THAI AGRICULTURAL STANDARD

TAS 10400-2004

DIAGNOSTIC TEST OF FOOT AND MOUTH DISEASE

National Bureau of Agricultural Commodity and Food Standards
Ministry of Agriculture and Cooperatives
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NOTIFICATION OF THE NATIONAL COMMITTEE ON
AGRICULTURAL COMMODITY AND FOOD STANDARDS
SUBJECT: THAI AGRICULTURAL COMMODITY AND FOOD STANDARD:
DIAGNOSTIC TEST OF FOOT AND MOUTH DISEASE

The resolution of the 1/2547 session of the National Committee on Agricultural Commodity and Food Standards dated 15 March B.E.2547 (2004) endorsed the Thai Agricultural Commodity and Food Standard entitled Diagnostic Test of Foot and Mouth Disease. This standard would be of benefits for quality improvement, facilitating trade and protecting consumers.

By virtue of the Cabinet Resolution on Appointment and Authorization of the National Committee on Agricultural Commodity and Food Standards dated 19 November B.E.2545 (2002), the Notification on Thai Agricultural Commodity and Food Standard entitled Diagnostic Test of Foot and Mouth Disease is hereby issued as a voluntary standard, details of which are attached herewith.

Notified on 3 August B.E.2547 (2004)

Mr. Somsak Thepsuthin
Minister of Agriculture and Cooperatives
Chairperson of the National Committee on Agriculture Commodity and Food Standards
The standard for the diagnosis of Foot and Mouth Disease is provided for the precision and accuracy of animal diagnostic laboratory.

This standard was originally drafted by the Department of Livestock Development and it was revised by the Committee of Animal Sanitary Standards and finally approved by the National Committee on Agricultural Commodity and Food Standards.

The establishment of this standard is based on the information of the following documents:


**Remark:**

The standard title has been revised from “Thai Agricultural Commodity and Food Standard (TACFS)” to “Thai Agricultural Standard (TAS)” in accordance with the enforcement of The Agricultural Standards Act B.E. 2551 (2008)
1. INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious viral disease of animals which is infectious and can spread rapidly. All species of cloven-hoofed animals and wildlife are affected including cattle, buffalo, sheep, goat, pig, elephant, camel and deer, leading to severe economic loss of livestock industries worldwide.

FMD is caused by FMD virus (FMDV) which is belonging to genus *Aphthovirus* in the family Picornaviridae. There are seven immunologically distinct serotypes – O, A, C, Asia1, SAT1, SAT2 and SAT3. In Thailand, there are three serotypes, O, A and Asia1. There is no cross protection among serotypes. Each serotype is divided into subtypes. All 64 subtypes within these serotypes are found. There are 11, 32, 5, 3, 6, 3 and 4 subtypes of O, A, C, Asia1, SAT1, SAT2 and SAT3 serotypes respectively.

Clinical signs of FMD infected animal are characterized by fever, depression, anorexia and salivation. The vesicles appear on the tongue, oral cavity and foot. They rupture rapidly and become erosion within 24 hours. Two to five days later, FMDV is found all over the body. The vesicles and wounds will be appeared on feet and coronary bands leading to lameness. In severe cases, lesions and wound can be found around mammary gland and teat of cow and on the snout of pig.

FMD can be transmitted via various routes such as by direct contact with secretion or excretion from acute infected animal including urine, feces, expired air, saliva, milk and vesicular fluid. Transmission can be also occurred by indirect contact with contaminated fomites or ingestion of contaminated feed.

After air-borne infection, FMD is found in pharynx (Burrows et al., 1971) where the virus replicate and spread to various organs via blood stream and lymphatic system. Before showing clinical signs, it was found that there was virus shedding through secretions and excretions of infected animals at high quantity. (Burrows, 1968; Burrows et al.,1981). Following recovery from acute infection, the amount of virus decreased after antibody response to virus infection and FMDV can be neutralized. Infected animal can be carrier and can spread the disease to susceptible animal. The carrier state in cattle can be persisted up to 2.5 years (Hedger, 1968). African buffalo have been shown to be carrier for 3 – 5 years (Hedger et al., 1972), 12 months in sheep (Sutmoller, 1970), and 4 months in goat (Singh, 1977) but no report of carrier state in pig (Panima et al., 1988).

Carrier animals can be identified by isolating FMDV from esophageal – pharyngeal fluid using tissue culture and typing by ELISA. Esophageal – pharyngeal fluid from ruminant is collected by using Probang cup.

FMD outbreaks can be controlled by various methods such as vaccination to build up immunity for the animals. FMDV vaccines must be closely matched with the serotype or
strain of FMDV in the field outbreaks. Control by movement restriction and quarantine animals for at least 21 days is another method that can limit spreading FMDV to neighboring area. Ring vaccination at 5 – 10 km radius shall be done.

Prevention of transmitting the disease via contaminated equipment and utensils as well as animal products can be done by using effective disinfectants for FMDV such as 2% sodium hydroxide, 4% sodium carbonate, glutaraldehyde, Iodophore (iodine compound) or 2% citric acid.

FMD can not be distinguished clinically from other vesicular diseases. Laboratory diagnosis is necessary for specific and accurate FMD diagnosis by serotyping. (Roeder and Le Blanc Smith, 1987).

Laboratory diagnosis includes virus detection from tongue, feet and coronary lesions by virus isolation using tissue culture typing by ELISA technique. Reverse transcription polymerase chain reaction (RT-PCR) is another available molecular biological technique to accurately detect RNA of FMDV. Many serological methods such as liquid phase blocking ELISA (LP ELISA) and virus infection associated agar gel immunodiffusion test (VIA-AGID) are used for FMDV antibody detection. LP ELISA is able to detect FMD serotype O, A, Asia1 and other serotype which is depending on the serotype of FMDV in the vaccine using in the region. The principle of LP ELISA is that antibody to FMDV in the serum was neutralized or blocked by specific serotype of FMDV. The remaining FMDV is detected by ELISA (Mc Vicar and Sutmoller, 1970). VIA-AGID test is used to detect antibody to VIA antigen in infected or vaccinated animals. The test is able to differentiate infected animals from vaccinated ones. False positive may be found in animals which were vaccinated with inactivated vaccine using BEI as inactivant. Indirect ELISA or non – structural ELISA (NS-ELISA) tests are developed to detect antibody to non – structural protein of FMDV. The NS-ELISA is more FMD accurate than VIA – ELISA and at present, the NS-ELISA is being used as a standard method for diagnosis in international trade.

2. SAMPLE COLLECTION

The samples for laboratory diagnosis are epithelium or vesicular fluid collected from FMD suspected animals. The samples of choice in cattle are lesions from tongue tissue, buccal mucosa, wounds from feet and hoofs. In pig, fluid filled vesicles wounds from tongue, snout, coronary band and hoof shall be collected.

At least one gram of epithelial tissue shall be collected from each animal. Additional samples shall be collected if the weight of each sample is not enough. The samples are kept in screw cap bottle, contained 50% glycerine buffer, capped tightly and sealed with adhesive tape to prevent leakage of the buffer. The bottles are clearly labeled and wrapped with many layers of paper before putting in suitable container or can and tightly sealed. For transportation of the samples to laboratory, the samples are kept cool in strong, unbreakable ice container. In case of sending samples via post, the samples shall be wrapped with many layers of paper, to prevent from breaking and leaking, before putting into unbreakable container or box together with details history of the samples.

For serum samples, serum is collected by using sterile glass syringe or dried and cleaned container. After blood clotting, the cleared serum is transferred to plastic vial. If serum containing red blood cell, centrifuge the serum to separate red blood cell and then
collect clear serum in plastic vial and seal tightly to prevent leaking. Then the serum shall be kept cool in ice cube container or -20 °C refrigerator while transport to laboratory.

3. LABORATORY DIAGNOSIS

Presumptive diagnosis of FMDV infection is carried out by observation of clinical signs including dullness, fever, salivation, frequently licking lip and lameness. The occurrences of fluid filled vesicles, which contain large amount of FMDV are appeared on the tongue. After that the virus will spread all over the body via blood stream and spread to hoof and hoof tissue. The rupture of these vesicles will be observed within 24 hours. The remaining shallow wound with slough off of tongue tissue are observed. FMD diagnosis shall be differentiated from swine vesicular stomatitis, vesicular exanthema and vesicular disease.

Virus isolation in tissue culture and ELISA typing are the definitive diagnosis for confirmation of FMD infection. The specimens used for laboratory diagnosis from piglets that died from FMD infection due to myocarditis, are blood, heart and other organs.

3.1 TISSUE EXTRACTION OF FMDV ANTIGEN

1) Dissect one gram of tissue and put into sterile mortar.

2) Dissect the tissue into small pieces with sterile scissors.

3) Grind with sterile sand.

4) Make 10% suspension by using M/25 phosphate buffer saline (PBS) or 0.4 M PBS (one gram of tissue plus 9 ml of PBS) as a diluent by weighing one gram tissue sample and add 9 ml of M/25 PBS into centrifuge tube.

5) Centrifuge at 1,000 x g or 2,500 rpm for 15-20 min.

6) Collect supernatant fluid and mix with 1’1’2’ trichlorotrifluoroethane at the ratio of 1:1, mix well for 20 min.

7) Centrifuge at 2,500 rpm for 20 min.

8) Collect supernatant fluid and dispense into two volumes which will be used for ELISA typing and virus isolation.

3.2 ENZYME LINKED-IMMUNOSORBENT ASSAY (ELISA) FOR FMDV TYPING

This method is a qualitative method for FMDV typing by indirect double antibody sandwich ELISA as following steps:

1) Coat plate with 50 µl/well of appropriate dilution of rabbit anti FMDV type O, A, Asia1 and normal rabbit sera which were properly diluted with coating buffer and dispense into flat bottom plate for 50 ml per each well. Place the plate on the orbital shaker, set at 200 rpm, in 30 °C incubator for 60 min or leave over night at 4 °C.

2) Wash the plates five times with PBS and tap for drying.

3) Prepare control antigen and antigen for virus typing in microtube by using typing diluent (ELISA diluent containing 3% bovine serum albumin) to make two fold serial dilution from 1:2, 1:4 and 1:8. Dispense into microplate, cover
4) Wash the plates five times with PBS and tap for drying.

5) Dispense 50 µl/well of appropriate dilution of guinea pig anti FMDV serum type O, A, Asia1 and normal guinea pig serum which were properly diluted and incubate at 37 °C on microplate shaker for 30 min.

6) Wash the plates five times with PBS and tap for drying.

7) Add 50 µl/well of appropriate dilution of horseradish peroxidase conjugate which were properly diluted with typing diluent and incubate at 37 °C on microplate shaker for 30 min.

8) Wash the plate five times and tap for drying.

9) Add one tablet of substrate TMB or 3, 3’5, 5’ tetramethyl benzindine into 60 ml of citrate acetate buffer, pH 5.6 which contain 0.05% H2O2 (30% H2O2 W/V) by adding 6 µl of 30% H2O2 into 12 ml of substrate solution per 1 plate. Dispense 100 µl of substrate solution into each well and leave for 20 min.

10) Add 50 µl/well of 1N H2SO4 to stop reaction of substrate. The wells that show yellow colour are positive to specific FMDV subtype.

11) The plates are read at 450 nm by ELISA reader. (Labsystem Multiskan®/Titutex Multiskan®). When orthophenyline diamine or OPD substrate is used, the plate shall be read at wavelength 492 nm.

3.3 VIRUS ISOLATION AND IDENTIFICATION.

Virus isolation by using cell culture is the most sensitive and accurate diagnosis but it is necessary to use the following tissue culture such as primary lamb kidney cell or bovine thyroid cell culture or baby hamster kidney cell line (BHK). However, cell line is less sensitive than primary cell culture.

3.3.1 Culture of BHK cell line (Stationary monolayer)

1) Confluent monolayer of BHK seed cell culture is prepared.

2) Discard the medium and wash the cell with PBS.

3) Add 0.1% Versene trypsin to trypsinize cell culture by using pipetting technique.

4) Pipetting trypsinized single cell in growth medium containing 5% bovine serum at prescribed volume.

5) Dispense five ml into 25 cm² tissue culture flask.

6) Incubate at 37 °C for two days.

3.3.2 Virus isolation

The virus isolation is carried out by inoculation of extracted tissue samples into cell culture to propagate the virus.

1) Discard the medium from tissue culture flask of confluent monolayer cell culture.
2) Add one ml supernatant of extracted samples in tissue culture flask and incubate at 37 °C for one hour.

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

4) Daily observe cytopathic effect (CPE) of cell culture for two days.

5) Harvest the virus by freeze – thawing cell culture and centrifuge at 2,500 rpm at 4 °C for 15 min. Collect supernatant fluid.

6) If there is no CPE observed in the first passage, another 2 passages are carried out until appearance of 100% CPE is observed. Harvest the infected fluid for confirmation by ELISA typing.

3.4 REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION (RT-PCR) FOR THE DETECTION OF FMDV ANTIGEN

3.4.1 Extraction of viral RNA

1) Add 0.25 ml of test sample to 0.75 ml Trizol LS in sterile 1.5 ml plastic tube, capped tightly.

2) Mix by gently turning the tube up and down and leave at the room temperature for five min to completely separate nucleoprotein complex.

3) Add 250 µl of chloroform and vortex mix for about 15 s and leave at room temperature for five min.

4) Centrifuge at 12,000 x g at 4 °C for 15 min and three layers of the solution are observed. Collect viral RNA from the upper layer.

5) Add 0.5 ml of isopropanol and vortex mixing and leave at room temperature for 10 min.

6) Centrifuge at 12,000 x g in 4 °C for 10 min. Discard the supernatant fluid from each tubes.

7) Wash RNA pellet with one ml of 75% ethanol and centrifuge at 10,000 x g in 4 °C for five min. Remove supernatant fluid from each tube, carefully not to dislodge or lose any pellet at the bottom of the tubes. Air-dry each tube at room temperature.

8) Re – suspend each pellet by adding 30 – 50 µl of RNase – free water or DEPC.H₂O.

9) Spin down and transfer to new eppendorf tubes, keep the extracted samples on ice if the next step is about to be performed or keep in -20 °C freezer.

3.4.2 Reverse transcription (Omnigene Thermal Cycler®)

1) Mix all reagents in Table 1 in 0.5 ml eppendorf tubes.
**Table 1** Reagents for reverse transcription reaction.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
<th>Volume / 1 Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC H(_2)O / RNase free water</td>
<td></td>
<td>9.0 µl</td>
</tr>
<tr>
<td>DNTPs</td>
<td>2.5 mM</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>5x buffer</td>
<td></td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Random primer / universal primer</td>
<td>25-50 pmol/µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Rnasin inhibitor</td>
<td>20-40 U/µl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>DTT</td>
<td></td>
<td>2.5 µl</td>
</tr>
<tr>
<td>MoMLV</td>
<td>0.1 M</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Viral RNA</td>
<td>200 U/µl</td>
<td>5.0 µl</td>
</tr>
</tbody>
</table>

2) Mixing by vortex mixer

3) Place the tubes in Thermal Cycler for PCR amplification and run the following programme.

- 90 °C for 2 min
- 42 °C for 60 min
- 95 °C for 5 min
- 4 °C for 10 min

4) Keep the product from 3) at 4 °C if the next step about to perform or keep at -20 °C freezer for longer period.

### 3.4.3 PCR amplification (Omnigene Thermal Cycler®)

1) Mix all reagents in Table 2 in 0.5 ml eppendorf tubes.

**Table 2** Reagents for PCR amplification reaction

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
<th>Volumes / 1 Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC H(_2)O / RNase free H2O</td>
<td></td>
<td>33.5 µl</td>
</tr>
<tr>
<td>DNTPs</td>
<td>10 mM</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>10x buffer</td>
<td></td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Primer 1 (universal)</td>
<td>25 pmol / µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Primer 2 (sense primer)</td>
<td>25 pmol / µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5 U/ µl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>RT product from 3.4.2</td>
<td></td>
<td>5.0 µl</td>
</tr>
</tbody>
</table>

2) Vortex mixing and add 1 drop (50 µl) of mineral oil into each tube.

3) Place in PCR Thermal Cycler and run the following programme.
94 °C for 4 min
94 °C for 1 min
55 °C for 1 min
72 °C for 1.3 min
72 °C for 5 min

\{ 30 cycles \}

4) Keep PCR product at 4 °C for short duration or at -20 °C for longer duration.

3.4.4 Detection of PCR products by agarose gel electrophoresis

1) Prepare 1.5% Agarose gel with punged wells and place in electrophoresis chamber.

2) Add running buffer to cover Agarose plate.

3) Add 5 µl of DNA marker and test samples, which are mixed with 6 x dye, into each well.

4) Cover the chamber, connect electrodes to power supply and supply electricity at 100 volts for 40 min.

5) Stain gel plates with ethidium bromide for 5 min and wash with water for 15 min.

6) Detect PCR band by UV transilluminator and PCR bands are photographed by using Polaroid Camera.

3.5 LIQUID PHASE BLOCKING ELISA FOR DETECTION OF ANTIBODY TO FMDV

Liquid phase blocking ELISA (LP ELISA) is a quantitative immunology of FMD by neutralization or blocking of known amount of virus by diluted test serum samples. The un-neutralized or unblocked virus is detected by indirect double antibody sandwich ELISA. The procedure is as followings.

1) ELISA plates are coated with 50 µl/well of rabbit trapping antibody and leave overnight at 4 °C.

2) Prepare virus serum mixture in U-shape plates, two fold serial serum dilution is prepared. Add equal volume of fix amount of virus for 50 µl/well. Leave overnight at 4 °C.

3) At the same time, prepare control panel contains antigen control, strong positive control serum, weak positive control serum and negative control serum in every plate of U-shape plates.

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

5) Add 50 µl/well of conjugated guinea pig detecting antibody place on plate shaker at 37 °C for one hour. Wash the plates four times with PBS.

6) Add 50 µl/well of horseradish peroxidase conjugate and shake in incubator at 37 °C for one hour. Wash the plates with PBS for four times.
7) Add one tablet of TMB or 3, 3’5, 5’ tetramethyl Benzindine substrate into 60 ml of citrate acetate buffer pH 5.6. Add 6 µl of 30% H₂O₂ into 12 ml of TMB substrate buffer into each plate then drop 100 µl into each well and leave at room temperature for 20 min (For OPD substrate, leave at room temperature for 15 min).

8) Stop reaction of substrate by adding 50 µl of 1 N H₂SO₄.

9) Optical density (OD) reading by ELISA reader (Labsystem Multiskan® / Titertek Multiskan®) at wavelength of 450 nm (For OPD substrate, OD at wavelength of 492 nm is used).

10) The antibody titers are calculated by determining from the highest value of serum dilution within the range of 50% OD value of control antigen where the OD value is 1.0 – 1.5.

3.6 DETECTION OF VIRUS INFECTIOUS ASSOCIATED (VIA) ANTIGEN BY AGAR GEL IMMUNODIFFUSION TEST (VIA-AGID)

The principle of the method is to detect VIA antigen in serum of animals infected with FMDV by using a reaction of specific antigen and antibody which is causing a movement of them in agar gel media and meet each other at certain point where the concentration is optimal to stop such movement. The precipitation line is formed by reaction of specific antigen and antibody in agar gel. The procedures are as followings.

1) Prepare 0.8% agarose in plastic petridish.

2) Make 6 holes, including one hole at the center, by using 6 – well, 4 nm φ gel puncher, remove the remaining gel in the hole by vacuum pump.

3) Add positive control serum in the opposite upper and lower wells. Put the test serum in the remaining wells and 50 µl of VIA antigen in the center well.

4) Cover the petridish and put in moisture chamber or in a plastic container, with water soaked cotton to produce moisture, cover the container and leave at room temperature. The precipitation lines are observed within five days.

3.7 INDIRECT ENZYME LINKED IMMUNOASSAY TESTS FOR DETECTION OF FMDV NON-STRUCTURAL PROTEIN ANTIBODY (NS ELISA TESTS)

This method is used to detect antibody to FMDV non-structural proteins which were genetically engineered as 3A, 3B, 3AB, 3ABC or 3D. The NS ELISA test is able to differentiate infected from vaccinated animals (Deigo et al., 1997; Mackay, 1998). At present 3ABC/3AB/3B of non-structural protein are developed for commercial test kit which is suitable and save time for laboratory diagnosis. They are available in many countries including Thailand.

The test procedures are as followings.

1) Dilute test serum samples with diluent from test kit.

2) Add the diluted serum samples into coated plate which are already pre-coated with antigen consisting of non-structural protein.

3) Place the plate on orbital shaker, incubate at 37 °C for one hour, and wash the plates four times with PBS – tween (PBST).
4) Add conjugated solution, prepared from horseradish peroxidase labeled anti-
species immunoglobulin.
5) Place on plate shaker, incubate at 37 °C for 30 min, wash 4 times with PBST.
6) Add substrate and leave at room temperature for 15 min.
7) Stop reaction by adding 1.25 N H$_2$SO$_4$.
8) OD reading by ELISA reader (Labsystem Multistan®, Titutex Multiskan®
Reader). The wavelength at 492 nm is used for OPD substrate and 450 nm for
TMB substrate.
ANNEX A
REAGENTS PREPARATION FOR ELISA TECHNIQUE

A.1 PREPARATION OF RABBIT ANTI FMDV SERA TYPES O, A AND ASIA1

Prepare purified, concentrated 146s antigen from each FMDV type. Inoculate rabbit intramuscularly (I/M) with 40 µg/animal of 146s antigen in complete Freund’s adjuvant. Proster with 20 µg of 146s / animal of 146s antigen in incomplete Freund’s adjuvant 28 days after first inoculation. Bleeding and collecting rabbit serum 10 days after second injection. Titration of the serum to determine the appropriate dilution for ELISA testing. Put aliquot serum into microtubes and keep at -20 °C.

A.2 PREPARATION OF DETECTING ANTIBODY

Prepare guinea pig anti FMDV types O, A and Asia1 by inoculation of guinea pig I/M with 20 µg / animal of 146s antigen in complete Freund’s adjuvant. Bleeding within 28 days post inoculation and collecting serum. The serum is titrated for appropriate dilution for ELISA testing. Put aliquot serum in microtubes and keep at -20 °C.

A.3 PREPARATION OF REAGENTS FOR ELISA TECHNIQUE

A.3.1 Coating buffer

0.05 M Carbonate bicarbonate buffer, pH 9.5
1) Na₂CO₃ (anhydrous)  1.59 g
2) NaHCO₃  2.93 g
Add distilled water (D.W.) to one liter and mix well

A.3.2 PBS for washing diluent

Stock 10 x PBS for 5 L
1) NaCl  400 g
2) KCl  10.0 g
3) Na₂HPO₄  57.5 g
4) KH₂PO₄  10.0 g
Dissolve in D.W. 5 L

A.3.3 Stock 10 x MgCl₂ solution

MgCl₂ .6H₂O  5.0 g
Dissolve in D.W. 500 ml
A.3.4 Stock 10 x CaCl₂ solution

1) Dissolve 6.65 g CaCl₂ (anhydrous) in 500 ml D.W.
2) Prepare 10 L of washing buffer by diluting 1 L PBS A in 1 L of D.W.
3) Dilute 100 ml of CaCl₂ in 900 ml D.W.
4) Dilute 100 ml of MgCl₂ in 900 ml D.W.
5) Mix the solution in 2, 3, 4 and adjust pH to 7.4.

A.3.5 ELISA diluent

1) Dilute 50 ml of 10 x PBS A in 350 ml D.W.
2) Dilute 5 ml of CaCl₂ (3.4) with 45 ml D.W.
3) Mix solution of 1) and 2)
4) Dilute 5 ml of MgCl₂ (3.3) with 45 ml D.W.
5) Mix solution of 3) and 4)
6) Add 10 g NaCl.
7) Add 1 ml of 1% phenol red
8) Add 0.25 ml of Tween 20
9) Adjust pH to 7.4 – 7.6.

A.3.6 Citrate – acetate buffer stock

1) Stock 1 M citric acid.
   
   Citric acid    20 g
   Dissolve in D.W. 100 ml

2) Stock 1 M sodium acetate
   
   Sodium acetate 8.2 g
   Dissolve in D.W. 100 ml

3) Adjust pH of 100 ml. 1 M sodium acetate to pH 5.6 by adding 4 ml of 1 M citric acid.

A.3.7 Blocked diluent for dilute guinea pig serum and conjugate dilution

High grade of bovine serum albumin (BSA) is not necessary to be used in most of the test. Gelatin or skimmed milk could be used instead of BSA. The blocking diluent for ELISA typing assay is 3% BSA and for LP ELISA is 3% – 5% skimmed milk.

Preparation of blocked diluent (500 ml)

1) PBST      500 ml
2) Add 0.5% gelatin (V / V) or 3% – 5% skimmed milk powder (W / V) or 3% bovine serum albumin (W / V) in PBST.
3) Adjust pH to 7.4 – 7.6.
A.3.8 Chromogen / TMB substrate solution (3, 3’, 5, 5’ tetramethyl benzindine)

For 1% stock solution
1) TMB substrate 0.1 g
2) DMSO (Dimethylsulfoxide) 10.0 ml
3) dissolve TMB substrate DMSO, put aliquot 1 ml / tube, keep at 4 °C.

For working solution
4) Preparation of

Solution A: D.W. 9.0 ml
1% TMB 0.1 ml
Citrate acetate buffer 1.0 ml

Solution B: Dilute H₂O₂ 25 μl (30% W/V) in D.W. 400 μl
Add 25 μl of solution B in solution A when need to use or prepare substrate solution by
dissolve 1 tablet of TMB in 60 ml of citrate acetate buffer and add H₂O₂ (30% W / V) 30 μl

A.3.9 Stopping solution (1 N H₂SO₄)

conc. H₂SO₄ (36 N) 1 ml
D.W. 35 ml

A.3.10 Disinfectant

4% Na₂CO₃ solution (W / V)
10% Citric acid (W / V)
5% Iodophore solution (V / V)
ANNEX B

PREPARATION OF VIA ANTIGEN AND SOLUTION

B.1 PREPARATION OF VIA ANTIGEN BY ION EXCHANGE CHROMATOGRAPHY

FMDV infected fluid is prepared by inoculation high multiplication of infection (MOI) of FMDV in tissue culture, incubate at 37 °C for 9 -12 h.

Harvest the fluid and centrifuge. Collect infected fluid and mix with DEAE Sephardic A- 50 leave at 4 °C overnight with shaking. Pack DEAE into glass column 2.6 cm x 40 cm Wash the column with washing buffer of 0.02 M Tris-HCl pH 7.6 mixed with 0.15 N NaCl. Separate VIA antigen binded with DEAE sephadex by using eluting buffer of 0.02 M Tris - HCl pH 7.6 mixed with 1 N NaCl. Connect column to the control machine which was connected with fraction collector and measure optical density at 254 nm. Collect VIA peak after passed through the column and concentrating and purifying by precipitation with saturated (NH₄)₂ SO₄ and centrifuge at 4 °C for one hour. Collect only the precipitin and dissolve with 0.02 M Tris - HCl pH 7.6 mixed with 0.15 N NaCl then transfer to cellophane tube and dialysis at 4 °C for 2 - 3 days to separate salt NH₄⁺ and SO₄ from VIA antigen and test for specificity and optimal working dilution of VIA antigen. Put aliquot in microtubes and keep at -20 °C for future test.

B.2 PREPARATION OF BUFFER FOR VIA – AGID TEST

B.2.1 Stock buffer 0.2 M Tris - HCl pH 7.6
1) Tris (Hydroxymethyl aminomethan) 24.2 g
2) Dissolve in Deionized water 1,000 ml
3) Adjust pH to 7.6 by using 0.5 N HCl drip by drop
4) Add NaN₃ from stock 10% W / V 2 ml

B.2.2 Preparation of Buffer A (0.02 M Tris - HCl pH 7.6 mix 0.15 N NaCl)
1) 0.2 M Tris - HCl pH 7.6 (from item 1 (2.1)) 100 ml
2) Dissolve in Deionized water 900 ml
3) Add NaCl (analar grade) 8.766 g
4) Add NaN₃ from stock 10% W / V 2 ml

B.2.3 Preparation of Buffer B (0.02 M Tris - HCl pH 7.6 mix 1.0 N NaCl)
1) 0.2 M Tris - HCl pH 7.6 (from item 1 (2.1)) 100 ml
2) Dissolve in Deionized water 900 ml
3) Add NaCl (analar grade) 58.44 g
4) Add NaN₃ from stock 10% W / V 2 ml
B.2.4 Preparation of Saturated (NH$_4$)$_2$SO$_4$

1) (NH$_4$)$_2$SO$_4$ powder 1,000 g
2) 0.02 M Tris - HCl pH 7.6 300 ml
3) Put into warm water bath until saturated and crystallised

B.2.5 Disinfectant

4% Na$_2$CO$_3$ solution (W / V)
0.5% Iodophore
10% Citric acid
ANNEX C
PREPARATION OF REAGENTS FOR TISSUE CULTURE

C.1 7% NaHCO₂
1) NaHCO₂  7  g
2) D.W.    100 ml
Sterile filtration, keep at room temperature

C.2 10 x PBS
1) NaCl    80  g
2) KCl     2   g
3) Na₂HPO₄ 11.5  g
   or Na₂HPO₄·2H₂O  14.0  g
   or Na₂HPO₄·12H₂O 28.9  g
4) KH₂PO₄  2.0  g
Sterile by autoclave, keep at room temperature

C.3 EDTA 1%
1) EDTA    1   g
2) PBS     100 ml
Sterile by autoclave, keep at -20 °C

C.4 Trypsin 1%
1) Trypsin x 250 1  g
2) PBS     100 ml
Sterile at 4 °C overnight, sterile filtration, keep at -20 °C

C.5 Trypsin - Versine (TV)
1) 1% Trypsin  12.5 ml
2) 1% EDTA    2.5 ml
3) Dissolve in PBS 85 ml
Sterile filtration, keep at -20 °C

C.6 Fungizone
1) Fungizone  50 ml
2) D.W.     100 ml
Filter, put aliquot in 3 ml vial, keep at -20 °C
C.7 Antibiotics

Penicillin 100 - 200 U/ml
Streptomycin 10,000 - 20,000 mg/ml
Kanamycin 500 - 10,000 U/ml
Fungizone 100 g/ml

Filter, put aliquot in 3 ml vial, keep at -20 °C

C.8 Growth medium (GM) containing 5% Bovine serum

1) MEM 93 ml
2) Bovine serum 5 ml
3) Antibiotic 1 ml
4) Fungizone 0.5 ml
5) 7% NaHCO$_2$ 1 ml

C.9 Maintenance medium (MM) containing 2% Bovine serum

1) MEM 97 ml
2) Bovine serum 2 ml
3) Antibiotic 1 ml
4) Fungizone 0.5 ml
5) 7% NaHCO$_2$ 2.5 – 3.5 ml

C.10 Freezing medium

1) MEM 7 ml
2) Bovine serum 2 ml
3) 7% NaHCO$_2$ 0.15 ml
4) DMSO 1 ml
The units and symbols used in this standard and the units recognized by the International System of Units (Le Système International d’Unités) or SI are as follows:

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