6 /ml (4×10^5 per well) in duplicate wells (see **Note 4**). This amount can be reduced if the response is too strong and leading to too many spots to count. In an experiment that cannot be repeated, it is advisable to set up two different cell concentrations (e.g., 2×10^5 and 4×10^5 cells per well) (see **Note 5**).
5. According to ELISpot plate layout, add 100 μl per well of the stimulants in culture medium: PHA, antigen, and control mock medium (see **Note 6**).
6. Incubate plates overnight (16 hours) at 37 °C in humidified atmosphere with 5% CO2. Keep the plate motionless during this step (see **Note** 7).
7. Remove cells and supernatant from plate (flick-off if the sample is no longer necessary or collect carefully by pipetting with the plate at an angle avoiding touching the bottom of the wells). If discarding samples that contain virus, these must be removed to waste containing validated disinfectant (also in following steps).
8. Add 150–200 μl ultra-pure water and keep at room temperature for 5 min.
9. Discard the water from the wells (flick-off and if necessary, tap gently over absorbent paper to remove residual water from the wells; if the samples contained virus, discard the paper into validated disinfectant and disinfect the working area that contacted the paper).
10. Dilute biotinylated detection antibody to 1 μg/ml in PBS, and add 100 μl to each well of the plate. If the plate contained virus, cover with a plate sealer.
11. Place plate on shaker (e.g., 600 rpm or equivalent) for 2 hours at RT.
12. Wash three times with 200 μl PBS.
13. Dilute streptavidin alkaline phosphatase 1:1000 in PBS and add 50 μl/well. If plate contained virus, cover plate with sticky plastic sealer.

14. Incubate 1 hour at RT on shaker.
15. Wash three times with 200 μl PBS.
16. Color development: dilute as needed the components of the alkaline phosphatase conjugate substrate kit. We prepare 5 ml of total substrate solution for one plate (50 μl/well). According to manufacturer instructions, dilute 200 μl of the "AP colour development buffer" (25× concentrate) in 5 ml ultra-pure water at RT, and mix well; just before use, add 50 μl of each "AP colour reagent A" and "AP colour reagent B," and mix gently. Add 50 μl per well and cover with plate sealer if necessary.
17. Place plate on shaker with gentle agitation at RT, and observe until spots are clearly visible (usually within 10 minutes but may take up to 20 minutes if rare positive cells are present).
18. Flick off substrate and stop color development by washing the wells with 150 μl ultra-pure water for 10 min in plate shaker with gentle agitation; replace the water once during these 10 min (as usual if working with infectious samples, discard contents to approved disinfectant, and protect the plate with sealer tape during washes).
19. Discard water. If no infectious samples were present, allow the wells to air-dry by inverting the plate over absorbent paper, taping gently to remove residual water from the wells, and leave the plate inverted at an angle overnight at RT.

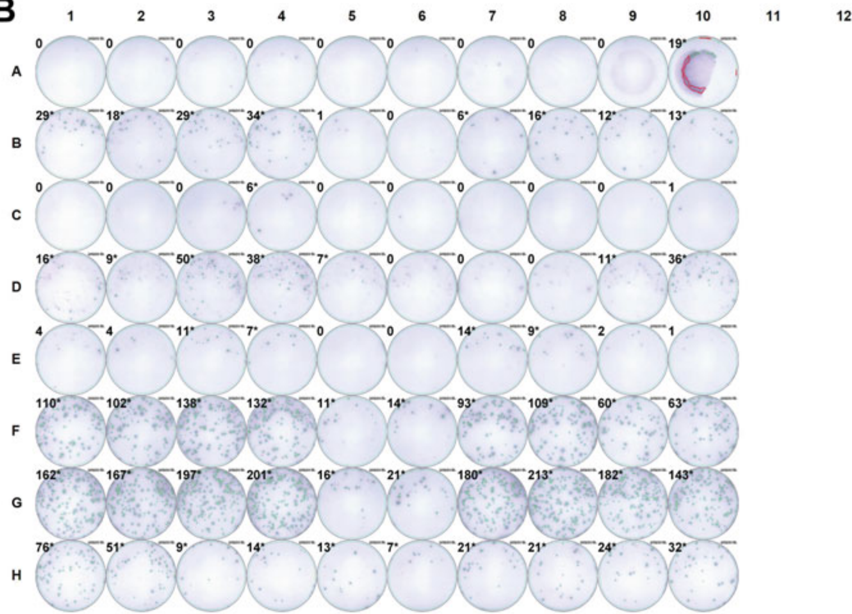
    If plate contained infectious samples, further submerge in 4% formaldehyde for 30 min after making sure the solution enters all wells. Wash with water a couple of times and allow to air-dry as described.
20. Read the results using an automated ELISpot plate reader and dedicated software (e.g., CTL Analyzer – LLC with ImmunoSpot 7.0 software). An example of an ELISpot assay with PBMCs collected during a vaccination experiment and its typical result can be observed in Fig. 1.

4 Notes

1. We use MultiScreen-IP Filter Plates from Millipore, and in combination with our capture antibody and coating buffer, we observed no need for pre-wetting of the wells with ethanol solution (usually 35%). Given the hydrophobicity of the PVDF membrane, this step may be necessary depending on the hydrophobicity of the capture antibody and may require optimization [5]. For example, we have seen significant background when using mixed cellulose ester membranes which may be ameliorated by the addition of a blocking step.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock	Mock		
B	Virus 1	Virus 1	Virus 1	Virus 1	Virus 1	Virus 1	Virus 1	Virus 1	Virus 1	Virus 1		
C	Virus 2	Virus 2	Virus 2	Virus 2	Virus 2	Virus 2	Virus 2	Virus 2	Virus 2	Virus 2		
D	PHA	PHA	PHA	PHA	PHA	PHA	PHA	PHA	PHA	PHA		
E	Ctrl Ag	Ctrl Ag	Ctrl Ag	Ctrl Ag	Ctrl Ag	Ctrl Ag	Ctrl Ag	Ctrl Ag	Ctrl Ag	Ctrl Ag		
F	Ag 1	Ag 1	Ag 1	Ag 1	Ag 1	Ag 1	Ag 1	Ag 1	Ag 1	Ag 1		
G	Ag 2	Ag 2	Ag 2	Ag 2	Ag 2	Ag 2	Ag 2	Ag 2	Ag 2	Ag 2		
H	Ag 3	Ag 3	Ag 3	Ag 3	Ag 3	Ag 3	Ag 3	Ag 3	Ag 3	Ag 3		



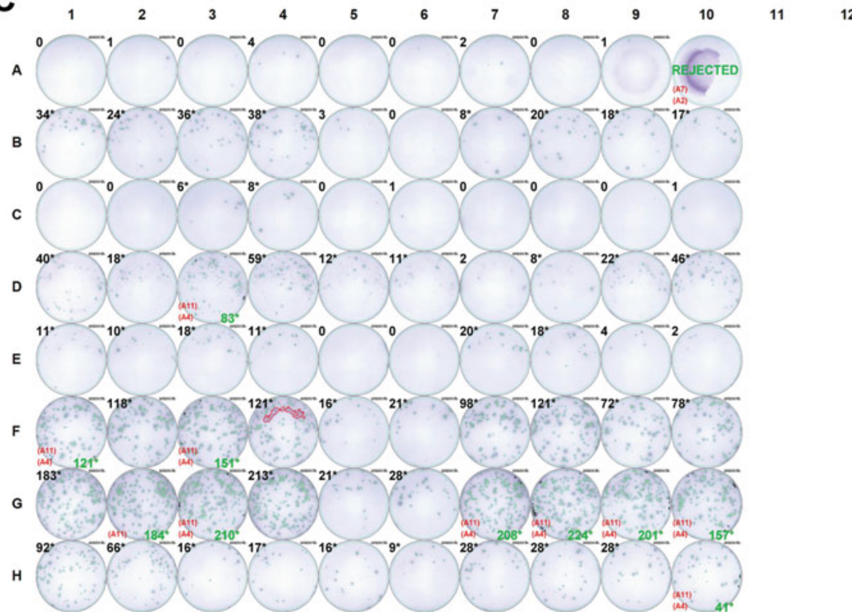


Fig. 1 ELISpot assay using PBMCs from five different animals from a vaccination experiment with Adenovirus and MVA vectors expressing viral proteins used for immunization. Cells collected from the animals were plated

It is vital not to allow the wells to dry or touch the bottom of the wells with the pipette tips at all times before the end of the assay to avoid damaging the well membrane, which will lead to intense background and unreadable results.

2. Coated plates with the capture antibody can be kept for several days stored at 4 °C. We routinely store plates in this way for up to 3 days when necessary.
3. After extensive washing of the capture antibody, it is usually recommended that plates are blocked and equilibrated for 2 hours at 37 °C with similar culture medium as the one that will be used with the cells (200 μl/well), or alternatively at 4 °C overnight (longer storage is not recommended due to the possibility of protein precipitation on the wells and lower spot resolution). We have found no clear advantage of doing this blocking step in our assay, but for different antibodies it may be necessary.
4. It is important to assess the viability as a measure of the quality of the cells and of the assay. We typically use trypan blue dye exclusion to count and assess the viability of our cell preparations of either fresh or frozen cells. If the freezing conditions are optimal, the cells usually retain their activity after thawing. However, working with freshly prepared cells is preferable for higher sensitivity since it cannot be excluded that the freeze-thaw process will not cause cell death and impairment of the cell's functionality. Especially when working with frozen cells, after thawing and centrifuging the cells in culture medium to remove DMSO, we typically allow the cells a resting time of a minimum 2 hours in culture medium at room temperature before counting and seeding on the ELISpot plate.
5. Cells per well: Seeding more than 400,000 cells per well in a 96-well plate might result in cell overstacking and diffuse spots because some of the cells may not contact directly the capture antibody surface. T-cell ELISPOT counts should be linear in the range of 100,000–400,000 cells per well. Cell density should be adjusted depending on the expected number of antigen-specific cells present in the cell sample and hence number of spots obtained. Different amounts of cells should be

Fig. 1 (continued) in duplicate and stimulated with mock medium, PHA, two different ASFV isolates (Virus 1 and Virus 2), a pool of peptides corresponding to a control non-ASFV protein (Ctrl Ag), and pools of peptides corresponding to different viral proteins (Ag 1, Ag 2, Ag 3). (**a**) Layout of the ELISpot plate where cells from different pigs (4×10^5 cells/well) were plated in duplicate wells. (**b**) Spot count result per well obtained with the aid of the ELISpot plate reader and software. User-defined settings were adjusted for spot recognition. (**c**) Spots per well result after further quality control analysis of the wells. Images of the plates were taken with a CTL Analyzer using ImmunoSpot software

seeded in replicate in case the number of spots per well is unknown. We routinely seed replicates of 400,000 and 200,000 cells per well.

6. Antigen concentration: the amount of ASFV observed to induce a clear response on PBMCs recall stimulation should be determined empirically. For the typical cell concentration in our assays, we have observed that 2×10^5 $TCID_{50}$ virus per well is optimal (MOI of 0.5–1 for $2–4 \times 10^5$ cells per well). We also test frequently peptides for T-cell antigenicity (e.g., pools of synthetic peptides corresponding to viral proteins used in subunit immunization). In this case we usually prepare peptide pools for a working concentration of up to 20 μg/ml per peptide in culture medium. We frequently pool up to 25 peptides from the same viral protein (total peptide mass of 500 μg/ml) and add 100 μl per well for a final concentration of 10 μg/ml per peptide.
7. It is important that the plates are not moved while the cells are incubated since this may result in diffuse spots. Cells will start to secrete the immunomediator which is captured by the antibody in proximity. Moving cells around in the well will result in the mediator being captured by extra areas of the antibody-coated surface.

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Chapter 13

Purification of African Swine Fever Virus

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Abstract

African swine fever virus is a cytolytic virus that leads to the apoptosis of both cultured cells and primary macrophages. Cell culture supernatants of virus-infected cells are routinely used for virological and immunological studies, despite differences in the biological behavior between such preparations and highly purified virus. In addition, more recent data suggests that exosomes containing viral proteins may be secreted from infected cells. While African swine fever virus can be purified through a number of methods, in our hands Percoll provides the most robust method of separating virus from cellular contaminants.

Key words African swine fever virus, Purification, Percoll, Density centrifugation, Vero cells

1 Introduction

Tissue culture supernatants from cultured cell lines and primary macrophages infected with African swine fever virus (ASFV) typically contain cellular contaminants (Fig. 1a) due to normal biological activity of the cells as well as the cytolytic nature of the virus. Appropriate controls from uninfected cell cultures can be successfully used to mitigate such problems; however, in some instances use of cell culture supernatants can prove problematic. For example, tissue culture supernatants of ASFV induce macropinocytosis, whereas highly purified virus does not [1]. Infected cells may also secrete exosomes containing viral proteins [2] which could complicate the results of immune assays using live virus. Lastly, studies focused on virion structure required highly purified preparations to generate high-quality reconstructions at the atomic level [3–5]. Sucrose gradient centrifugation [6, 4] or a combination of iodixanol and sucrose has been successfully used to purify ASFV; however, in our hands use of colloidal silica particles coated with polyvinylpyrrolidone (sold as Percoll®) that was described nearly 40 years ago [7] remains the most reproducible. This method

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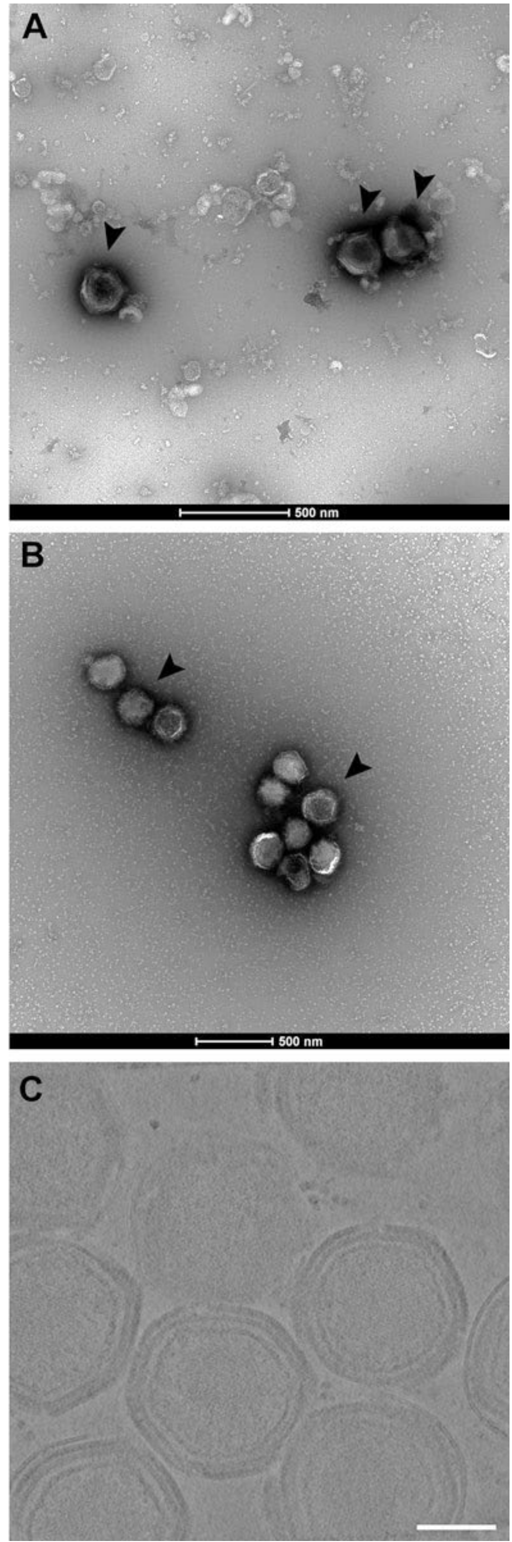

Fig. 1 Electron microscopy analysis of non-purified and purified virus inocula. Vero cells were infected with Ba71V and incubated until complete cell

removes practically all cellular contaminants (Fig. 1b) and has been successfully used for many studies including proteomic analysis of viral particles [8] and cryo-electron microscopy analysis of ASFV virions (Fig. 1c) [5]. Here we describe conditions for bulk culture of tissue culture-adapted ASFV in the laboratory as well as concentration and purification of the virions using Percoll® gradients.

2 Materials

2.1 Cell and Virus Preparation

1. Biological safety cabinet.
2. Polypropylene conical tubes (50 mL).
3. Benchtop centrifuge (capable of spinning 50 mL conical tubes).
4. Pipette gun.
5. Serological pipettes (10 and 25 mL).
6. Tissue culture grade phosphate-buffered saline, divalent cation-free (PBS).
7. Tissue culture grade trypsin-EDTA.
8. Vero cells (*see* **Note 1**).
9. Badajoz 1971 Vero-adapted African swine fever virus (Ba71v) seed stock (*see* **Note 2**).
10. Humidified incubator with carbon dioxide supply.
11. Tissue culture flasks, 175 cm^2 (T175).
12. Roller bottle incubator, or adaptor for rotating roller bottles in standard tissue culture incubator.
13. Roller bottles, 850 cm^2 (*see* **Note 3**).
14. Cell media (Dulbecco's modified Eagles media supplemented with 10% fetal bovine serum, 2 mM glutamine, 25 mM HEPES, 100 μg/mL penicillin, 100 U/mL streptomycin (*see* **Note 4**).

Fig. 1 (continued) detachment. Cell supernatants were harvested and clarified by low-speed centrifugation. Supernatants were either concentrated using ultracentrifugation or concentrated and purified using a double round of Percoll purification. Concentrated non-purified (Panel **a**) and concentrated purified stocks (Panel **b**) were then fixed using glutaraldehyde and analyzed by negative stain electron microscopy. Arrows point to ASFV virus particles. Panel **c** shows a computational slice through a cryo-tomographic volume depicting individual virions. The two protein and lipid layers along with the genome enclosed within are clearly visible. Scale bars are shown on each panel

15. Virus media (Dulbecco's modified Eagles media supplemented with 2% fetal bovine serum, 2 mM glutamine, 25 mM HEPES, 100 μg/mL penicillin, 100 U/mL streptomycin) (*see* **Note 4**).

2.2 Purification

1. Gradient media (45% Percoll® diluted in PBS).
2. Ultracentrifuge.
3. Swing-out ultracentrifuge rotor (*see* **Note 4**).
4. Angled ultracentrifuge rotor (*see* **Note 4**).
5. Appropriate ultracentrifuge tubes for rotors (*see* **Note 5**).
6. Long needle (50 mm) and syringes.
7. Pipettes.
8. Aerosol-resistant tips.
9. 2 mL polypropylene tubes.

3 Method

All steps other than centrifugation should be carried out within a biological safety cabinet using aseptic technique. Follow your laboratory's procedures and protocols for the safe handling and storage of African swine fever virus (see Chapter 1).

3.1 Virus Preparation

Pre-warm all media (*see* **Note 6**).

1. Set up two 175 cm^2 tissue culture flasks of Vero cells following standard culture practice as outlined by your cell supplier (*see* **Note 1**).
2. Once cells have reached 95–100% confluency, remove media using a serological pipette, add 10 mL PBS, and wash cell monolayer by gently rocking the flask. Remove PBS with serological pipette and repeat. Add 5–10 mL of trypsin-EDTA, and return T175 flasks to incubator for 3–5 min.
3. Once cells have detached, collect into 50 mL polypropylene tube, wash T175s with cell media, and add media and remaining cells to 50 mL polypropylene tube containing trypsin-EDTA and cells.
4. Pellet cells by centrifugation in a benchtop centrifuge ($400 \times g$, 5 min, 4 °C), remove supernatant, and resuspend cells. Add 20 mL cell media, and then add 1 mL of the cell suspension to 20 T175 flasks containing 40–50 mL of cell media. Evenly distribute the cells and return the flasks to the incubator.
5. T175s should be approaching 100% confluency around 4 days later. At this point repeat step 1 for all of the flasks. Each T175 flask should be used to seed one 850 cm^2 roller bottle containing at least 150 mL of cell media. Ensure lid is firmly closed,

then gently turn the bottle by hand to distribute the cells evenly, and then place in roller bottle incubator with continuous rotation.

6. Observe cell growth daily. Transfer bottles to biological safety cabinet, and open and close lids to allow buildup of gas to escape. Return bottles to incubator. Once cells are 90–95% confluent, they will be ready to infect with virus.
7. Dilute 5×10^8 $TCID_{50}$ of virus seed stock into 200 mL of virus media (*see* **Note 7**). Remove culture media from roller bottles using serological pipette and pipette gun (*see* **Note 8**), and then add 10 mL of diluted virus seed stock to each bottle. Firmly close bottle and return to the incubator, and allow virus to absorb for 2–3 h. Add 35 mL fresh virus media to each bottle, and then return to incubator.
8. De-gas cells daily and monitor cells until 90–95% of the cells are exhibiting cytopathic effect. This should typically take 3 days.
9. Remove supernatant and cell debris to 50 mL conical tubes using serological pipettes. Pellet debris in benchtop centrifuge ($1000 \times g$, 20 min, 4 °C). Collect and combine supernatants into two T175 flasks, and discard the pellets (*see* **Note 9**). Remove an aliquot of virus for titration, and store virus at 2–8 °C until ready for purification (*see* **Note 10**).

3.2 Purification

1. Concentrate virus by ultracentrifugation (24,000 g, 90 min, 4 °C) (*see* **Notes 5** and **11**). Remove and discard supernatant (*see* **Note 12**).
2. Using a needle and syringe, gently resuspend virus pellets in 5 mL 45% Percoll® solution, and combine in 50 mL conical tubes.
3. Transfer virus-Percoll® mixture to ultracentrifuge tubes suitable for fixed angle rotor, and use 45% Percoll® solution to balance the tubes within the tolerance of your ultracentrifuge (typically 0.1 g).
4. Add tubes to fixed angle rotor within biological safety cabinet, seal lid, and transfer to ultracentrifuge. Run gradients in ultracentrifuge ($41{,}000 \times g$, 30 min, 4 °C) with no brake; this may take up to 2 h to stop.
5. Open rotor within biological safety cabinet and remove tubes. Virus should appear as a white diffuse band just above the curved bit of the bottom of the tube. Remove virus bands, and combine in 50 mL conical tubes (*see* **Note 13**).
6. Repeat steps 11 and 12.
7. Transfer virus band to tube suitable for swinging bucket rotor, adjust volume, and balance with PBS (rather than 45% Percoll®) (*see* **Note 14**). Transfer tubes to rotor buckets in

biological safety cabinet, move to rotor, and then centrifuge virus and Percoll® (100,000 × *g*, 2 h, 4 °C).

8. Open buckets in biological safety cabinet. Percoll® will have formed a slightly glassy looking pellet at the bottom of the tube with the white virus band on top of it (*see* **Note 15**). Remove the virus with a pipette, and transfer to 2 mL tubes, aliquot as required, and store at −80 °C (*see* **Note 16**).

4 Notes

1. We routinely use Vero cells for the growth of Ba71v; however, other Vero adapted strains [9, 10] as well as combinations of different viruses and continuous cell lines have been described in the literature [11–13] which may be suitable. If the virus of interest will grow to a titer of at least 1×10^7 $TCID_{50}$/mL in Vero cells, then it likely to be a suitable candidate. Carry through of cellular debris after using other cell lines would need to be checked using electron microscopy.
2. We use T175s with filter caps to grow up seed stocks of Ba71v and 850 cm^2 roller bottles without filters for bulk virus culture. Roller bottles have the advantage over tissue culture flasks of supporting higher cell densities and allowing smaller volumes of cell culture media for a given number of cells, simplifying the subsequent centrifugation steps. This protocol describes our standard 20 roller bottle prep, but we have had success with smaller numbers of bottles, and we would suggest using 5 in the first instance.
3. We use sealed roller bottles, and therefore inclusion of HEPES in the media is important to maintain buffering capacity in the absence of CO_2.
4. All of the steps in this protocol can be carried out with an ultracentrifuge; however, access to a superspeed centrifuge and suitable rotors and tubes vastly simplifies the initial concentration of virus from tissue culture supernatant. We have successfully used fixed angle superspeed centrifugation in 50 mL tubes (15,000 × *g*, 3 h, 4 °C) and 250 mL bottles (11,500 × *g*, 6 h, 4 °C) in the place of ultracentrifugation. For biosafety reasons, use rotors or buckets that have O-rings. Reusable tubes and bottles with O-rings for superspeed centrifuges are available from most manufacturers.
5. Closed tubes have clear biosafety benefits. Clear tubes are advantageous for collecting the virus bands, but we routinely use polypropylene tubes for running Percoll® gradients without any problems.

6. Although using pre-warmed media for cell culture should be standard practice, it is particularly important when using closed cap roller bottles to avoid the buildup of pressure and so prevent bloating of the bottle.
7. It should be possible to obtain a virus titer of around 1×10^7 TCID50/mL from a T175 infected with Ba71v. Therefore, sufficient seed stock for 20 roller bottles can be produced by infecting 1 or 2 T175s of Vero cells.
8. It is a lot quicker to decant media from the roller bottles than to manually pipette the media out; however, avoiding contamination at this stage of the process outweighs the tedious nature of the task. If this cannot be avoided, carefully disinfect the rims of the roller bottles with 70% ethanol after decanting.
9. Cell debris will likely contain live virus; however, in our experience 90–99% of the virus is in the supernatant after Vero cells cultured in roller bottles are infected with Ba71v. This may be different for other combinations of cells and viruses and should be checked. To extract cell-associated virus, resuspend the pellet in virus media, freeze at −80, thaw at room temperature, and repeat two or three times before pelleting the debris by centrifugation.
10. ASFV is relatively stable at 4 °C in protein-rich media; however, the shorter the time between preparation of the tissue culture supernatant and purification, the better. We have prepared virus from ten rollers 1 week and then from another 10 the week after and run the purifications the week following that.
11. We have successfully concentrated virus in both swing out and fixed angle rotors at the indicated speed.
12. A clear pellet of virus should be visible after the centrifugation. If desired an aliquot of the supernatant can be taken for titration; 90–99% of the virus should have been concentrated by centrifugation (*see* Chapters 5 and 14 for methods for titrating ASFV).
13. Depending on the tube type, it may be easier to remove some of the gradient from the top of the tube before trying to collect the virus band. It may also help to remove the top of the tube if not using open top tubes.
14. As ASFV is stable at 4 °C, the procedure can be paused at this point and the virus stored in a fridge overnight.
15. The white virus band will move if the tube is angled slightly, whereas the Percoll® will not.
16. Samples prepared using this protocol are likely to contain significant amounts of Percoll® (*see* Fig. 1b); this can be removed by gel filtration through Sephacryl S-1000 columns [7].

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Chapter 14

African Swine Fever Virus Plaque Assay and Disinfectant Testing

Lorraine Frost and Carrie Batten

Abstract

Quantifying the titer of African swine fever virus is critical for disease control, viral infection studies, and disinfectant efficacy tests. Techniques such as real-time PCR and virus isolation provide an understanding as to whether viral genome is present or gives a qualitative assessment of live viral presence in a sample respectively, but neither provide a quantitative measurement of live virus. Here we describe a plaque assay for the titration of a Vero-adapted African swine fever virus strain (BA71V) and describe how to apply this method to determine disinfectant efficacy.

Key words African swine fever virus, Plaque assay, Disinfectant

1 Introduction

African swine fever (ASF) is a fatal disease of domestic pigs and wild boar, with lethality near 100% with highly virulent strains. ASF Virus (ASFV) has spread, since its introduction to Georgia in 2007 to include much of Europe from 2014, into China and the other parts of Asia in 2018, and most recently to the Dominican Republic in July 2021. The economic impact of ASFV necessitates its inclusion as an OIE notifiable disease. There is no vaccine available for ASFV, and therefore application of strict biosecurity measures including disinfection of farms and transport vehicles is one of the main recommendations provided by FAO for control [1].

In this chapter we describe one use of the ASFV plaque assay, for application as a disinfectant efficacy test. The plaque assay remains the favoured method for quantifying viral concentration of replication-competent virions [2]. The plaque assay described here uses a Vero-adapted African swine fever virus strain (BA71V) and has yielded similar results to that of the hemadsorption assay with the added benefit of using a continuous cell line as opposed to

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primary cells (porcine bone marrow cells) which are required for the hemadsorption assay, thus negating the requirement for the culling of an animal to perform the assay [3]. The identification of appropriate disinfectants for use against ASFV was a gap identified by the European Food Safety Authority (EFSA) in a 2019 scientific report [4]. The confirmation that commercially available disinfectants are effective against ASFV is therefore an important aspect of choosing the correct biosecurity regime for both farms and transport vehicles to prevent further spread.

2 Materials

2.1 African Swine Fever Virus Culture in Vero Cells

1. Vero cells (African green monkey cells, ECACC 84113001).
2. Badajoz 1971 Vero cell-adapted strain of African swine fever virus [3].
3. Cell culture media – Dulbecco's modified Eagle's medium (DMEM) + 1% L-glutamine +10% heat-inactivated fetal bovine serum (FBS). To one 500 mL bottle of DMEM, add 5 mL L-glutamine and 50 mL FBS.
4. 1× sterile phosphate-buffered saline (PBS).
5. T-175 tissue culture flasks.
6. 5, 10, and 25 mL serological pipettes.
7. 6-well tissue culture plate.
8. Hemocytometer.
9. 1.5 or 2 mL cryovials.

2.2 Plaque Titration on Vero Cells

1. Cell culture media – Dulbecco's modified Eagle's medium (DMEM) + 1% L-glutamine +10% heat-inactivated fetal bovine serum (FBS). To one 500 mL bottle of DMEM, add 5 mL L-glutamine and 50 mL FBS.
2. Tissue culture grade water.
3. 3.4% Avicel solution: Add 17 g Avicel (formulation Rc 591) to 500 mL distilled water. Stir for 2–3 h. Autoclave to sterilize prior to use. Store at room temperature until needed.
4. Overlay medium: 1.375× Eagle's overlay +4% FBS+ 1% Avicel solution +0.1% pen/strep +0.1% L-glut. To 161 mL Eagle's overlay, add 10 mL FBS, 74 mL 3.4% Avicel solution, 2.5 mL 100× penicillin/streptomycin +2.5 mL 100× L-glutamine.
5. 1% Crystal violet stock solution: Dissolve 1 g crystal violet in 80 mL distilled water and 20 mL absolute ethanol. Store at room temperature until needed.

6. 0.1% Crystal violet working solution: 25 mL 1% crystal violet stock solution in 45 mL absolute ethanol and 180 mL distilled water. Store at room temperature until needed.
7. Phenol red: 0.1% phenol red in distilled water. Store at 4 °C.
8. 0.1% phenol red solution: 6 mL 0.5% phenol red diluted in tissue culture grade water.
9. PBS sample diluent: PBS + 1% pen/strep, 1% L-glutamine, 1% FBS, 0.01% phenol red. To 100 mL PBS, add 1 mL 100× penicillin/streptomycin, 1 mL 100× L-glutamine, 1 mL FBS, 1 mL 0.1% phenol red.
10. WHO hard water (400 ppm): 3.622 mmol calcium chloride +1.4494 mmol magnesium chloride in 1 L water, acceptable pH 6.50–7.50, filtered. Dissolve 0.402 g calcium chloride in 100 mL tissue culture grade water. Dissolve 0.138 g magnesium chloride in 100 mL tissue culture grader water. Combine calcium chloride and magnesium chloride solutions, and make up to 1 L with polished water.
11. 1× phosphate-buffered saline (PBS).
12. 10, 25 mL serological pipettes.
13. Pipettes and aerosol-resistant pipette tips.
14. 42 °C shaker incubator.
15. 37 °C CO_2 incubator.

3 Method

Propagation and seeding of Vero cells should be performed under aseptic conditions. Plates should be transferred to a BSL-3Ag facility; the facility should be risk assessed and able to handle ASFV. All work should be carried out within a biological safety cabinet (BSC) using personnel trained to handle BSL-3Ag viruses. ASFV strain BA71V is used as a reference virus control; other ASFV strains may be used provided they produce plaques in Vero cells.

3.1 Preparation of Vero Cell Plates and Virus

1. Prepare Vero cells in cell culture media. Seed appropriately sized flasks at a density to ensure full confluence on day of plating. Cells are plated in six-well plates, in growth media at a seeding density of 1.5×10^5 cells/mL for use after 24 h or 1.0×10^5 cells/mL for use after 48 h. Cells should be 70–90% confluent on day of use.
2. A T-175 flask will provide approximately 20 virus aliquots (approx. 20 mL). Obtain a 1 mL aliquot of previously propagated ASFV BA71V.
3. Remove culture medium from Vero cells.

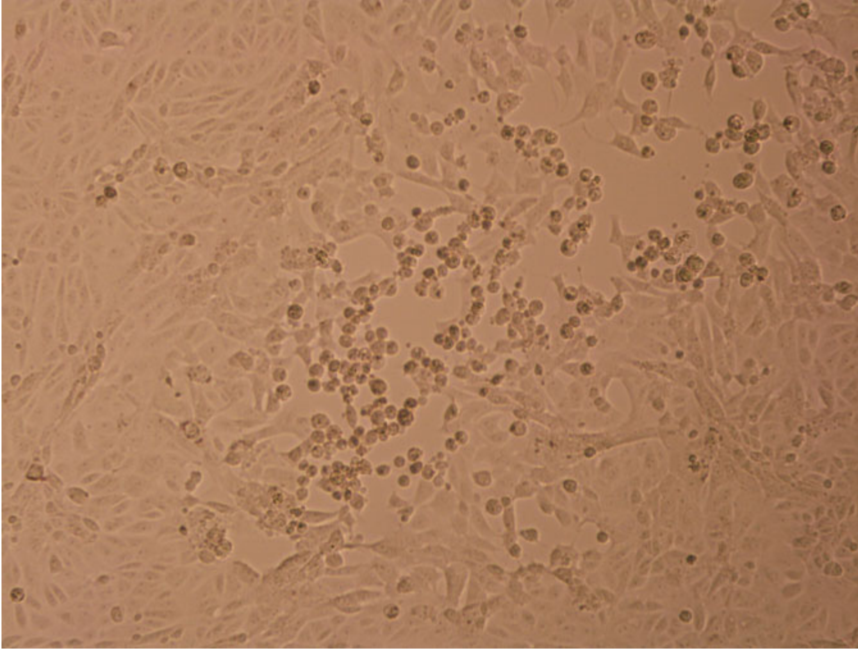

Fig. 1 Propagation of ASF reference virus control BA71V on Vero cells, image showing cytopathic effects (CPE); the virus is ready for harvest when 100% CPE is observed

4. Using aseptic technique and a 10 mL serological pipette, wash the cell monolayer twice with approximately 10 mL PBS. Rock the flask to wash the cells and gently pour off PBS.
5. Add 1 mL of ASFV BA71V and 20 mL of cell culture media (described in materials section). Place vented lid on flask.
6. Incubate flask at 37 °C, 5% CO_2 and observe regularly for cytopathic effect (CPE) (Fig. 1).
7. ASFV should be harvested when extensive CPE is observed, i.e., the cells have detached from the flask, typically around 72 h after inoculation.
8. Pipette the infected cell lysate into a centrifuge tube, e.g., 25 mL conical tube, and centrifuge at $1000 \times g$ for 10 min at 4 °C to remove cell debris.
9. Collect 18 mL of supernatant from each tube and store at 4 °C.
10. Resuspend the remaining 2 mL and cell pellet by vortexing.
11. The resuspended cell pellet should be freeze-thawed three times at −80 °C, vortexing between each freeze/thaw cycle.
12. Following freeze/thaw cycles, the resuspended cell pellet should be resuspended by vortexing and subject to a further centrifugation at $1000 \times g$ for 10 min at 4 °C.
13. Combine the supernatant with the 18 mL stored at 4 °C, and mix using a serological pipette.

14. Adequately label 1.5 mL cryovials, and aliquot 1 mL supernatant to each cryovial.
15. Store aliquots at −80 °C until required.

3.2 Plaque Assay

1. Obtain previously seeded six-well plates, and transfer into 37 °C, 5% CO_2 incubator within the BSL-3Ag facility.
2. Prepare overlay medium, and place in a conical flask in 42 °C shaking incubator until needed.
3. Samples are titrated in individual glass bijous. Add 0.9 mL of PBS sample diluent to labelled glass bijous (*see* **Note 1**), ensuring enough bijous are available to serially dilute to the maximum dilution required (*see* **Note 2**). Place bijous at 4 °C for at least 1 h.
4. If performing a titration of ASF reference virus control BA71V or an unknown ASF virus, skip steps 5–11.
5. If performing a disinfectant test, place a cool block within the MBSC, and adjust to required temperature.
6. Label microcentrifuge tubes for disinfectant test samples, ASF virus-positive control (*see* **Note 3**), formaldehyde control, disinfectant control (*see* **Note 4**), and neutralization control. Add 800 μl WHO hard water to each microcentrifuge tube with an additional 100 μl WHO hard water in the virus control-labelled tube.
7. Disinfectants should be diluted to 10× the intended final dilution in WHO hard water (*see* **Note 5**).
8. Transfer 100 μl of 10× disinfectant to appropriate microcentrifuge tubes for testing, in addition to one microcentrifuge tube for a disinfectant control and one for a neutralization control (*see* **Note 6**).
9. Prepare 0.56% formaldehyde in WHO hard water, and transfer 100 μl microcentrifuge tube labelled formaldehyde control (*see* **Note** 7).
10. Add 100 μl cell culture media to microcentrifuge tubes labelled disinfectant and neutralization control.
11. Obtain ASF reference virus control BA71V stock stored at −80 °C and thaw. Transfer 100 μl to each microcentrifuge tube except those labelled disinfectant and neutralization control. A timer should be set to the appropriate contact time to begin immediately once the virus stock is added to the first tube (*see* **Note 8**).
12. Remove six-well cell culture plates from the incubator, label appropriately, and remove medium from each well by pipetting.

13. Wash plates once with PBS with a volume 60–70% of the original cell culture medium volume. Leave cells in PBS until ready to transfer sample.
14. Following contact time, transfer 100 μl of contents of each microcentrifuge tube to each appropriately labelled bijou, to make a 10^{-1} dilution. To the neutralization control bijou, additionally transfer 100 μl of ASF virus-positive control after transferring from the neutralization microcentrifuge tube, and mix by pipetting.
15. With a new pipette tip, transfer 100 μl of the 10^{-1} dilution to the next bijou, to make a 10^{-2} dilution, and mix by pipetting.
16. Using a new pipette tip for each transfer, continue to serially transfer 100 μl until maximum dilution, and mix by pipetting.
17. Remove PBS from six-well plates by pipetting.
18. Transfer 200 μl of each serially diluted sample to triplicate wells of a six-well plate for each log dilution, and rock back and forth thoroughly to ensure even and complete spread of inoculum over well.
19. Place the lid on the plate and incubate for 1 h in 37 °C, 5% CO_2 incubator.
20. At the end of the 1 h incubation, transfer the six-well plates to the MBSC, and rock plates again.
21. Using a serological pipette, transfer 2 mL overlay medium to each well, ensuring that the overlay is mixed periodically throughout the transfer time.
22. Return the plates to 37 °C, 5% CO_2 incubator for 6 days.
23. Following incubation, remove overlay by pipetting, and discard to suitable disinfectant.
24. Add a volume of crystal violet stock solution to cover the entire well, and leave stain for 30 min.
25. Remove the stain by pipetting and discard to suitable disinfectant, and rinse the cell sheet with tap water to ease plate reading.
26. Remove plates from MBSC and observe for plaques (Fig. 2).
27. Calculate titers: $\text{Pfu/mL} = \log^{10}(\text{number of plaques}/3) \times 5 \times$ serial dilution.

3.3 Result Determination

1. ASF virus-positive control – titer calculated using above equation (*see* **Note 9**).
2. Disinfectant log reduction – titer of ASF virus-positive control minus titer of disinfectant test sample. $4\log^{10}$ reduction = 99.99% reduction, 3 $\log^{10}$ reduction = 99.9% reduction, 2 $\log^{10}$ reduction = 99.0% reduction, 1 $\log^{10}$ reduction = 90.0% reduction.

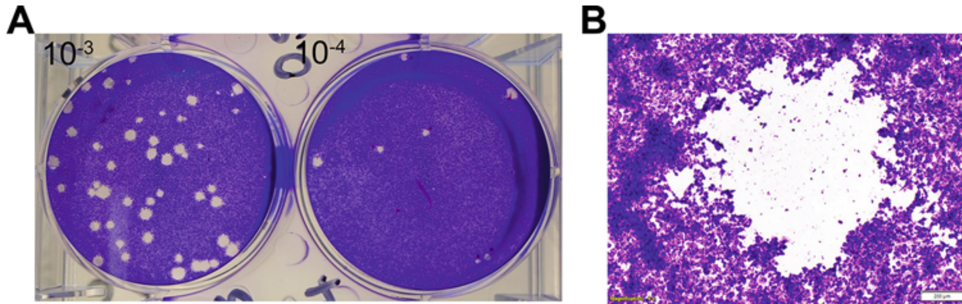

Fig. 2 (**a**) Wells of a six-well plate showing tenfold dilution of ASF reference virus control BA71V, plaques visualized at 10^{-3} (left) and 10^{-4} (right) dilution. (**b**) Appearance of plaque at higher magnification. The monolayer is stained with crystal violet, which stains living cells; dead cells are unstained, which can be seen as the plaque

3. Formaldehyde control reduction – titer of ASF virus-positive control minus titer of formaldehyde control. Should be determined for each batch of formaldehyde and reduction maintained as no greater than 2 standard deviations from mean.
4. Neutralization control – difference between ASF virus-positive control titer and neutralization control titer should not exceed $0.5\log^{10}$. If difference is greater than $0.5\log^{10}$, contact time cannot be determined.
5. Disinfectant control – determination of cytotoxicity of disinfectant under test.

4 Notes

1. If performing a disinfectant test, label bijous suitably for titration of disinfectant test sample, ASF virus-positive control, formaldehyde control, neutralization control, and disinfectant control. Only add 0.8 mL (instead of 0.9 mL) of PBS sample diluent to the first bijou labelled neutralization control.
2. Maximum dilution required should be re-evaluated after growth of all working stock of ASF reference virus control BA71V (or suitable alternative), and range adjusted depending on titer of virus control.
3. The ASF virus-positive control is a 1/10 dilution of ASF reference virus control BA71V.
4. The disinfectant control is the intended final dilution of the disinfectant without the addition of virus. This control will be used to evaluate the cytotoxicity of the disinfectant.
5. Fetal bovine serum (FBS) or bovine albumin can be added to WHO hard water as a soiling agent.

6. For each dilution tested for each disinfectant, there should be a disinfectant control prepared to assess cytotoxicity of the disinfectant. A neutralization control should also be prepared to confirm the disinfectant is effective within the contact time of the test and does not continue to decrease the virus titer throughout the remainder of the testing.
7. A formaldehyde control should be prepared for each test to ensure consistent results between tests performed at different times.
8. Consumables from this step onwards should be discarded to a suitable concentration of disinfectant, validated for use with ASFV. Some national authorities have created approval lists for ASFV disinfectants, for example, USA: USDA document – Disinfectants Approved for Use Against African Swine Fever Virus in Farm Settings (https://www.aphis.usda.gov/animal_health/emergency_management/downloads/asf-virus-disinfectants.pdf). Alternatively, the laboratory may validate a disinfectant internally by proving the disinfectant effectively inactivates ASFV.
9. The titer of the ASF virus-positive control should be as high as possible – ideally above $5.5\log^{10}$. This would ensure that the disinfectant can demonstrate at least a $4\log^{10}$ reduction within the test.

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Chapter 15

Bioorthogonal Labelling of African Swine Fever Virus-Infected Cells

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Abstract

Bioorthogonal labelling of living cells enables the incorporation of small, chemically inert units (alkynes or azides) into nascent chains of biomolecules allowing the tracking of DNA synthesis, transcription, and translation in a temporal-spatial manner without compromising their integrity. This chemical labelling method can be used to replace traditional radiolabelled nucleosides, ribonucleosides, or amino acids with the added benefit of enabling visualization using confocal or super-resolution microscopy. Here, we outline our recently published methods for labelling nascent DNA and polypeptides in cells infected with African swine fever virus.

Key words African swine fever, Bioorthogonal labelling, Click chemistry, Virus, Confocal microscopy

1 Introduction

African swine fever virus (ASFV) is a large double-stranded DNA virus with a complex multi-step replication cycle. After a poorly understood initial nuclear step [1], ASFV replicates mainly in perinuclear regions in the cytoplasm and viral genes do not contain introns [2]. ASFV encodes its own transcriptional machinery that is packaged into the virion allowing transcription immediately after virus entry. Gene expression occurs in four stages: immediate-early and early before the onset of viral DNA replication, intermediate, and late afterwards additionally dependent on early viral protein synthesis [3]. Most of viral replication and the entire assembly and morphogenesis process of ASFV virions happens at perinuclear regions, so-called viral factories. An accumulation of membrane precursors at perinuclear regions represents the first stages of factory formation. Different components of the host cell such as mitochondria, cytoskeleton, ribosomes, and different membranous compartments are drastically rearranged around and recruited to

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the viral factories to support the viral replication processes [4]. Finally, newly synthesized ASFV particles are transported along microtubules to the plasma membrane (PM) [5] and exit the cell by budding or are propellered out of the cell on actin projections [6]. Many of these processes have been studied biochemically with radiolabelled amino acids as well as through in situ hybridization using radiolabelled nucleosides; parallel studies using confocal microscopy and electron microscopy have uncovered additional information on the localization of these processes, particularly with respect to morphogenesis. Bioorthogonal labelling, sometimes referred to as click chemistry, allows to combine these two approaches, enabling the visualization of protein and nucleic acid synthesis in combination with high-resolution microscopy techniques [7, 8]. We have adapted these methods to ASFV [9] and present protocols for labelling infected cells with amino acid and nucleic acid analogues as well as methods to detect them by immunofluorescence and in cell lysates.

2 Materials

All Protocols

1. Biological safety cabinet.
2. Vero cells.
3. Culture media (Dulbecco's Modified Eagle's medium (DMEM) with glutamine supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin).
4. Virus media (Dulbecco's Modified Eagle's medium (DMEM) with glutamine supplemented with 2% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin).
5. Phosphate-buffered saline, divalent cation-free (PBS).
6. Serological pipettes and pipette gun.
7. Pipettor and aerosol-resistant tips.
8. 2 ml screw-cap tubes.
9. Sterile deionized water.
10. 50 mM copper(II)sulfate ($CuSO_4$) in water.
11. 10 mM tris(3-hydroxypropyl-triazolylmethyl)amine (THPTA) in DMSO.
12. Fresh 1 M sodium L-ascorbate in water (*see* **Note 1**).
13. 1 M aminoguanidine hydrochloride in water.
14. Vortex.

2.1 Protein Labelling for Microscopy

1. Protein Labelling Media: L-methionine-free DMEM supplemented with 2% dialyzed fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin (*see* **Note 2**).
2. L-Homopropargylglycine (HPG).
3. Glass coverslips (13 mm diameter).
4. 24-well plates.
5. PFA: 4% formaldehyde solution in PBS (*see* **Note 3)**.
6. Water bath set to 37 °C.
7. Airtight plastic box.
8. Permeabilization solution: 0.2% Triton X-100 in PBS.
9. Plate shaker.
10. Blocking solution: 10% normal goat serum, 0.2% cold water fish skin gelatin, 50mM Tris, 150 mM sodium chloride, pH 7.5.
11. Primary anti-virus or anti-cellular markers and appropriate secondary antibodies conjugated to fluorescent dyes (*see* **Note 4**).
12. Lightproof box, or aluminum foil to protect plate from light.
13. Dye azide: 10 mM dye conjugated to 5-carboxamido-(6-azidohexanyl), bis(triethylammonium) salt diluted in DMSO (*see* **Note 5**).
14. Nuclear dye: 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in water or 1 μM To-Pro-3 in PBS.
15. Glass microscope slides.
16. Antifade mount suitable for confocal or super resolution microscopy as appropriate.

2.2 Labelling Protein Lysates

1. 6-well plates.
2. Protein labelling media: L-methionine-free DMEM supplemented with 2% dialyzed fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin (*see* **Note 2**).
3. L-Homopropargylglycine (HPG).
4. Lysis buffer: 1% sodium dodecyl sulfate, 250 U/ml Benzonase, protease inhibitor cocktail in PBS.
5. 2 ml polypropylene tubes.
6. 1 ml syringe.
7. Ice.
8. Refrigerated microcentrifuge.
9. −80 °C freezer.
10. Bicinchoninic acid (BCA) assay kit.
11. TCEP: 2 mM tris(2-carboxyethyl)phosphine hydrochloride in water.

12. Biotin azide: 10 mM PEG4 carboxamide-6-azidohexanyl biotin in DMSO (*see* **Note 5**).
13. Aluminum foil.
14. Rotary shaker.
15. EDTA stop: 0.5 M ethylenediaminetetraacetic acid, sodium salt diluted in water, pH 8.0.
16. Chloroform.
17. Methanol.
18. Sample loading buffer: 62.5 mM Tris-HCl, pH 6.8, 2.5% SDS, 0.002% bromophenol blue, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol.
19. SDS-PAGE apparatus (*see* **Note 6**).
20. Immunoblot apparatus (*see* **Note 6**).

2.3 DNA Labelling

1. 5-Ethynyl-2′-deoxycytidine (EdC) or 5-ethynyl-2-′-deoxyuridine (EdU).
2. Glycine quench: 100 mM glycine in PBS.
3. Glass coverslips, 13 mm diameter.
4. 24-well plates.
5. PFA: 4% formaldehyde solution in PBS (*see* **Note 3**).
6. Water bath set to 37 °C.
7. Airtight plastic box.
8. Permeabilization solution: 0.2% Triton X-100 in PBS.
9. Plate shaker.
10. Blocking solution: 10% normal goat serum, 0.2% cold water fish skin gelatin, 50mM Tris, 150 mM sodium chloride, pH 7.5.
11. Primary anti-virus or anti-cellular markers and appropriate secondary antibodies conjugated to fluorescent dyes (*see* **Note 4**).
12. Lightproof box, or aluminum foil to protect plate from light.
13. Dye azide: 10 mM dye conjugated to 5-carboxamido-(6-azidohexanyl), bis(triethylammonium salt) diluted in DMSO (*see* **Note 5**).
14. Nuclear dye: 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in water or 1 μM To-Pro-3 in PBS.
15. Glass microscope slides.
16. Antifade mount suitable for confocal or super resolution microscopy as appropriate.

3 Methods

All steps prior to fixation or cell lysis should be carried out within a biological safety cabinet using aseptic technique. Follow your laboratory's procedures and protocols for the safe handling and storage of African swine fever virus (*see* Chapter 1).

3.1 Bioorthogonal Labelling of Newly Synthesized Protein in ASFV-Infected Cells for Microscopy

1. Prepare subconfluent Vero cells grown on glass coverslips in 24-well plates.
2. Remove media from cells, and add sufficient Ba71v diluted in virus media to achieve a multiplicity of infection of at least 3 infectious units per cell and return plates to incubator. Allow virus to enter cells, typically 1 h is sufficient, then remove inoculum and replace with fresh virus media.
3. Allow infection to proceed until desired time point, i.e., to visualize virus factories 12–18 h.
4. Remove medium, wash cells twice with PBS and replace with Protein Labelling Media, return to incubator, and leave for 45 min to starve the cells.
5. Add fresh met-free media containing 0.5 mM HPG, return to incubator, and leave for 30 min (*see* **Note 7**).
6. *Optional.* For a pulse-chase experiment. Remove HPG-containing media and replace with fresh virus media (*see* **Note 8**).
7. Remove media from cells and wash twice with PBS. Add 4% PFA and incubate for 30 min at room temperature. Put the plate in an airtight box and then float on water bath set to 37 °C, and incubate for another 30 min. At this point the virus on the coverslip can be considered inactivated (*see* **Note 9**).
8. Wash coverslips three times with PBS and then add 500 μl permeabilization solution (*see* **Note 10**), and incubate for 15 min with shaking at room temperature.
9. Wash coverslips three times with PBS and then add 500 μl blocking buffer, and incubate for 60 min with shaking at room temperature.
10. Add primary antibodies diluted in blocking buffer, and incubate for 30 min with shaking at room temperature.
11. Wash coverslips three times with PBS, place one shaker for at least 5 min at room temperature, and then wash three times with PBS.
12. Add secondary antibodies diluted in blocking buffer, and incubate for at least 20 min with shaking at room temperature in the dark.

13. Wash coverslips three times with PBS, place one shaker for at least 5 min at room temperature, and then wash three times with PBS.
14. Prepare fresh 1 M sodium L-ascorbate and then use this to prepare fresh microscopy reaction buffer in the order listed, and vortex after each component is added.
15. Microscopy reaction buffer (PBS containing 0.01 mM dye azide, 1 mM $CuSO_4$, 10 mM sodium L-ascorbate, 10 mM aminoguanidine hydrochloride, and 1 mM THPTA).
16. Vortex for 2 min, and immediately add microscopy reaction buffer to the cells (*see* **Note 11**). Incubate for 2 h with shaking at room temperature in the dark.
17. Wash cells twice with PBS, return to shaker for 5 min, and repeat **step 15** twice more.
18. Rinse cells twice with PBS.
19. Add nuclear dye to cells, and incubate for 15 min with shaking at room temperature in the dark.
20. Rinse cells with water, and mount coverslips on microscope slides with appropriate mounting medium.

3.2 Bioorthogonal Labelling of Newly Synthesized Protein in ASFV-Infected Cells for Immunoblot

1. Prepare subconfluent Vero cells grown in six-well plates.
2. Remove media from cells, and add sufficient Ba71v diluted in virus media to achieve a multiplicity of infection of at least 3 infectious units per cell, and return plates to incubator. Allow virus to enter cells, typically 1 h is sufficient, and then remove inoculum and replace with fresh virus media.
3. Allow infection to proceed until desired time point.
4. Remove medium and replace with met-free media, return to incubator, and leave for 45 min to starve the cells.
5. Add fresh met-free media containing 1 mM HPG, and return to incubator and leave for 60 min (*see* **Note 6**).
6. Place 2 ml screw tubes for each well on ice.
7. Remove HPG dilution from cells, and then wash monolayer twice with PBS. Add 200 μl lysis buffer, incubate plate for 20 min on ice, tilt plate, and collect supernatant into cold 2 ml tube by pipetting up and down.
8. Apply another 100 μl of lysis buffer to each well, and collect remaining cells by pipetting up and down, and add to 200 μl lysate already collected.
9. Vortex tubes thoroughly for 5 min, and then remove insoluble material by centrifugation (15,000 × *g*, 15 min, 4 °C), and transfer to a new tube on ice. Store lysate at −80 °C until ready for the next step.

10. Determine protein concentration using BCA assay, and transfer 100 μg of protein to a fresh 1.5 ml tube wrapped in foil (*see* **Note 12**).
11. Add biotin azide to a concentration of 0.5 mM; vortex (*see* **Note 13**).
12. Add $CuSO_4$ to a final concentration of 1 mM; vortex.
13. Add TCEP to a final concentration of 2 mM; vortex.
14. Add THPTA to a final concentration of 0.2 mM; vortex.
15. Incubate (protected from light) on a rotating mixer for 1.5 h at room temperature.
16. Add EDTA to a final concentration of 10 mM; vortex.
17. Precipitate the proteins by adding 3 volumes of water to the tubes, followed by 4 volumes of methanol, and then 1 volume of chloroform. Mix vigorously by vortexing for 2 min.
18. Separate aqueous, organic, and protein layers by centrifugation (>10,000 × *g*, 5 min, room temperature).
19. The samples should have resolved into three layers. Carefully remove approximately 80% of the top (aqueous layer) and discard. Add 1 ml methanol to each tube. Mix vigorously by vortexing for 2 min.
20. Pellet proteins by centrifugation (>10,000 × *g*, 5 min, room temperature).
21. A pellet should be visible on the side or the bottom of the tube. Remove at least 95% of the supernatant, and then allow to air dry for approximately 10 min (*see* **Note 14**).
22. Resuspend sample in 50 μl sample loading buffer for analysis by SDS-PAGE electrophoresis followed by immunoblotting with a streptavidin conjugate.

3.3 Bioorthogonal Labelling for Nucleic Acids

1. Prepare subconfluent Vero cells grown on glass coverslips in 24-well plates.
2. Remove media from cells, and add sufficient Ba71v diluted in virus media to achieve a multiplicity of infection of at least 3 infectious units per cell, and return plates to incubator. Allow virus to enter cells, typically 1 h is sufficient, and then remove inoculum and replace with fresh virus media.
3. Allow infection to proceed until desired time point, i.e., to visualize virus factories 12–18 h.
4. Wash cells once with PBS.
5. Dilute EdC to a final concentration of 50 μM, or EdU to a final concentration of 5 μM in virus media, and add to cells.
6. Incubate for 30 min at 37 degrees and 5% CO_2.

7. Remove EdC/EdU dilution from the cells, and wash twice with PBS.
8. Add 4% PFA and incubate for 30 min at room temperature. Put the plate in an airtight box, and then float on water bath set to 37 °C, and incubate for another 30 min. At this point the virus on the coverslip can be considered inactivated (*see* **Note 9**).
9. Wash coverslips three times with PBS, and then add 500 μl glycine buffer, and incubate for 5 min at room temperature.
10. Wash coverslips three times with PBS, and then add 500 μl permeabilization solution, and incubate for 15 min with shaking at room temperature.
11. Prepare fresh 1 M sodium L-ascorbate, and then use this to prepare fresh microscopy reaction buffer. Vortex for 2 min and immediately add to the cells (*see* **Note 11**). Incubate for 2 h with shaking at room temperature in the dark.
12. Wash coverslips three times with PBS, place on shaker for 5 min, and then repeat **step 12** twice more.
13. Add 500 μl blocking buffer to each coverslip, and incubate for 60 min with shaking at room temperature.
14. Add primary antibodies diluted in blocking buffer, and incubate for 30 min with shaking at room temperature.
15. Wash coverslips three times with PBS, place one shaker for at least 5 min at room temperature, and then wash three times with PBS.
16. Add secondary antibodies diluted in blocking buffer, and incubate for at least 20 min with shaking at room temperature in the dark.
17. Wash coverslips three times with PBS, place on shaker for at least 5 min at room temperature, and then wash three times with PBS.
18. Add nuclear dye to cells, and incubate for 15 min with shaking at room temperature in the dark.
19. Rinse cells with water, and mount coverslips on microscope slides with appropriate mounting medium.

4 Notes

1. Sodium ascorbate solution and the click chemistry reaction buffers need to be prepared fresh each time. Prepare click reaction buffers in the order in which they are written.
2. It is crucial that dialyzed FCS for the starvation and labelling with HPG as standard FCS may contain traces of methionine. This is readily available from many suppliers.

3. Best results are achieved using freshly thawed homemade 4% formaldehyde solutions. Commercial formaldehyde solutions are typically stabilized with methanol and in our hands do not perform as well as the solutions we prepare ourselves.
4. The choice of primary and secondary antibodies is entirely down to the researcher. We achieved good results with bioorthogonal labelling when using both viral and cellular markers and using primary antibodies raised in mice and rabbits, as well as with serum from ASFV-recovered pigs.
5. Prepare single-use aliquots and store at −20 °C. We have successfully used Alexa 488- and Alexa 555-conjugated azides for microscopy.
6. The choice of SDS-PAGE and immunoblotting protocols is down to the researcher; we have used the bis-Tris system for SDS-PAGE and wet-blotting for transferring proteins to membranes.
7. For analysis by microscopy, we have successfully detected newly synthesized proteins using incubation periods ranging from 10 min to 1 h, but we settled on a time of 30 min for most of our work. For analysis by in-gel fluorescence or immunoblot, we have incubated with 1 mM HPG for 60 min; however, it is likely that other conditions may be used.
8. We successfully incorporated HPG to nascent virions by pulsing for 60 min and then incubating in fresh virus media for 3 h.
9. Typically coverslips are fixed in formaldehyde for 1 h at room temperature; however, a detailed study suggested this may not be sufficient for reproducible and complete inactivation of the prototype poxvirus vaccinia, whereas 30 min at room temperature followed by 30 min at 37 °C is [10]. A risk assessment for the transfer of samples out of primary containment should consider the possible presence of virus on the parts of the plate that were not in contact with formaldehyde.
10. **Steps 8** through **13** are our standard protocol for indirect immunofluorescence which we have used in our experiments for bioorthogonal labelling; it is likely that other protocols will be equally successful. If you only wish to view the localization of the bioorthogonal label, then **steps 10** to **13** can be skipped.
11. The reaction works best when CuSO4 levels are at their highest, which will start to decrease once all of the components are added to the buffer.
12. The volumes of each reagent added between **steps 8** to **11** of Subheading 3.2 will depend on the concentration of protein in each sample; therefore, preparation of a spreadsheet to rapidly calculate the volumes required will greatly speed up this process. Alternatively adjust the volumes of the lysates to match the most dilute, although this will use up more of the reagents.

13. A dye-conjugated azide can be used in place of biotin-azide if your laboratory has the capacity to read in-gel fluorescence; we describe biotin here as immunoblotting is more widely available. For in-gel fluorescence, we have successfully used a dye-azide conjugate at a final concentration of 0.1 mM in these reactions.
14. Do not overdry the protein pellet, but ensure that all of the organic solvents have evaporated.

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Chapter 16

Whole Genome Sequencing of African Swine Fever

Ha Thi Thanh Tran, Anh Duc Truong, and Hoang Vu Dang

Abstract

Next-generation sequencing (NGS) technologies have been powerfully applied in both research and clinical settings for the understanding and control of infectious disease. It enables high-resolution characterization of viral pathogens in terms of properties that include molecular epidemiology, genotype, serotype, and virulence. However, a beginner's NGS protocol for characterization of African swine fever virus (ASFV) is lacking. Here, we present detailed step-by-step methods for obtaining NGS data from ASF virus (ASFV) using the Illumina platform. The protocol has been performed with respect to ASFV DNA genome extraction, qualification of DNA, library preparation, quality control, de novo assembly, and data quality control. The protocol represents a step-by-step and reproducible method for producing high-quality sequencing data. The key advantages of this protocol include the protocol being very simple for users with no experience of genome sequencing and reproducibility of the protocol for other DNA genome viruses.

Key words Next-generation sequencing, Whole genome sequencing, African swine fever, DNA genome virus, Genome extraction

1 Introduction

African swine fever (ASF) is a fatal viral disease that affects pigs of all ages and breeds that is reportable to the World Health Organization for Animal Health (OIE). ASF virus (ASFV) is highly virulent and remains a global threat because of the lack of a vaccine or drugs and the ability of the virus to survive in various environmental conditions [1]. Additionally, as a highly contagious virus, ASFV infection of pigs typically results in up to 100% of morbidity, and the mortality of ASF is dependent on the virulence of the virus, the host, and transmission cycles [2]. Since the first confirmed outbreak of ASFV in China in 2018, the disease has spread to Mongolia, Vietnam, Cambodia, Hong Kong, Korea (Dem People's Rep. of), Laos, Myanmar, Philippines, Korea (Rep. of), Timor-Leste,

Ha Thi Thanh Tran and Anh Duc Truong contributed equally with all other contributors.

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Indonesia, Papua New Guinea, India, and Malaysia [3]. From early February 2019, an ASF outbreak in Vietnam was reported officially, and within 7 months, ASF had spread across the whole country. Approximately six million pigs on infected farms and households have been culled, indicating the socioeconomic impact on the pig industry [4, 5].

So far, approximately 120 near-complete genome sequences (including unverified ones and sequences from patents) have been deposited into the GenBank [6]). Scientists who try to produce additional complete sequences of biologically relevant ASFV strains are frequently thwarted by technical problems, such as the purity of ASFV DNA and applying the most suitable next-generation sequencing (NGS) method for their production. To further research on epidemiological investigations, advance research, and vaccine development, it should have a simple method for full genome sequencing of the ASFV. A number of different next-generation sequencing platforms have been used successfully to generate reads suitable for ASFV genome assembly [7–11]. Here we describe an easy-to-follow, step-by-step protocol for preparing ASFV DNA suitable for sequencing using Illumina's technology which we have used to generate genomes of isolates of ASFVs from seven agricultural regions of Vietnam.

2 Materials

2.1 DNA Extraction

1. Biological safety cabinet.
2. Centrifuge tube 15 mL.
3. Centrifuge tube 50 mL.
4. Tip pipette 10 μL.
5. Tip pipette 1000 μL.
6. Tip pipette 200 μL.
7. Ethanol, 99.9%.
8. 2-Propanol (isopropanol).
9. DNA extraction kit (*see* **Note 1**).
10. ASF samples of interest (*see* **Note 2**).
11. Proteinase K.
12. 1.5 mL or 2 mL screw cap DNA/RNA free tubes.
13. Microcentrifuge (with rotor for 2 mL tubes).
14. Vortexer.
15. Water baths or heating blocks at 70 °C and 56 °C.
16. Spectrophotometer.
17. Nuclease-free water.

2.2 Real-Time PCR

1. qPCR master mix (*see* **Note 3).**
2. Primer 1: 50 pmol/μL in nuclease-free water (5'-CTG-CTC-ATG-GTA-TCA-ATC-TTA-TCG-A-3′ positive strand).
3. Primer 2: 50 pmol/μL in nuclease-free water (5′-GAT-ACC-ACA-AGA-TC(AG)-GCC-GT-3′ negative strand).
4. Probe: 5 pmol/μL in nuclease-free water (5′-[6-carboxy-fluorescein (FAM)]-CCA-CGG-GAG-GAA-TAC-CAA-CC(CY)-AGT-G-3′-[6-carboxy-tetramethyl-rhodamine (TAMRA)]) (*see* **Note 4**).
5. Optical Flat 8-Cap/well strips 0.2 mL.
6. Centrifuge capable of spinning plates.
7. Real-time PCR system.

2.3 Agarose Gel Electrophoresis

1. Erlenmeyer flask.
2. Agarose, electrophoresis grade.
3. 50 × TAE (40 mM tris-acetate, 1 mM EDTA) (*see* **Note 5**).
4. TAE (4 mM Tris-acetate, 0.1 mM EDTA) (*see* **Note 5**).
5. Microwave.
6. Ethidium bromide (*see* **Note 6**).
7. Electrophoresis equipment and power supply.
8. DNA ladder.
9. Gel loading buffer (0.25%(w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 30% (v/v) glycerol in water).

2.4 Library Preparation and Sequencing

1. Library preparation kit (*see* **Note** 7).
2. Sequencing kit (*see* **Note** 7).
3. Flow cell (*see* **Note** 7).
4. Sequencer (*see* **Note** 7).

2.5 Assembly

1. Bioinformatics software applications suitable for removing adaptor sequences and low-quality reads, assembling and manipulating contigs, mapping against reference sequences, and analyzing assembled genomes (*see* **Note 8**).

3 Methods

3.1 DNA Extraction

The use of appropriate aseptic techniques and the wearing of appropriate personal protective equipment are strongly recommended to maintain acceptable work health and safety standards and minimize exposure to harmful agents. All work with live ASFV should be carried out in a class II biological safety cabinet.

Equilibrate all samples and perform all centrifugation steps at room temperature (15–25 °C).

Check for precipitates in buffers ATL and AL and redissolve by incubating at 56 °C if required.

1. Heat two water baths or heating blocks: one to 56 °C for use in step 1 and one to 70 °C for use in step 3 (*see* **Note 8**).
2. Add 20 μL proteinase K to a 1.5 mL screw-cap microcentrifuge tube and then add 200 μL of sample containing ASF virus to 1.5 mL screw-cap microcentrifuge tube.
3. Add 180 μL buffer ATL, mix by vortexing, and then incubate at 56 °C until completely lysed (1–3 h). Vortex occasionally during incubation (*see* **Note 9**).
4. Add 200 μL buffer AL. Mix thoroughly by vortexing for 15 s.
5. Incubate at 70 °C for 10 min. Briefly centrifuge the tube to remove drops from the lid (*see* **Note 10**).
6. Add 200 μL ethanol (96–100%). Vortex for 15 s. Briefly centrifuge the tube to remove drops from the lid.
7. Pipette the mixture onto the QIAamp Mini spin column (in a 2 mL collection tube). Centrifuge at 6000 × g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
8. Place the QIAamp Mini spin column in a new 2 mL collection tube, and add 500 μL buffer AW1. Centrifuge at 6000 × g (8000 rpm) for 1 min. Discard the flow-through and collection tube.
9. Place the QIAamp Mini spin column in a new 2 mL collection tube, and add 500 μL buffer AW2. Centrifuge at full speed (20,000 × g; 14,000 rpm) for 3 min. Discard the flow-through and collection tube.
10. Recommended: Place the QIAamp Mini spin column in a new 2 mL collection tube (not provided), and centrifuge at full speed for 1 min. This eliminates the chance of possible buffer AW2 carryover.
11. Place the QIAamp Mini spin column in a new 1.5 mL microcentrifuge tube, add 200 μL buffer AE or distilled water (*see* **Note 11**), and incubate at room temperature for 1 min. Centrifuge at 6000 × g (8000 rpm) for 1 min to elute the DNA.
12. Optional: Repeat step 10 for increased DNA yield with a further 200 μL buffer AE or distilled water.

3.2 Real-Time PCR to Confirm the Presence ASF DNA After Extraction

Handle and store all reagents, buffers, and plates on ice.

1. In a sterile 1.5 mL microcentrifuge tube, prepare the PCR mixture described below for each sample. Prepare the master mix for the number of samples to be assayed, but allow for one extra sample or add an additional 10% of each reagent.
   (a) 7.5 μL nuclease-free or sterile water.
   (b) 12.5 μL PCR reaction master mix.
   (c) 1 μL Primer 1 (50 pmol).
   (d) 1 μL Primer 2 (50 pmol).
   (e) 1 μL Probe (5 pmol).
2. Add 22 μL PCR reaction mix to one well of an optical reaction plate for each sample to be assayed.
3. Add 3 μL of extracted DNA template or blank extraction control, and securely cover each well with a cap.
4. Spin the plate for 1 min in a suitable centrifuge to mix the contents of each well.
5. Place the plate in a sequence detection system for PCR amplification (real-time PCR machine equipped with FAM fluorescence channel), and run the following program (*see* **Note 12**):
   (a) One cycle at 50 °C for 2 min.
   (b) One cycle at 95 °C for 10 min.
   (c) Forty cycles at 95 °C for 15 s, 58 °C for 1 min.
6. Assign a cycle threshold (Ct) value to each PCR reaction from a scan of all amplification plots (a plot of the fluorescence signal versus cycle number). Negative test samples, uninfected negative, or extraction blank controls should have a Ct value >40.0. Positive test samples and controls should have a Ct value <40.0, and strongly positive samples have a Ct value <30.0 (*see* **Notes 13** and **14**).

3.3 DNA Sample QC Using Electrophoresis

3.3.1 Preparation of the Gel

1. Prepare a 1% (w/v) solution of agarose in TAE buffer in an Erlenmeyer flask. The volume of the buffer should be appropriate to the size of the gel, but should not be greater than 1/3 of the capacity of the flask.
2. Swirl to mix and then melt the agarose/buffer mixture. This is most commonly done by heating in a microwave but can also be done over a Bunsen flame. At 30 s intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved (*see* **Note 15**).
3. Allow the agarose to cool either on the benchtop or by incubation in a 65 °C water bath (*see* **Note 16**).
4. Add ethidium bromide (EtBr) to a concentration of 0.5 μg/mL (*see* **Note 6**). Alternatively, the gel may also be stained after electrophoresis in running buffer containing 0.5 μg/mL EtBr

for 15–30 min, followed by destaining in running buffer for an equal length of time.

5. Place the gel tray into the casting apparatus. Alternatively, one may also tape the open edges of a gel tray to create a mold. Place an appropriate comb into the gel mold to create the wells.
6. Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box. Alternatively, the gel can also be wrapped in plastic wrap and stored at 4 °C until use.

3.3.2 Setting Up of Gel Apparatus and Separation of DNA Fragments

1. Add loading dye to the DNA samples to be separated. Gel loading dye is typically made at 6X concentration (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) (*see* **Note 17)**.
2. Program the power supply to desired voltage (1–5 V/cm between electrodes).
3. Add enough running buffer to cover the surface of the gel (*see* **Note 18**).
4. Attach the leads of the gel box to the power supply. Turn on the power supply and verify that both gel box and power supply are working.
5. Remove the lid. Slowly and carefully load the DNA sample (s) into the gel. An appropriate DNA size marker should always be loaded along with experimental samples.
6. Replace the lid to the gel box (*see* **Note 19**).
7. Turn on the power. Run the gel until the dye has migrated to an appropriate distance.

3.3.3 Observing Separated DNA Fragments

1. When electrophoresis has completed, turn off the power supply, and remove the lid of the gel box.
2. Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.
3. Remove the gel from the gel tray and expose the gel to UV light (*see* **Note 20**). This is most commonly done using a gel documentation system. DNA bands should show up as orange fluorescent bands (Fig. 1). Check for the presence of high molecular weight DNA (lanes 1 to 11 on Fig. 1) (*see* **Note 21**).
4. Properly dispose of the gel and running buffer per institution regulations.

3.4 Spectrophotometric Determination of DNA Concentration

The concentration and purity of DNA samples is another important part of the QC step. The example given below is for a NanoDrop 2000c; other spectrophotometers may require slightly different protocols (*see* **Note 22**).

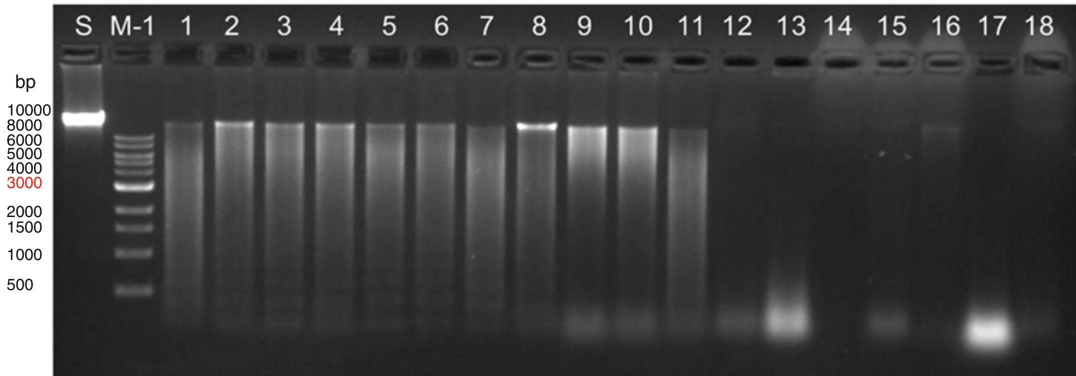

Fig. 1 Example agarose gel electrophoresis QC results. 500 nL ASFV DNA were subjected to electrophoresis for 40 min at 100 V in a 1% agarose gel in TAE buffer. Remarks: S, standard sample (50 ng); M-1, NEB 1 kb DNA ladder; Samples 1–18

1. To begin, clean the upper and lower optical surfaces of the microvolume spectrophotometer sample retention system by pipetting 2–3 μL of clean deionized water onto the lower optical surface.
2. Close the lever arm, ensuring that the upper pedestal comes in contact with the deionized water. Lift the lever arm and wipe off both optical surfaces with a clean, dry, lint-free lab wipe.
3. Open the NanoDrop software and select the Nucleic Acid application. Use a small-volume, calibrated pipettor to perform a blank measurement by dispensing 1 μL of buffer onto the lower optical surface. Lower the lever arm and select "Blank" in the Nucleic Acid application.
4. Once the blank measurement is complete, clean both optical surfaces with a clean, dry, lint-free lab wipe.
5. Choose the appropriate constant for the sample that is to be measured.
6. Dispense 1 μL of nucleic acid sample onto the lower optical pedestal and close the lever arm. Because the measurement is volume independent, the sample only needs to bridge the gap between the two optical surfaces for a measurement to be made.
7. Select "Measure" in the application software. The software will automatically calculate the nucleic acid concentration and purity ratios. Following sample measurement, review the spectral output.
8. The software will automatically calculate the nucleic acid concentration and purity ratios. Following sample measurement, review the spectral image to assess sample quality.

9. To accurately assess sample quality, 260/280 or 260/230 ratios should be analyzed in combination with overall spectral quality. Pure nucleic acids typically yield a 260/280 ratio of 1.8 ~ 2.0 for DNA. The 260/230 purity ratio is a second measure of DNA purity with values for a "pure" nucleic acid commonly in the range of 2.0–2.2. Purity ratios that are significantly lower than the expected values may indicate the isolation technique used may require further optimization (*see* **Notes 23 and 24**).

3.5 Illumina Library Prep and Sequencing

Protocols for library preparation and sequencing will be dependent on the particularities of the kits and sequencers, and if the readers are running their own samples, they should follow these protocols closely (*see* **Note 7**). The following is an overview of the process.

1. Genomic DNA is randomly sheared into short fragments.
2. Fragments are end repaired, A-tailed, and then ligated to Illumina adapters.
3. The fragments with adapters are PCR amplified, size selected, and then purified.
4. Quality control performed on the library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection.
5. Quantified libraries will be pooled and sequenced on Illumina platforms, according to effective library concentration and data amount required.

3.6 Assembly

Many different bioinformatics tools are available, and the following is a simplistic overview of the process (*see* **Note 8**). Generating high-quality ASFV genome sequences can be difficult due to the presence of repeat regions and homopolymers, and care must be taken at all stages, preferably by comparison to a known reference sequence.

1. Remove low quality reads and reads containing adapters (e.g., BBTools).
2. Assemble filtered reads into contigs (e.g., SPAdes).
3. Compare contigs to reference sequences (e.g., VBRC).

4 Notes

1. We use the QIAamp DNA mini kit to extract nucleic acid from our samples following a modified protocol; however, other commercially available extraction kits may be just as effective.
2. Host contamination is a particularly acute issue for ASFV whole genome sequencing; therefore, it is critical that your

sample contains a large amount of viral sequence. The sample should have a titer of at least 10^6 HAD50/mL and preferably higher. Concentration of tissue culture supernatants by centrifugation can help (*see* Chapter 13). We cultured and titered virus on porcine pulmonary alveolar macrophages (*see* Chapter 4).

3. We use the PerfeCTa Multiplex qPCR SuperMix from Quantabio; however, other reagents are likely to be suitable. Volumes of reagents and/or primer concentrations may need to be varied appropriately for other reagents.
4. The nucleotides sequences of primer and probe were listed above that are based on OIE protocol [12] or our modified protocol [13] for diagnosis of ASFV.
5. Make sure to use the same buffer as the one in the gel box (do not mix different buffers and do not use water). TAE can be substituted for 50× TBE (45 mM Tris-borate, 1 mM EDTA) if preferred.
6. EtBr is a suspected carcinogen and must be properly disposed of per institution rules. Gloves should always be worn when handling gels containing EtBr. Alternative dyes for the staining of DNA are available; however, EtBr remains popular one due to its sensitivity and cost.
7. Acquisition and maintenance of next-generation sequencing platforms can be very expensive, and if these are unavailable in your institution or difficult to access, many companies offer sequencing as a service which can be very cost-effective. We have successfully generated data from ASFV DNA samples using a commercial company who employed Illumina's NovaSeq 6000 system, to generate 150 bp paired end reads with the NovaSeq XP 4-Lane Kit v1.5 after library prep with NEBNext® Ultra™ IIDNA Library Prep Kit.
8. A whole range of different bioinformatics applications are available, and many will be suitable for analyzing ASFV NGS data. For example, BBTools available from the Joint Genome Institute can be used to trim reads, SPAdes can be used to assemble contigs, and these can then be manipulated using Geneious Prime. Analysis of final, assembled sequences can be undertaken using the applications available at the Viral Bioinformatics Research Centre [14].
9. The method that follows was optimized for preparation of viral DNA from tissue culture supernatant; refer to the handbook that comes with your kit for variations that may be appropriate to other sample types if required.
10. Do not add proteinase K directly to buffer ALT, add proteinase K first to the sample, and then add ATL.

11. The combination of proteinase K, guanidine hydrochloride, sodium dodecyl sulfate, and heating at 70 °C will likely inactivate ASFV; however, this should be tested as required by your institutional biosafety protocols (*see* Chapter 14).
12. For long-term storage of DNA, elute in Buffer AE in preference to water and place at −30 to −15 °C.
13. If a purpose-built thermal cycler is not available, an ordinary thermal cycler can be used and the PCR products analyzed by endpoint fluorescence readers or alternatively by electrophoresis on a 1.5% agarose gel. A product of 250 bp is expected.
14. Depending on the real-time PCR system, the Ct value, quantification cycle (Cq), and crossing point (Cp) were used as a same value.
15. Real-time quantitative PCR can be used to determine the precise number of copy numbers (*see* Chapter 6), and this could be compared to a similar qPCR assay targeting a host target to estimate contamination with swine DNA.
16. Wear eye protection and heat-proof gloves. Best practice is to microwave for 30–45 sec, stop and swirl, and then continue towards a boil. Keep an eye on it; the solution has a tendency to boil over.
17. Pouring boiling agarose solution is likely to warp the gel tray.
18. Loading dye helps to track how far your DNA sample has traveled and also allows the sample to sink into the gel.
19. It is important to use the same running buffer as the one used to prepare the gel.
20. Electrophoresis apparatus may be designed such that it is impossible to connect the cathode and the anode incorrectly; however, if not, 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.
21. When using UV light, protect your skin by wearing safety goggles or a face shield, gloves, and a lab coat.
22. Automated electrophoresis systems can also be used for analysis of DNA samples.
23. 260/280 nm readings are an important measure of sample quality; however, fluorometers can give more accurate measures of the concentration of DNA.
24. For next-generation sequencing, contaminant-free, high-molecular weight DNA with an absorbance (260 nm/280 nm) ratio between 1.8 and 2.0 is considered high-quality template.

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