UNOFFICIAL TRANSLATION

THAI AGRICULTURAL STANDARD

TAS 10400-2012

DIAGNOSIS OF FOOT AND MOUTH DISEASE

National Bureau of Agricultural Commodity and Food Standards
Ministry of Agriculture and Cooperatives
50 Phaholyothin Road, Ladyao, Chatuchak, Bangkok 10900
Telephone (662) 561 2277 www.acfs.go.th

Published in the Royal Gazette (General) Vol. 129, Special Section, 1734. (Ngo),
dated 16 November B.E.2555 (2012)
Technical Committee on the Elaboration of Thai Agricultural Standard for Diagnosis of Foot and Mouth Disease

1. Ms. Chawewan Viriyapak  
   Department of Livestock Development  
   Chairperson

2. Mr. Chaisiri Mahantachaisakul  
   National Bureau of Agricultural Commodity and Food Standards  
   Member

3. Ms. Noppawan Buamithup  
   Bureau of Disease Control and Veterinary Services,  
   Department of Livestock Development  
   Member

4. Mr. Panithan Thongtha  
   National Institute of Animal Health,  
   Department of Livestock Development  
   Member

5. Associate Professor Kittisak Ajariyakhajorn  
   Faculty of Veterinary Science, Chulalongkorn University, and  
   Thai Association of Veterinary Laboratory Diagnosticians  
   Member

6. Ms. Patchara Paugthes  
   Faculty of Veterinary Medicine, Kasetsart University  
   Member

7. Associate Professor Jatuporn Kajaysri  
   Faculty of Veterinary Medicine, Mahanakorn University of Technology  
   Member

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   Faculty of Veterinary Medicine, Mahidol University  
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    Swine Raisers Association of Thailand  
    Member

12. Mr. Viraj Viboonsirikul  
    Thai Swine Veterinary Association  
    Member

13. Ms. Wilai Linchongsubongkoch  
    Member

14. Ms. Kwanhatai Thongpalad  
    Office of Standard Development,  
    National Bureau of Agricultural Commodity and Food Standards  
    Member and Secretary
Regarding the Ministry of Agriculture and Cooperatives notification on the Thai Agricultural Standard entitled Diagnosis of Foot and Mouth Disease (TAS 10400-2547) which has been published in the Royal Gazette Vol. 121, special section 120 (Ngo), dated October 22, B.E. 2547 (2004), the said standard is amended to the changing of disease situation. The Agricultural Standards Committee deems it necessary to revise the TAS 10400-2547 to be a laboratory guideline for diagnosis of Foot and Mouth Disease.

This standard is based on the following documents:


NOTIFICATION OF THE MINISTRY OF AGRICULTURE AND COOPERATIVES

SUBJECT: THAI AGRICULTURAL STANDARD:
DIAGNOSIS OF FOOT AND MOUTH DISEASE
UNDER THE AGRICULTURAL STANDARDS ACT B.E. 2551 (2008)

Whereas the Agricultural Standards Committee deems it necessary to establish an agricultural standard for Diagnosis of Foot and Mouth Disease as a voluntary standard in accordance with the Agricultural Standards Act B.E. 2551 (2008) to promote agricultural commodity to meet its quality and safety standards.

By virtue of Section 5, Section 15 and Section 16 of the Agricultural Standards Act B.E. 2551 (2008), the Minister of Agriculture and Cooperatives hereby issues this Notification on Establishment of Thai Agricultural Standard: Diagnosis of Foot and Mouth Disease as follows:

1. The Notification of the National Committee on Agricultural Commodity and Food Standards entitled the establishment of The Thai Agricultural Standard for Diagnosis of Foot and Mouth Disease (TAS 10400-2004), dated 3 August B.E. 2547 (2004) is repealed.

2. The Thai Agricultural Standard for Diagnosis of Foot and Mouth Disease (TAS 10400-2012) is established as a voluntary standard, details of which are attached herewith.

Notified on 24 September B.E. 2555 (2012)

(Mr. Theera Wongsamut)
Minister of Agriculture and Cooperatives
1. SCOPE

This agricultural standard provides essential detail for the diagnosis of foot and mouth disease (FMD) caused by virus of the genus *Aphthovirus*, family Picornaviridae by using laboratory diagnostic methods. This standard is applied to the cloven–hoofed animals and its products.

2. DEFINITIONS

For the purpose of this standard:

2.1 Diagnosis means investigation of the etiology in conjunction with disease diagnosis.

2.2 Specificity of a diagnostic test means the efficiency to identify negative test results of the uninfected samples.

2.3 Sensitivity of a diagnostic test means the efficiency to identify positive test results of the infected samples.

2.4 Positive control set means a test panel containing the standard microbes to be studied for comparison with the unknown samples.

2.5 Negative control set means a test panel that does not contain the standard microbes to be studied for comparison with the unknown samples.

3. DIAGNOSIS

Presumptive diagnosis of FMD can be observed by clinical signs and lesions (Appendix A); however, these clinical observations are unable to confirm the disease caused by FMD virus (FMDV). Therefore, it is necessary to confirm the case of FMD by using laboratory tests and distinguish from other diseases which present similar clinical signs such as swine vesicular disease, vesicular stomatitis or vesicular exanthema.

Laboratory diagnosis for FMD can be performed by several methods. Each method provides different efficacy and purpose of use.

For example, the detection of FMD virus in carrier animals by virus isolation, oesophageal-pharyngeal fluid is collected and inoculated onto cell culture for viral multiplication, then FMDV serotypes are identified by Enzyme-linked immunosorbent assays (ELISA) typing or reverse transcription-polymerase chain reaction (RT-PCR) method.

Detection of antibody against different serotypes of FMDV can be conducted by several methods i.e., liquid phase blocking ELISA (LP ELISA) and virus neutralization test (VNT) which determine the level of antibody against FMDV’s types according to the vaccine’s strains administered or those naturally infected. In addition, the detection of antibody against the non-structural protein (NSP) of FMDV has been developed by the method of indirect ELISA or NSPs-ELISA in serum samples.
Such method is advantageous to precisely differentiate between infected and vaccinated animals. It is recently used as the standard procedure for detecting FMDV worldwide.

3.1 SAMPLE COLLECTION AND TRANSPORTATION

Sample collection shall be appropriate and complied with the procedures and rapidly transport to the laboratory since they are directly affecting the test result. Samples shall be packed according to the biosafety and biosecurity principles. The package shall be maintained in good condition until it arrives at the laboratory in order to prevent the leakage of virus to the surroundings.

Samples collected from lesions of infected or suspected animal, including serum samples are suitable for the diagnosis of FMD. Such samples provide good specificity, sensitivity and high accuracy of the test result.

3.1.1 Tissue samples

(1) Sample the tissue from vesicles or lesions on the tongue epithelium, buccal mucosa, coronary band, interdigital area and udder of infected or suspected animal. For pig, tissue samples may also be collected from snout lesion.

Sampling areas shall be cleaned with clean water. Disinfectants such as tincture iodine and gentian violet that may interfere with the test result are prohibited. At least 1 g of tissue sample shall be obtained. If inadequate amount of tissue is collected from one animal, additional tissue samples shall be obtained from others and shall be collected individually and separately.

(2) Put tissue samples in bottle containing 50% glycerin buffer or other suitable transport media.

- Add the transport medium into the bottle until the tissues are fully submerged.
- Tightly close the tissue bottle cap and seal with tape to prevent leakage.
- Clearly label the bottle.
- Wipe external surface of tissue bottles, packaging boxes and hands of the collector with proper FMDV disinfectants in accordance with the biosafety and biosecurity principles to prevent the spread of virus.

(3) Place the tissue bottles in a specific container containing ice packs. Such container is certified under the requirements of the United Nations (UN) or the International Air Transport Association (IATA) or equivalent (illustrations of the package for shipment are shown in Appendix G). History of infected animal shall also be accompanied with the shipment. Transport the samples to the laboratory as soon as possible.

3.1.2 Vesicular fluid

If unruptured vesicles which are normally found in early stage of infection, vesicular fluid is collected from a vesicle using sterile needle and syringe. Transfer the fluid into a clean bottle and place on ice packs prior to packing and transport to the laboratory.

3.1.3 Oesophageal–pharyngeal (OP) fluid samples

For ruminants, an OP fluid can be collected by using a probang cup to scrape of mucosa from oropharyngeal area of infected animal.
3.1.4 Serum samples

(1) Use dry, clean and sterile needles and blood collecting tubes.

(2) Incline the collecting tubes after drawing blood from animal in order to allow effective separation of serum and platelet. The collecting tubes should be placed in room temperature for at least 1 h.

(3) Transfer at least 2 ml of serum into clean and sterile plastic tube. Avoid mixture of red blood cell or the occurrence of hemolysis. Tube shall be tightly capped and carefully sealed to prevent leakage.

(4) Clearly label the sample tubes. Pack sample tubes in a sealed plastic bag and store on ice packs during transport. If samples need to be withheld for a period of time, they shall be kept in freezer at -20°C.

3.2 LABORATORY DIAGNOSTIC METHODS

Packaging or containers including accompanying documents shall be checked upon the arrival of samples. Examination of diagnostic techniques is selected according to the purpose and sample types as follows;

The detection of viral antigen or genetic materials, with the purposes to confirm suspected cases, early detection of carriers for disease control and surveillance as well as identification of FMDV’s serotypes, consists of indirect double antibody sandwich ELISA, virus isolation/identification and RT-PCR.

The detection of antibody against FMD virus, with the purposes to examine animal health prior to movement including importation and exportation, disease control and surveillance, confirmation of suspected animals, recognition of disease-free status and evaluation of vaccine’s efficacy, is based on two types of viral antigens as follows;

- Viral structural protein (SP) by LP ELISA or virus neutralization test
- Viral non-structural protein (NSP) by indirect ELISA

3.2.1 Sample preparation

Samples of vesicular fluid and serum can be diagnosed directly whilst tissue sample needs to be extracted before diagnosis as follows:

(1) Weigh tissue sample and cut into small pieces using sterile scissors.

(2) Grind the sample in sterile sand in a sterile pestle and mortar.

(3) Add 0.4 M phosphate buffered saline (PBS) in grinded sample giving a 10% suspension (w/v), for example, adding 9 ml of PBS into 1 g of sample.

(4) Centrifuge to clarify the suspension on a refrigerated centrifuge at 700 g for 15-20 min.

(5) Collect supernatant and filter by 0.45 μm syringe filter then transfer filtered supernatant into multiple tubes for further diagnosis.
3.2.2 ELISA typing

3.2.2.1 Principle

ELISA typing is a qualitative assay for the identification of viral serotype by using indirect double antibody sandwich ELISA. Samples for this assay are suspension of tissue extracts and vesicular fluid.

3.2.2.2 Test procedure

(1) Coating of ELISA plate

- Prepare working dilutions of rabbit trapping antibody against the suspected types of FMDV and normal rabbit serum using coating buffer (0.05 M carbonate bicarbonate buffer; pH 9.5) then dispense 50 µl per well of ELISA plate.

- Place coated ELISA plate on an orbital shaker at 200 revolutions per minute (rpm) in a 37°C incubator for 60 min or incubated overnight at 4°C.

(2) Wash the ELISA plate five times with PBS and tap the plate dry.

(3) Prepare control antigen and test sample in microtubes make three 2 fold serial dilution by using ELISA diluent and add into ELISA plate and incubate at 37 °C on microplate shaker at 200 rpm for 60 min.

(4) Wash the ELISA plate five times with PBS and tap the plate dry.

(5) Prepare working dilutions of guinea pig detecting antibody against the suspected types of FMDV and normal guinea pig serum by diluting with blocked diluents (ELISA diluents containing 3% BSA or 5 % skimmed milk) and dispense 50 µl/well of dilution. Place on microplate shaker in a 37°C incubator for 30 min.

(6) Wash the ELISA plate five times with PBS and tap the plate dry.

(7) Add 50 µl/well of working dilution of horseradish peroxidase conjugate using blocked diluent and incubate at 37 °C on microplate shaker for 30 min.

(8) Wash the ELISA plate five times with PBS and tap the plate dry.

(9) Add 100 µl of 3, 3', 5' tetramethylbenzidine (TMB) substrate or solution (Appendix C, item C.3.9) into each well and leave for 20 min at room temperature.

(10) Add 50 µl/well of 1N H₂SO₄ to stop reaction of substrate.

3.2.2.3 Interpretation of the test results

The wells containing positive samples to specific FMDV serotype are shown in yellow and the optical density is read by ELISA reader at 450 nm.

If orthophenylene diamine (OPD) substrate is used, the optical density shall be read at 492 nm.
3.2.3 Virus isolation and identification

3.2.3.1 Principle

For amplifying the virus in cultured cells, the procedure is needed to be carried out in a biosafety level three (BSL-3) laboratory. Tissue suspension or vesicular fluid is used in this procedure.

Virus isolation by using cell culture is reliable diagnosis, but it is necessary to use primary cells prepared from lamb kidney cell or bovine thyroid cell. Cell line from baby hamster kidney (BHK) can be used but lesser sensitive than primary cell.

Preparation of primary cell is described in Appendix E.

3.2.3.2 Test procedure

(1) Prepare of cultured cell as described in Appendix E. After the confluent monolayer cell is formed; e.g., two days for cell line or may be longer for primary cell, discard the medium from tissue culture flask culture.

(2) Add 1 ml supernatant of tissue extracts in tissue culture flask and incubate at 37 °C for one hour to allow absorption.

(3) Add maintenance medium (MM) and incubate at 37 °C.

(4) Observe cytopathic effect (CPE) of cell culture for the first two days (Appendix G).

(5) Harvest the virus by freeze – thaw and centrifuge at 1,000 g at 4 °C for 15 min to separate supernatant from cell debris.

(6) Inoculate supernatant into cultured cell by repeating steps (1) to (5) for another two passages (totally three repetitions). Confirm the result by ELISA typing of the supernatant as described in the Section 3.2.2.
3.2.4 RT-PCR

3.2.4.1 Principle

RT-PCR is a molecular-based technique for detection genomic fragment of FMDV. The procedure consists of RNA extraction, cDNA transcription, amplification of targeted DNA to a detectable level by using specific primers and, finally, separation of DNA bands by electrophoresis to determine PCR product. Supernatant of tissue extracts or OP fluid are used in this procedure.

3.2.4.2 Test procedure

3.2.4.2.1 RNA extraction using Trizol reagent

(1) Take 250 µl of test sample in a 1.5 ml sterile microcentrifuge tube, add 750 µl Trizol reagent and mix by gently turning the tube up and down. Leave at room temperature for 5 min.

(2) Add 250 µl of chloroform, mix by gently turning the tube up and down and leave at room temperature for 5 min.

(3) Centrifuge 6,000 g, 15 min using the refrigerated centrifuge (4°C). Three layers of the solution can be observed. Gently collect 500 µl of supernatant from the upper most layer and put into a new 1.5 ml microcentrifuge tube.

(4) Add 500 µl of isopropanol and mix by gently turning the tube up and down for RNA precipitation. Leave at room temperature for 10 min.

(5) Centrifuge 6,000 g, 15 min using the refrigerated centrifuge (4°C). The RNA will be visible as white pellet at the bottom of the tube.

(6) Discard the supernatant from the tube. Wash RNA pellet with 1 ml of 75% ethanol and centrifuge 6,000 g for 15 min by using the refrigerated centrifuge (4°C).

(7) Discard the supernatant from the tube. Keep RNA pellet.

(8) Leave RNA pellet to dry and add 30 µl of RNase – free water to re-suspend RNA pellet.

(9) Proceed to step in Section 3.2.4.2.2. or store in the freezer at -20°C for further testing.

Remark: Commercial kits for RNA extraction may also be used.
3.2.4.2.2 Reverse transcription (RT)

(1) Prepare the RT Master mix in 0.5 ml PCR tube as described in Table 1

**Table 1** Compositions of the RT Master mix

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration</th>
<th>Volume/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-free water</td>
<td>2.5 μl</td>
<td></td>
</tr>
<tr>
<td>5X Buffer</td>
<td>5.0 μl</td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM*</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>DTT</td>
<td>100 mM</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>40 U/μl**</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Reversed transcriptase primer (random hexamer)</td>
<td>20 pmol/μl</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Reversed transcriptase enzyme (M-MLV)</td>
<td>200 U/μl</td>
<td>1.0 μl</td>
</tr>
<tr>
<td><strong>Total (μl)</strong></td>
<td>15 μl</td>
<td></td>
</tr>
</tbody>
</table>

Remarks: *millimolar (mM); ** unit per microliter; U/μl

(2) Add 10 μl of RNA suspension from (8) of Section 3.2.4.2.1 and mix by using vortex mixer.

(3) Spin down the sample using microcentrifuge.

(4) Place the PCR tube in a thermocycler and set the program at 42 °C for 60 min and 95 °C for 5 min then cDNA (RT product) will be obtained for testing in the next step or store in freezer at -20 for further testing.

3.2.4.2.3 Polymerase chain reaction (PCR)

(1) Prepare the PCR Master mix in 0.5 ml PCR tube as described in Table 2

**Table 2** Compositions of the PCR Master mix

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration</th>
<th>Volume/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-free water</td>
<td>28.5μl</td>
<td></td>
</tr>
<tr>
<td>10X Buffer</td>
<td>5.0 μl</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25 mM</td>
<td>3.0μl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10mM</td>
<td>1.0μl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>20 pmol/μl</td>
<td>1.0μl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>20 pmol/μl</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>5 U/μl</td>
<td>0.5μl</td>
</tr>
<tr>
<td><strong>Total (μl)</strong></td>
<td>40μl</td>
<td></td>
</tr>
</tbody>
</table>
Table 3 Oligonucleotide Primer 1, Primer 2 and product length

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5'-3'</th>
<th>Location</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F</td>
<td>GCCTGGTCTTTCCAGGTCT</td>
<td>3D</td>
<td>328</td>
</tr>
<tr>
<td></td>
<td>(Forward primer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1R</td>
<td>CCAGTCCCCTTCTCAGATC</td>
<td>3D</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Reverse primer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP5</td>
<td>AGGACAAAAGCGCTGTTCCGC</td>
<td>3D</td>
<td>454</td>
</tr>
<tr>
<td></td>
<td>(Forward primer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP6</td>
<td>TCAGGGTTGCAACCGACCGC</td>
<td>3D</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Reverse primer)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(2) Add 10 μl of cDNA (RT product) from (4) of Section 3.2.4.2.2 and mix by using vortex mixer.
(3) Spin down the sample using microcentrifuge.
(4) Place the PCR tube in a thermocycler and set the program as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>4 min</td>
<td>1</td>
</tr>
<tr>
<td>94°C</td>
<td>60 min</td>
<td>30</td>
</tr>
<tr>
<td>55°C</td>
<td>60 min</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>90 min</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

3.2.4.2.4 Separation of DNA band by electrophoresis

(1) Prepare an 1.5% agarose gel with sample loading spaces and place an agarose gel in electrophoresis chamber.
(2) Add 1X TBE buffer into a chamber until an agarose gel is completely covered.
(3) Add DNA ladder into a loading space as a marker.
(4) Add 5 μl/well of mixed solution of samples and loading dye into loading spaces on an agarose gel.
(5) Cover the chamber lid and run the electrophoresis at 100 V for 25 min (The condition may be adjusted as appropriate).
(6) Stain an agarose gel with 0.5 μg/ml ethidium bromide for 10 min. Other florescence dye may be used.
(7) Place an agarose gel on an UV transilluminator where the DNA bands stained with ethidium bromide will be illuminated.

3.2.4.3 Interpretation of the results

Positive or negative result may be determined by the product length of each sample when compared to a DNA marker, positive/negative control as follows:
(1) Primer 1F/1R
- If product band is visible at 328 bp, it defines as positive.
- If product band is not visible at 328 bp, it defines as negative.

![Image of 1F/1R PCR gel with lane labels]

**Figure 2** Detection of Foot and Mouth Disease virus using RT-PCR with 1F/R Primers

(2) Primer AP5/AP6
- If product band is visible at 454 bp, it defines as positive.
- If product band is not visible at 454 bp, it defines as negative.

![Image of AP5/AP6 PCR gel with lane labels]

**Figure 3** Detection of Foot and Mouth Disease virus using RT-PCR with AP5/AP6 Primers
3.2.4.4 Caution

To prevent any contamination, working areas, equipment and tools shall be cleaned with RNase and DNase-neutralizing agents (Alkaline chemicals, e.g. NaOH), gloves shall be worn during operation and working areas shall be separated.

3.2.5 LP ELISA

3.2.5.1 Principle

Liquid phase blocking ELISA (LP ELISA) is a quantitative immunology of FMD in serum samples. Principle of test is to detect a specific reaction between antigen and antibody or neutralization of antigen to inhibit the viral infectivity. Two fold serial dilution of serum is performed, then reacted with the certain amount of virus under the condition of suitable duration and temperature. The remaining virus or the quantity of neutralized virus is determined by enzyme immunoassay: an indirect double antibody sandwich ELISA.

3.2.5.2 Test procedure

(1) Prepare working dilution of rabbit trapping antibody against the suspected types of FMDV using coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.5). Coat ELISA plate with 50 μl/well of the prepared solution. Incubate at 37°C for 1 h or left overnight at 4°C.

(2) Prepare virus-serum mixture in U-shape plate using two-fold serial dilution of serum and the reference virus of the suspected types of FMDV. Add 50 μl/well of equal volumes of serum and certain amount of virus (optimal dilution of virus determined by titration). Incubate at 37°C for 1 h or left overnight at 4°C.

(3) Meanwhile, prepare control sets for each type of FMDV consist of antigen, strong positive serum (C++), weak positive serum (C+) and negative serum (C-) and add to U-shape plate.

(4) Transfer 50 μl/well of virus – serum mixture into the coated plates. Place on orbital shaker and incubate at 37 °C for 1 h. Wash the plates for four times with PBS.

(5) Add 50 μl/well of conjugated guinea pig detecting antibody against the suspected types of FMDV in each plate and place on plate shaker at 37 °C for 1 h. Wash the plates four times with PBS.

(6) Add 50 μl/well of horseradish peroxidase conjugate and shake in incubator at 37 °C for 1 h. Wash the plates four times with PBS.

(7) Add 100 μl of TMB substrate solution (Appendix C, item C.3.9) into each well and leave for 20 min at room temperature.

(8) Add 50 μl/well of 1N H2SO4 to stop reaction of substrate.

3.2.5.3 Interpretation of the results

(1) Optical density (OD) reading by ELISA reader at wavelength of 450 nm, If orthophenyline diamine (OPD) substrate is used, the optical density shall be read at 492 nm.

(2) The antibody titers are calculated by determining from the highest dilution of serum within the range of 50% OD value (50% OD max) of control antigen where the OD value is 1.0 – 1.5.
3.2.6 Virus neutralization test (VNT)

3.2.6.1 Principle

VNT is a quantitative immunology of FMD in serum samples. Principle of test is to detect a specific reaction between antigen and antibody or neutralization of antigen to inhibit the viral infectivity

- Two-fold serial dilution of serum is performed on tissue culture plate which the MEM is used as a diluent.
- Prepare 100 TCID\textsubscript{50} (50% tissue culture infective dose) of the standard virus type and mix with equal amount of serum. The virus neutralization reaction is occurring under the suitable duration and temperature.
- Evaluate the complete neutralization or the remaining virus by observing CPE of cultured cells under inverted microscope or staining cultured cell with 0.1% crystal violet in the 10 % formalin-PBS solution. The cultured cell; e.g., BHK-21, IB-RS-2, lamb kidney cell or pig kidney cell may be used in this method.

Prepare the stock of virus by inoculating the virus onto the monolayer cell line. Virus shall be titrated 2 or 3 times by using the tissue culture method until the constant titer level is achieved. Aliquot 1 ml of virus in the microtube. In order to prolong the viral viability during storage, 50% glycerine should be added and stored at -20°C. For tested serum, complement and non-specific protein must be inactivated prior to the test, by incubating the serum sample in the water bath at 56°C for 30 min.

Remark: this procedure requires the BSL3 laboratory

3.2.6.2 Test procedure

(1) Serum is diluted in a two-fold serial dilution using MEM diluents starting from a 1:4 dilution, followed by the 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256; consequently. Add to the tissue culture plate (96-wells microplate) using at least two wells per dilution (duplicate).
(2) Prepare control serums of positive, negative and cell control in the microplate.

(3) Add 50 μl/well of previously titrated virus (final suspension of virus and serum should be calculated to contain 100 TCID₅₀). Thoroughly mixed serum and virus by gentle tapping the microplate and cover with lid.

(4) Incubate at 37 °C for 1 h in CO₂ incubator (provided with 5% CO₂ for the stability of pH atmosphere).

(5) Prepare a cell suspension at 10⁶ cells/ml using growth medium containing MEM, 10% bovine serum, 3% NaHCO₃ and antibiotic(s). Add 50 μl/well of a cell suspension and cover with lid.

(6) Incubate at 37 °C for 2-3 days in CO₂ incubator. Observe CPE of cell culture under inverted microscope within 48 h due to cultured cells will gradually die out that may affect the result.

(7) In case the microplate needs to be rechecked for the result, the plate should be fixed and stained with 0.1% crystal violet in 10 % formalin-PBS for 30 min and rinse the plate with clean water.

(8) To verify the concentration of prepared virus suspension at 100 TCID₅₀ by back titration of virus control, the viral titer of the virus control should range between 1.5-2.5 TCID₅₀.

### 3.2.6.3 Interpretation of test result

The serum titer is determined at the highest dilution of serum that can neutralize 50% of the virus (50% neutralizing end-point). The serum titer is in logarithmic value and convert serum titer into the numeric value by antilogarithm.

The example of serum titer calculation (Sample of S-2 (Figure 5))

The highest dilution of serum that is able to neutralize 50% of the virus in this example is between 1:8 (2³) and 1:16 (2⁴) therefore the interpretation is 2³.⁵.

**Calculation methods**

If:

\[
\text{Serum titer} = X
\]

\[
X = 2^{3.5}
\]

The log is applied

\[
\log X = \log 2^{3.5}
\]

\[
\log X = 0.301 \times 3.5 \text{ (where log 2 = 0.301)}
\]

\[
\log X = 1.0535
\]

Antilog is taken

\[
X = 11.310
\]

then, serum titer = 1:11
3.2.7 Indirect ELISA for the detection of antibody of FMD virus non-structural protein

3.2.7.1 Principle

This method is to detect antibody against non-structural proteins of FMDV which are developed by genetic engineering such as 3A, 3B, 3AB, 3ABC, 2A, 2B, 2C or 3D. Generally, FMDV consists of structural proteins (VP1, VP2, VP3 and VP4) and non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D). In infected animal, antibodies against structural and non-structural proteins are produced and can be detected in its serum. These antibodies will not be found in the vaccinated animals with inactivated vaccine. Therefore, this method will benefit to differentiate vaccinated from infected animal (DIVA).

3.2.7.2 Test procedure

At present, test kits of non-structural protein, 3ABC, 3AB, 3B or 2B have been developed and used worldwide including Thailand for the convenience and rapid testing.

Test kits shall be validated by the Regional Reference Laboratory for Foot and Mouth Disease in South East Asia (RRL) or the body recognised by the World Organization for Animal Health (OIE).

Examples of test kit approved by the RRL for Foot and Mouth Disease in South East Asia are appeared in Appendix F.
Appendix A

Epidemiology, pathogenesis and clinical signs of foot and mouth disease

(Section 3)

A.1 Epidemiology

Foot and mouth disease (FMD) is a highly contagious disease which is able to spread rapidly among cloven–hoofed animals, such as cattle, buffalo, goat, sheep, pig, deer, camel and other animals e.g. elephant. FMD caused by a virus in the genus *Aphthovirus*, family Picornaviridae. There are 7 serotypes including O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3. Only serotypes O, A, and Asia1 have been reported in Thailand. Cross immunity of each serotype has not been reported. FMD serotypes are classified into 64 subtypes; 11 subtypes within O, 32 subtypes within A, 5 subtypes within C, 3 subtypes within Asia 1, 6 subtypes within SAT 1, 3 subtypes within SAT 2 and 4 subtypes within SAT 3. Because of the highly contagious nature of FMD that resulted in severe economic loss and impact on international trade, the World Organisation for Animal Health (OIE) has listed FMD in the Terrestrial Animal Health Code. In addition, FMD has also been listed in the Animal Epidemics Act B.E.2499 (1956) of Thailand.

A.2 Pathogenesis

FMD has several routes of transmission i.e., direct contact with sick or carrier animal, exposure to the excretion or secretion of infected animal such as urine, feces, saliva, milk and vesicular fluid, viral exposure via inhalation or ingestion of contaminated feed.

The viral exposure via respiratory tract, a primary site of infection and replication of the mucosa of pharynx then virus will distribute throughout the body via blood vessel and lymphatic system before animal exhibit clinical signs.

If the animals expose the virus orally, high amount of virus will be shed from infected animals via their excretions and secretion. The amount of virus in the infected animal is decreasing when the production FMDV specific antibody is increasing. Recovered animal may be a carrier which can spread the virus to other animals. For example, it is found that cattle can be a potential carrier for 24 months but not for pig.

Methods to detect of FMDV in carrier animals can be performed by collecting OP fluid and inoculation onto cell culture for viral multiplication (virus isolation) and follow by serotype identification using ELISA typing, RT-PCR or real time RT-PCR.

A. 3 Clinical signs

Clinical signs of infected animal are shown by depression, anorexia, excessive salivation and presence of vesicles on tongue, oral cavity, buccal cavity, coronary band, interdigital and/or paws. Vesicles are usually ruptured within 24 h, followed by sloughing of epithelium of tongue and oral cavity.
Within 2-5 days, viremia will occur and generalise throughout the body that can be observed by the appearance of vesicles and lesions around paws and coronary band. At this stage, the infected animals may show the sign of lameness. In severe cases, lesions can be found on udders and nipples of cows as well as on the snout in pig (illustrations of clinical signs and lesions are showed in Appendix G).
Appendix B

Virus infection associated antigen agar gel immunodiffusion test (VIA-AGID)

B.1 Principle

VIA-AGID is a method for detecting antibody to virus infections associated (VIA) antigen in serum sample of animals that have been infected with FMDV. This method is simple, lesser expensive and capable to differentiate infected from vaccinated animal. Nevertheless, serum sample from multiple vaccinated animal with BEI inactivated vaccine may result in false positive. Majority of FMD vaccines produced in Thailand are BEI inactivated vaccine; as a result, VIA-AGID is not recommended in routinely vaccinated animals. However, it can be applied to other cloven–hoofed animals such as wildlife.

Precipitation line of specific reaction of antigen-antibody is visualized by the diffusion of antigen and antibody toward each other through an agarose gel matrix. The reaction stops when the optimal concentration of antigen-antibody complex is obtained. Terminated point is observed by the appearance of gel precipitate or precipitation line.

B.2 Procedure

1. Prepare 0.8% agarose gel in the petri dish.
2. Punch 6 holes on agarose gel in circle and one hole at the center using 4 mm diameter gel puncher and remove the cut off gels by vacuum pump.
3. Add positive control serum on the upper and lower wells of the opposite sides. Add VIA antigen in the center well. The other leftover wells are the test samples. All wells contain 50 µl (Figure B.1).
4. Cover the petri dish and place in a chamber with moist cotton. Close the chamber and place at room temperature. Observe the result of the precipitation line within 1 to 5 days.

B.3 Interpretation

If the serum sample contains antibodies against VIA antigen, precipitation line will be visible as illustrated in Figure B.1. Samples of S1 and S4 are the positive result presenting in Figure B.1.

![Figure B.1 Interpretation result of VIA-AGID](image)

<table>
<thead>
<tr>
<th>Ag</th>
<th>VIA antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>positive control</td>
</tr>
<tr>
<td>S1</td>
<td>sample 1 (positive)</td>
</tr>
<tr>
<td>S2</td>
<td>sample 2 (negative)</td>
</tr>
<tr>
<td>S3</td>
<td>sample 3 (negative)</td>
</tr>
<tr>
<td>S4</td>
<td>sample 4 (positive)</td>
</tr>
</tbody>
</table>
Appendix C

Preparation of antibodies and chemicals used for ELISA technique

(Section 3)

C.1 Preparation of rabbit trapping antibody

Preparation of rabbit trapping antibody by injection of purified and concentrated 146S antigen of FMDV in rabbit as follows;

- Intramuscular injection (I/M) of 40 μg per rabbit of 146S antigen of FMDV mixed with complete Freund’s adjuvant.
- After 28 days, boost with 20 μg of 146S antigen of FMDV mixed with incomplete Freund’s adjuvant.
- Collect blood 10 days after boosting and separate serum.
- Titration of the serum to determine the working dilution for ELISA testing. Aliquot serum into microtubes and stock at -20 °C.

C.2 Preparation of guinea pig detecting antibody

Preparation of guinea pig detecting antibody by injection of purified and concentrated 146S antigen of FMDV in guinea pig as follows;

- Intramuscular injection (I/M) of 20 μg per guinea pig of 146S antigen of FMDV mixed with complete Freund’s adjuvant.
- Collect blood 28 days after injection and separate serum.
- Titration of the serum to determine the working dilution for ELISA testing. Aliquot serum into microtubes and stock at -20 °C.

C.3 Chemicals preparation

C.3.1 Coating buffer

Prepare 0.05 M carbonate bicarbonate buffer pH 9.5 as described:

(1) Na₂CO₃ (anhydrous)  1.59  g
(2) NaHCO₃        2.93 g

Add distilled water (DW) to 1 L and mix well

C.3.2 PBS for washing diluent

Prepare stock solusion of 10X PBS A for 5 L as described:

(1) NaCl             400  g
(2) KCl             10  g
(3) Na₂HPO₄          57.5 g
(4) KH₂PO₄          10 g

Dissolve all chemicals in 5 L distilled water
C.3.3 Prepare stock solution of 10X MgCl₂ as described:

\[
\text{MgCl}_2 \cdot 6\text{H}_2\text{O} \quad 5 \text{ g}
\]
Dissolve in 500 ml distilled water

C.3.4 Prepare stock solution of 10X CaCl₂ as described:

\[
\text{CaCl}_2 \text{ (anhydrous)} \quad 6.65 \text{ g}
\]
Dissolve in 500 ml distilled water

C.3.5 Prepare 10 L of washing buffer as described

1. Dilute 1 L of PBS A (Section C.3.2) with 7 L of distilled water
2. Dilute 100 ml MgCl₂ stock solution (Section C.3.3) in 900 ml distilled water
3. Dilute 100 ml CaCl₂ stock solution (Section C.3.4) in 900 ml distilled water
4. Mix well the solutions in (1), (2) and (3) and then adjust pH to 7.4

C.3.6 ELISA diluent (PBST)

1. Dissolve one pack of commercial 0.01M PBS with 1 L of deionized water
2. Add 1% phenol red 1 ml
3. Add Tween 20 0.25 ml
   Add HCl/NaOH for adjusting pH to 7.4 - 7.6

C.3.7 Prepare a block diluent as described:

Skimmed milk can be used instead of bovine serum albumin (BSA). 3% BSA is used for ELISA typing while 3-5% skimmed milk is used for LP ELISA.

1. ELISA diluent 100 ml
2. Skimmed milk powder 5 g (W/V) or 3 g of BSA (W/V)
   Add HCl/NaOH for adjusting pH to 7.6

C.3.8 Citrate-acetate buffer stock solution

1. Stock 1 M citric acid stock
   Citric acid 20 g
   Dissolve in distilled water 100 ml
2. Stock 1 M sodium acetate
   Sodium acetate 8.2 g
   Dissolve in distilled water 100 ml
3. Adjust pH of 100 ml of 1 M sodium acetate to pH 5.6 by adding 4 ml of 1 M citric acid.
C.3.9 Chromogen/TMB substrate solution (3, 3', 5, 5' tetramethylbenzidine)

Prepare 1% TMB (w/v) stock solution as described:

(1) TMB substrate 0.1 g
(2) DMSO (dimethylsulfoxide) 10 ml
(3) Dissolve TMB substrate in DMSO, aliquot 1 ml/tube and keep at 4 °C
(4) For working solution, e.g. preparing 10 ml of substrate solution as follow:

<table>
<thead>
<tr>
<th>Solution A:</th>
<th>distilled water 9 ml</th>
<th>1% TMB 0.1 ml</th>
<th>Citrate acetate buffer 1 ml</th>
</tr>
</thead>
</table>

Solution B: Dilute H₂O₂ (30% w/v) in distilled water 400 µl
Add 25 µl of solution B into 10 ml solution A.

In case of TMB tablet is used:
TMB substrate 1 tablet
Dissolve with citrate-acetate buffer 60 ml
Add H₂O₂ (30% w/v) 30 µl

C.3.10 Stopping solution

Prepare 1N H₂SO₄ as described:

(1) Conc. H₂SO₄ (36 N) 1 ml
(2) Distilled water 35 ml

C.3.11 Disinfectants for FMDV

The following solutions can be used:

(1) 4% Na₂CO₃ solution (w/v)
(2) 10% citric acid (w/v)
(3) 2.5 % Iodophor as iodine 2.5% (w/v), recommend to dilute 1:200 before use
(4) 0.1 % glutaraldehyde

Or other chemicals which have been proved to be effective
Appendix D

Preparation of antibodies and chemicals used for VIA-AGID technique

D.1 Preparation of VIA antigen using ion exchange chromatography

(1) Culture FMD virus in cell lines using high multiplicity of infection (MOI). Incubate in 37°C incubator for 9-12 h.

(2) Harvest the virus by centrifuging the cell precipitate from the supernatant, mix the supernatant with DEAE Sephadex A-50 and shake overnight at 4°C. Pack DEAE sephadex in the glass column (2.6 cm in diameter, 40 cm tall).

(3) Rinse the glass column with washing buffer that contains 0.02 M Tris-HCl pH 7.6 mixed with 0.15N NaCl. Separate the VIA antigen captured-DEAE Sephadex using eluting buffer which contains 0.02 M Tris-HCl pH 7.6 mixed with 1N NaCl.

(4) Connect the glass column to the monitor linked to the fraction collector and measure optical density (OD) at 254 nm.

(5) Collect VIA peak and then purify and concentrate by precipitating with saturated (NH₄)₂SO₄. Centrifuge at 4°C for 1 h and collect the precipitate part.

(6) Dissolve the precipitate in 0.02 M Tris-HCl, pH 7.6 mixed with 0.15 N NaCl.

(7) Transfer solution (6) into cellophane tube and dialysis at 4°C for 2-3 days in order to separate NH₄²⁺ and SO₄²⁻ salt from VIA antigen. Test the specificity and working dilution of VIA antigen by by agar gel immunodiffusion (AGID).

(8) Aliquot VIA antigen in microtubes and keep at -20°C for future test.

D.2 Preparation of chemicals for VIA-GRID test

D.2.1 Stock buffer 0.2 M Tris-HCl pH 7.6

(1) Tris (hydroxymethyl aminomethan) 24.2 g

(2) Dissolve in deionized water 900 ml

(3) Adjust pH to 7.6

(4) Add NaN₃ (10% w/v stock solution) 2 ml

(5) Add deionized water to bring the total volume up to 1 L.

D.2.2 Prepare washing A (0.02 M Tris-HCl pH 7.6 mixed with 0.15 N NaCl) as described:

(1) 0.2 M Tris-HCl, pH 7.6 (from D.2.1) 100 ml

(2) Dissolve in deionized water 800 ml

(3) Add NaCl (analytical grade) 8.766 g

(4) Add NaN₃ (10% w/v stock solution) 2 ml

(5) Add deionized water to bring the total volume up to 1 L.
D.2.3 Prepare eluting B (0.02 M Tris-HCl pH 7.6 mixed with 1 N NaCl) as described:

1. 0.2 M Tris-HCl, pH 7.6 (from D.2.1) 100 ml
2. Dissolve in deionized water 800 ml
3. Add NaCl (analytical grade) 58.44 g
4. Add NaN₃ (10% w/v stock solution) 2 ml
5. Add deionized water to bring the total volume up to 1 L.

D.2.4 Prepare saturated (NH₄)₂SO₄ as described:

1. (NH₄)₂SO₄ powder 1,000 g
2. 0.02 M Tris-HCl pH 7.6 300 ml

Put in warm water bath until saturated and crystallised.

D.2.5 Disinfectants for FMDV
The following solutions can be used:

1. 4% Na₂CO₃ solution (w/v)
2. 10% citric acid (w/v)
3. 2.5% Iodophor as iodine 2.5% (w/v), recommend to dilute 1:200 before use
4. 0.1% glutaraldehyde

Or other chemicals which have been proved to be effective.
Appendix E

Preparation of cultured cell line and the relevant chemicals

(Section 3.2.3)

E.1 Chemicals preparation

E.1.1 7% NaHCO₃

(1) NaHCO₃  7 g
(2) Distilled water  100 ml
Sterilized by filtration and keep at room temperature.

E.1.2 10X PBS stock solution

(1) NaCl  80 g
(2) KCl  2 g
(3) Na₂HPO₄  11.5 g
or Na₂HPO₄·2H₂O  14 g
or Na₂HPO₄·12H₂O  28.9 g
(4) KH₂PO₄  2 g
(5) Add distilled water up to volume 1 L
Sterilized by autoclave and keep at room temperature. Prepare 1 X PBS solution before use.

E.1.3 1% EDTA

(1) EDTA  1 g
(2) 1X PBS solution  100 ml
Sterilized by autoclave and kept at -20°C

E.1.4 1% Trypsin

(1) Trypsin  1 g
(2) 1X PBS solution  100 ml
Stir at 4°C overnight, sterilized by filtration and kept at -20°C

E.1.5 Trypsin-Versine (TV)

(1) 1% Trypsin  12.5 ml
(2) 1% EDTA  2.5 ml
(3) 1X PBS solution  85 ml
Sterilized by filtration and kept at -20°C
E.1.6 Fungizone stock solution
   (1) Fungizone 50 mg
   (2) Dissolve in distilled water 100 ml
   Sterilized by filtration, aliquot 3 ml/tubes and keep at -20°C

E.1.7 Antibiotic stocks
   Penicillin 10,000 U/ml (unit per milliliter)
   Streptomycin 10,000 mg/ml
   Kanamycin 10,000 mg/ml
   Dissolve in distilled water 100 ml
   Sterilized by filtration, aliquot 3 ml/tubes and keep at -20°C

E.1.8 Stock 2.92% L-glutamine, prepare for 100 ml
   (1) L-glutamine 2.92 g
   (2) Dissolve in distilled water 100 ml
   Sterilized by filtration, aliquot 20 ml/bottle and keep at -20°C

E.1.9 Growth medium (GM), prepare for 100 ml
   (1) MEM 92 ml
   (2) Bovine serum 5 ml
   (3) Antibiotics 0.5 ml
   (4) Fungizone 0.5 ml
   (5) 7% NaHCO₃ 1 ml
   (6) 2.92% L-glutamine 1 ml

E.1.10 Maintenance medium (MM), prepare for 100 ml
   (1) MEM 95.5-96 ml
   (2) Antibiotics 0.5 ml
   (3) Fungizone 0.5 ml
   (4) 7% NaHCO₃ 2-2.5 ml
   (5) 2.92% L-glutamine 1 ml

E.1.11 Freezing medium, prepare for 10 ml
   (1) MEM 6.75 ml
   (2) Bovine serum (20%) 2 ml
   (3) 7% NaHCO₃ (1.5-2%) 0.15 ml
   (4) DMSO (10%) 1 ml
(5) 2.92% L-glutamine 0.1 ml

E.1.12 0.45% trypsin

(1) trypsin (analytical grade) 0.45 g
(2) PBS buffer 100 ml
(3) Stir on magnetic stirrer at 4\(^\circ\)C until trypsin is completely dissolved.
Filter the solution and then warm the solution in water bath before use. The solution can be stored at -20\(^\circ\)C, thaw at 37\(^\circ\)C before use.

E.1.13 Growth medium for primary cell culture, prepare for 100 ml

(1) Hank Earle Lactalbumin Yeast extract (HELY) medium 87 ml
(2) Foetal calf serum 10 ml
(3) Antibiotics 1 ml
(4) NaHCO\(_3\) 1 ml
(5) L-glutamine 1 ml

E.2 Cell culture preparation

E.2.1 Primary cell

Primary cell that suitable and sensitive for FMDV culture can be prepared from calf’s thyroid cell or lamb’s kidneys. Selection of those young animals and absence of FMDV antibody, for instance, thyroid cell from 7 day-old calf or kidney of lamb at age less than 2 months.

The preparing of primary cell from calf’s thyroid cell, Aseptic technique is required in all steps. Equipment and all solutions shall be sterilized. Specific consideration should be taken as follows:

(1) Collect calf’s thyroid with avoidance of bacterial contamination. Place thyroid on the Petri dish with coverlid. Chill during transport to laboratory.

(2) Prepare and digest of cultured cells under laminar flow cabinet. Wash thyroid 3 times with PBS solution containing antibiotic. Transfer thyroid to a new Petri dish by using forceps.

(3) Remove connective tissue and fiber surrounding the gland with scissors. Wash thyroid with PBS solution containing antibiotic and transfer to a new Petri dish.

(4) Chop thyroid into fine pieces with a new scissors. Add 50 ml of PBS solution, shake gently in order to separate unwanted tissues and then pour out the supernatant which only the cell pellet is remained.

(5) Transfer cell pellet into the trypsinizing flask. Add 20 ml of warm 0.45% trypsin solution (37 \(^\circ\)C), shake gently and pour out the supernatant through the opening of sidearm of the trypsinizing flask.

(6) Add 50 ml of 0.45% trypsin into flask and placed the flask in 37 \(^\circ\)C water bath. Stir the solution with magnetic stirrer for 15 min which allows trypsin to digest the cell pellet into a single cell which suspended in the solution.
(7) Transfer the digested single cells into the stocking bottle and add 20 ml of foetal calf serum. Viability of the cells can be maintained at 4°C.

(8) Add 50 ml of 0.45 % trypsin into the same flask in order to digest the remaining cells. Place the flask in 37 °C water bath and stir with magnetic stirrer for 15 min. Transfer the digested cells into the stocking bottle in (7). Repeat the process in (6) until the cells are mostly digested and left only white connective tissue.

(9) Transfer the suspension in stocking bottle in (7) into 50 ml centrifuge tube and centrifuge at 400 g for 5 min.

(10) Discard the supernatant and re-suspend the remaining cell precipitate with approximately 5-10 ml growth medium. Mix the suspension with pipette and then add more growth medium until 100 ml of volume is obtained.

(11) Count the number of cells which should be 4x10^4 cell/ml. Transfer 1 ml of the cells into each test tube or 5 ml into culture flask, depending on the purpose of testing. Incubate the cells at 37 °C for 7-10 days.

(12) Daily observed cell growth. Replace new growth medium after 3 or 4 days of culture, 50% growth of primary cell should be observed at this period. If 80% - 90% confluent monolayer is obtained, the cell line is suitable for virus culture and can be used for the virus isolation.

**E.2.2 Monolayer BHK cell line**

(1) Subcuture the confluent monolayer as seed cell by discarding old culture medium.

(2) Rinse the cells with PBS and discard PBS.

(3) Add 0.1% Trypsin-Versine to digest and use pipetting technique to segregate cells into single cells.

(4) Transfer the digested single cells into new growth medium containing 5% bovine serum.

(5) Distribute cell suspension into culture bottle. The volume of cell suspension is adjusted according to the size of culture bottle, for example, 5 ml of cell suspension should be added into 25 cm² culture bottle.

(6) Incubate the bottle at 37°C for 2 days.
Appendix F

Test kit: NS kit

(Section 3.2.7)

There are several NS test kits that have been validated and currently used in Thailand for examples:

**F.1 UBI® FMDV NS EIA**

The test kit is based on the detection of antibody against 3B non-structural protein of FMDV by indirect ELISA technique. Test procedures according to the manufacturing protocols are as follows:

Allow the reaction plate wells, the dilution microplates or the strips as well as reagents and test serum to reach room temperature (15 to 30°C) for 30 min, but do not expose to direct sunlight or elevated temperatures (>30°C).

1. Dilute test serum and control serum (reactive and non reactive control) into 1:21, add 200 µl of specimen diluent in each well of the dilution plate then add 10 µl of test serum or control serum into the plate.

2. Transfer 100 µl of diluted test serum and control serum to the reaction microplates which are pre-coated with 3B non-structural protein antigen (provided by the manufacturer).

3. Incubate the plate at 37°C for 1 h and wash the plate 4 times with PBST.

4. Prepare working conjugate solution at 1:101 by using the conjugate diluent, add 100 µl of conjugate solution to all wells. The volume of conjugate diluent is used according to the preparation of working dilution for each test.

5. Incubate the plate at 37°C for 1 h and wash the plate 4 times with PBST.

6. Add 100 µl of substrate solution into each well and leave the plate at room temperature for 15 min.

7. Add 100 µl of stop solution (1 M H$_2$SO$_4$) to each well to stop the reactions.

8. Measure the optical density (OD) by ELISA reader at wavelength of 450 nm.
Figure F.1 Illustration of formate plate and diagram for 3B NS test

(9) Interpretation of the results

Calculations of mean OD value of the FMDV non-reactive control serum (NRC) and reactive control serum (RC) are as described:

(9.1) The mean OD value of non reactive control (NRC) should be in range $\leq 0.15$, as showed in the following example:

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.102</td>
</tr>
<tr>
<td>B1</td>
<td>0.113</td>
</tr>
<tr>
<td>Total</td>
<td>0.215</td>
</tr>
<tr>
<td>Average NRC</td>
<td>0.215/2 = 0.108</td>
</tr>
</tbody>
</table>

(9.2) Determine the mean OD value of reactive control (RC) should be in range $\geq 0.7$ and $\leq 1.9$, as showed in the following example:

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>1.245</td>
</tr>
<tr>
<td>D1</td>
<td>1.191</td>
</tr>
<tr>
<td>Total</td>
<td>2.436</td>
</tr>
<tr>
<td>Average RC</td>
<td>2.436/2 = 1.218</td>
</tr>
</tbody>
</table>

(9.3) Determination of cut off value

$$\text{cut off value} = (0.23) \times \text{RC}$$

Example: $\text{RC} = 1.218$

$$\text{cut off value} = 0.23 \times 1.218 = 0.282$$

(9.4) Compare the test serum OD value to the cut off value as indicated in the interpretation result in (9.3) The test serum OD values greater than or equal to the cut off value are considered as positive. The test serum OD values less than the cut off value are considered as negative.
F.2 PrioCKECK® FMDV-NS

The test is based on the detection of antibody against 3ABC non-structural protein of FMDV by blocking ELISA technique. Test procedures according to the manufacturing protocols are as follows:

1. The plates are coated with 3ABC specific monoclonal antibody follow by adding the specific 3ABC antigens, and then dry the plate for ready to use.

2. Dilute the test serum and control serum into 1:5 dilution by adding 80 µl of ELISA buffer (provided by the manufacturer) to 3ABC coated plate.

3. Add 20 µl of test serum, strong positive, weak positive and negative serum which provided by the manufacturer. Shake the plate gently, the format plate is shown in Figure F2.

4. Incubate the plate overnight (16-18 h) at 20-25 °C.

5. Wash the plate 6 times with washing buffer provided by the manufacturer and tap the plate until dry.

6. Add 100 µl of conjugate solution to all wells, the working dilution of conjugate is prepared at 1:101 by using conjugate diluent which provided by the manufacturer.

7. Close the lid and place the plate at room temperature for 1 h, wash the plate 6 times with washing buffer and tap the plate until dry.

8. Add 100 µl of chromogen/substrate solution provided by the manufacturer to all wells. Place the plate at room temperature for 20 min.

9. Add 100 µl of stop solution to all wells to stop the reactions.

10. Measure the optical density (OD) by ELISA reader at wavelength of 450 nm

11. Interpretation of the results

   The OD value at 450 nm of each sample is expressed as percent inhibition (% PI) comparing to the negative control (OD\text{450 max}) as described:

   \[
   \text{% PI} = \frac{\text{OD}_{450 \text{ test sample}}}{\text{OD}_{450 \text{ Max}}} \times 100
   \]

12. Determine of the cut off value or validation criteria are considered from these limited control values as follows:

   - OD max (C1-C2) > 1.0
   - Mean % PI of weak positive > 50 %
   - Mean % PI of strong positive > 70 %

13. Reporting the results

   - PI < 50% considered as negative result indicated antibodies to non structural protein of FMDV are absent.
   - PI > 50% considered as positive result indicated antibodies to non structural protein of FMDV are present.
**Figure F.2** Illustration of format plate and diagram for 3ABC NS test
Appendix G

Illustrations of foot and mount disease diagnosis

(Section 3 and Appendix A)

Figure G.1 The clinical signs showing depression and excessive salivation

Figure G.2 The clinical signs showing ulcerative lesions due to the ruptures of vesicles of oral cavity and tongue epithelium (red arrow).
Figure G.3 The clinical sign showing vesicles at the snout of pig (red arrow).

Figure G.4 The clinical sign showing vesicles at the coronary band of pig hoof (red arrow).

Figure G.5 Myocarditis in young animals infected with FMDV showing white striped appearance in myocardium (tiger heart) (red arrow).
Figure G.6 Tissue samples in bottles

Figure G.7 Primary container (a) and secondary container (b) for tissue samples before being packed into outer box or standard box (c).

Figure G.8 Packing of samples for transportation with ice packs (red arrow)
Figure G.9 Normal cells (G.9.1) and dead cells with CPE (G.9.2); transformation of long-fibroblastic living cells (G.9.1) to round, smooth-thick membrane cells (G.9.2) occurs during cell death. Under microscope (400X), degenerate cytoplasm results in the reflection of cells. Dead cells do not attach to the surface of cultured flask and suspend in culture medium.

Courtesy: the Regional Reference Laboratory for Foot and Mouth Disease in South East Asia (Figure G.1 to G.9)
Appendix H

Units

Units and symbols used in this standard and the SI unit (International System of Units or Le Système International d’Unités) approved to be used are:

<table>
<thead>
<tr>
<th>Items</th>
<th>Unit</th>
<th>Unit Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass</td>
<td>gram</td>
<td>g</td>
</tr>
<tr>
<td></td>
<td>milligram</td>
<td>mg</td>
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