STANDARD OPERATING PROCEDURE

Title: Liquid Phase Blocking ELISA (LPBE) for detection of antibodies against Foot-and-Mouth disease virus (FMDV)

1. MATERIALS

1.1. Equipment

Bench Centrifuge	Any manufacturer
Carrier plates	U-well polypropylene plates, any make
Cryotubes	Approved make
ELISA plates	Nunc, cat no. 442404; U-well - 96 well, any make. Note: it will be important to test representative plates of new batch of plates for their suitability before stockpiling.
ELISA plate reader	96 well spectrophotometer capable of reading at 490nm, linked to a PC for data capture. Any manufacturer
Hot Room/Incubator	Maintained between 35°C and 39°C
Pipettes and tips	Sterile, 10ml plastic, any manufacturer. Digital, single and multi-channel, ranging from 1µl-1000µl, multi-stepper, and filter tips.
Reagent Reservoirs	Any manufacturer, plastic
Rotary Shaker	Any manufacturer (Plates should be placed in a suitable box or container before being put on the shaker)
Universals	Sterile, plastic, any manufacturer
Waterbath	Any manufacturer, maintained at 54-58°C

1.2. Reagents and Consumables

Water	De-ionised or distilled,
Phosphate Buffered Saline (PBS)	This may be stored at room temperature until the expiry date indicated on the label of the container
Phenol red (0.1% solution)	Add to PBST if required to aid visualisation
Normal bovine serum	Non-immune adult bovine serum, any make
Glycerol	For -20^{o}C storage of virus, any make, sterilised.
FMDV antigen/virus	Cell culture derived FMDV virus or inactivated antigen of the appropriate serotype. Store stock between –90^{o}C and -50°C, if stock is glycerinated it can be stored at between -30^{o}C and -5^{o}C.
Control sera (reference serum)	Anti-FMD serotype specific strong positive antiserum Anti-FMD serotype specific weak positive antiserum Anti-FMD antibody negative serum Store between –30^{o}C and -5^{o}C
Trapping antibody	Serotype specific rabbit anti-FMDV serum. Before use, the rabbit serum is diluted 1/5 with PBS and stored at -30°C to -5°C. When in use, an aliquot can be stored at +1°C to 8°C.

Detecting antibody	Serotype specific guinea pig anti-FMD serum. Before use, the serum is blocked by adding an equal volume of normal, non-immune bovine serum and incubating for 1 hour +/- 5 minutes at room temperature. The blocked serum is then diluted 1/5 with PBS and aliquoted. The stock of pre-blocked and pre-diluted (final 1/10) serum is kept at -30°C to -5°C. When in use, an aliquot can be stored at +1°C to 8°C.
Conjugate	Horseradish peroxidase conjugated rabbit anti-guinea pig immunoglobulin (DAKO- P0141 or equivalent). Block conjugate by adding an equal volume of normal bovine serum (NBS) to it and incubating for 1 hour +/- 5 minutes at room temperature. The blocked conjugate is further diluted up to 1/5 with PBS (*e.g*. 1ml conjugate + 1ml NBS + 3ml PBS) and then glycerinated by adding an equal volume of sterile glycerol (final 1/10). The stock of pre-blocked/diluted and glycerinated conjugate is kept at -30°C to -5°C. When in use, an aliquot can be stored at +1°C to 8°C.
Coating buffer	Dissolve the contents of one carbonate/bicarbonate capsule (Sigma C-3041 or equivalent) in 100ml filtered deionised water to give a 0.05 M carbonate/bicarbonate buffer. This may be stored at +1°C to 8°C for 1 month.
Wash Buffer	Store at room temperature until the expiry date indicated on the label of the container
Diluent (PBST**)**	PBS containing 0.05% Tween-20 and 2% (v/v) Phenol red. Note: Tween-20 is highly viscous. This may be stored at +1°C to 8°C for 3 months.
Blocking buffer	PBST containing 5% skimmed milk powder (Marvel). Make fresh for each test and keep at room temperature during use
Phosphate-citrate buffer	Dissolve the contents of one Phosphate-Citrate Buffer tablet (Sigma P-4809 or equivalent) in 100ml filtered de-ionised water to give 0.05 M phosphate citrate buffer. This may be stored at room temperature for 2 weeks.
Chromogen	Dissolve 1 30mg OPD tablet (Sigma, P-8412) in 55mls of phosphate-citrate buffer. Use immediately but any excess can be stored at -30°C to -5°C in the dark for 3 months. Thaw and use immediately. As OPD is potentially carcinogenic, the operator must always wear gloves when preparing and handling this solution.
Substrate solution	Add 1/2000 dilution of 30-33% hydrogen peroxide to the Chromogen solution just before use. OPD is potentially carcinogenic so gloves must be worn when preparing and handling this solution
Acid Stopper solution	1.25M sulphuric acid. Add 68 ml of concentrated Sulphuric acid to 932 ml of filtered de-ionised water. The exact volume of acid required will vary with the purity of the preparation used. Store at room temperature**.** NOTE: Always add acid to water, never water to acid! NOTE: Wear safety glasses.
Samples	Biological samples (*e.g*. blood or serum samples), stored at either +1°C to +8°C pending assay or longer term at -5°C to -30°C
Plate lids/sealers	

1.3. Chemicals

Sulphuric acid	BDH 102760B or equivalent
Tween-20 non-ionic detergent	Sigma P-1379 or equivalent

Ortho-phenylenediamine Dihydrochloride (OPD, chromogen)	Suitably sized tablets, Sigma P-8412 or equivalent Store at +1°C to +8°C in dark
Hydrogen peroxide (substrate)	30% v/v (Sigma H-1009 or equivalent). Store at +1°C to +8°C in dark

2. PROCEDURE

2.1. Prior to the test, calculate the number of plates required with reference to the total number of samples to be tested.

NOTE: plate lids or sealers should be used as appropriate throughout the test procedure.

Preparation of sera

2.2. Prepare sera if required.

Test Procedure Day 1

2.3. Prior to the test, referring to **Appendix 1**, choose the plate layout or design as appropriate. The spot test (**a**) is only used for outbreak/herd surveillance samples. Half titration (**b**) can be used for testing samples where the majorities are expected to be negative, *e.g*. import-export testing. Full titration (**c**) is required for those expected to be positive *e.g*. when measuring immune status post vaccination or those giving reactions on screening.

2.3.1. Coating of ELISA plates

Prepare a 1/1000 or as appropriate working dilution of trapping antibody in coating buffer in a volume sufficient for the number of plates required (11 ml per plate *e.g.* 11µl of rabbit antibody stock in 11ml of coating buffer for 1/1000 dilution). Add 100µl to each well required, tap the plates gently to ensure uniform dispersion, cover the plate and place in a humidity cabinet at room temperature overnight (12 to 24hrs). Coated plates can be stored at room temperature for up to 5 days. Alternatively, plates can be coated at 35°C to 39°C for 1 hour +/- 5 minutes.

2.3.2. Addition of the control/test serum to a carrier plate for **titration**

Reference sera and test sera are titrated in duplicate wells against a reference antigen/virus in a carrier plate.

2.3.3. Add 50µl of PBST to all wells except the negative control wells (G3, G4, H3, H4 or as appropriate per plate layout). Add an extra 37.5µl of PBST to all wells in Row A. If doing a half plate titration also add 37.5µl to E5-E12.

2.3.4. Addition of the control sera: Add 12.5µl of the strong positive control to wells A1+A2 or as appropriate. Add 12.5µl of the weak positive control to wells A3+A4 or as appropriate. Referring to the plate layout, make a twofold dilution series by transferring 50µls from row to row consecutively discarding 50 µl from the last dilution. Add 50µl of the negative control (1/16 dilution of ABS with PBST) to wells G3, G4, H3 and H4 or as appropriate.

2.3.5. Addition of the test sera: referring to the plate layout, dispense 12.5µl of test sample to rows A (for the full titration plate layout) or rows A and E (for the half titration plate layout) columns 5-12 or as appropriate in duplicate. Using a multichannel pipette, make a twofold dilution series by mixing and transferring 50µls from row to row consecutively, discarding 50µl from the last dilution. Go to step 2.4.

2.3.6. Addition of the control/test serum to a carrier plate for the **spot test**

Addition of the control sera: dilute a strong positive serum 1/16 with PBST. Add 50µl to wells A, B, 1+2 or as appropriate. Dilute a weak positive serum 1/16 with PBST; add 50µl to wells C, D, 1+2 or as appropriate. Dilute negative serum 1/16 with PBST; add 50µls to wells E, F, 1+2 or as appropriate. Add 50µls of PBST to wells G1, G2, H1 and H2 or as appropriate (antigen controls).

NOTE: The final dilution of the control sera after addition of virus is 1/32. The strong positive serum may be titrated during a test for reference purposes either on a test plate or separate control plate.

2.3.7. Addition of the test sera: make a 1/22 serum dilution (5µl+105µl PBST). Add 25µl PBST and 25µl of 1/22 dilution to duplicate wells of a carrier plate. Go to step 2.4.

2.4. Dilute the virus/antigen with PBST to an optimal working concentration (allowing at least 6ml per plate). Add 50µl to each well of the carrier plate.

2.5. Stack the plates and cover the top plate with a lid. Incubate the plates at +1°C to 8°C overnight with continual shaking on an orbital shaker. Alternatively, plates can be incubated at 35°C to 39°C for 1 hour +/- 5 minutes with continual shaking.

Test Procedure Day 2

2.6. Wash the coated ELISA plates three times using a plate washer or wash manually: empty the well contents then flood the plates with wash buffer (PBS). After filling, discharge the contents. Repeat with two more washes. Slap the inverted plates onto a lint-free absorbent surface to remove residual contents.

2.7. Using a multichannel pipette, transfer 50µl of antigen/serum mixture from the carrier plates to the corresponding wells of the coated ELISA plates.

NOTE: For titrations, transfer row A first, followed by subsequent dilutions.

2.8. Stack the plates and cover the top plate with a lid and place into a tin or plastic airtight box. Incubate plates at 35°C to 39°C for 1 hour +/- 5 minutes on an orbital shaker.

2.9. Calculate the amount of blocking buffer needed for the plates. Allow 12ml per plate. (1gram Marvel to 20ml PBST) Dilute type-specific guinea pig antiserum in blocking buffer (6ml per plate).

2.10. Wash plates as 2.6.

2.11. Add 50µl of the diluted guinea pig antiserum to all wells of all plates. Stack the plates and cover the top plate with a lid. Incubate the plates at 35°C to 39°C for 1 hour +/- 5 minutes on an orbital shaker.

2.12. Dilute the conjugate in blocking buffer (6ml per plate).

2.13. Wash plates as 2.6.

2.14. Add 50µl of diluted conjugate to all wells of all plates. Stack the plates and cover the top plate with a lid. Incubate the plates at 35°C to 39°C for 1 hour +/- 5 minutes on an orbital shaker.

2.15. Wearing gloves prepare the OPD solution (6ml per plate). As described under section 1.2 Reagents and consumables.

2.16. Wash plates as step 2.6. Wash for a fourth time to ensure that the conjugate has been completely removed if using a plate washer. Ensure that the bottom of the plates is clean and dry.

2.17. Just before use, add the appropriate amount of 30-33% hydrogen peroxide to the prepared chromogen solution. Wearing gloves, add 50µl of substrate/chromogen solution to all wells of all plates including blank plates/wells. Incubate plates at room temperature for 15 +/-5 minutes or until in the opinion of the operator, an OD of between 0.4 and 2.5 is obtained in the antigen control wells.

2.18. During the colour development, turn on the ELISA plate reader and allow to warm up if required. Check that the appropriate interference filter (490nm) is in place.

2.19. Wearing gloves and safety glasses stop further colour development by adding 50µl of acid stopper solution (1.25M sulphuric acid) to all wells of all plates, including blank plates/wells, in the same order as the addition of substrate/chromogen. NOTE: This solution is a strong acid and can cause severe burns. Avoid contact with skin and eyes. After the addition of the stopping solution, immediately discard the tips from the multichannel pipette as the acid may cause corrosion

2.20. Before reading the micro plates ensure that there are no bubbles in any of the wells or any condensation on the bottom of the plate as this can cause optical aberrations. If necessary, rupture any bubbles with a clean pipette tip and wipe the bottom of the plate clean with a soft cloth. Ensure that there is no condensation or that there are no smudges (*e.g.* fingerprints) on the bottom of the micro plates.

2.21. Different photometers may have different initiation procedures. Please consult the manual for the photometer being used. Ensure the correct filter (490 nm for this procedure) is present before reading the plates

2.22. Blank the plate reader as appropriate. Place an ELISA plate in the plate reader and initiate the reading sequence.

2.23. Annotate all printout sheets as appropriate. For each plate, indicate the type of virus/antigen used and the batch numbers of sera tested. Calculate and assess the results as detailed in section 3 below.

3. RESULTS

3.1. Calculate the median optical density (OD) of the 4 antigen control wells of each plate as follows: a) identify the highest and lowest OD readings and ignore these two values b) calculate the average OD of the remaining 2 wells. This is the median OD of the reaction (antigen) control which represents the maximum value for the test *i.e*. the 100% control value. Divide the median OD of antigen control wells by 2. This is the OD value representing 50% inhibition of the reaction control.

3.2. Calculate the percentage inhibition of all wells using the following formula:

$$Percent\ Inhibiton\ (PI) = 100 - \frac{OD\ of\ test\ serum\ well}{mediam\ OD\ antigen\ control} \times 100$$

3.3. **Titration test**

3.3.1. Score the well positive if the PI is equal to or greater than 50. Consider the well negative if the PI is less than 50.

3.3.2. Calculate the titres of reference sera and test samples referring to **Appendix 2**. The end-point is defined as the dilution at which half of the wells show 50% inhibition.

3.3.3. Accept the assay if the results meet the following criteria

a. The titre of the reference serum should not fluctuate more than two-fold of the running mean.

b. The negative control should be <50% inhibition.

c. The titre of strong positive control sera should be >128.

d. The titre of weak positive control sera should be in the range of 45-128.

NOTE: Test results may still be accepted when either the weak positive or strong positive fall out of the range of criteria, on the condition that all other controls pass and at the discretion of the line manager or an experienced, competent operator.

3.3.4. Interpretation of results:

a. Imported /exported/post vaccination samples

The titre of a sample ≥45	positive	antibodies against FMDV present in a sample
The titre of a sample < 45	negative	no antibodies against FMDV present in a sample

b. Suspected FMD cases

The titre of a sample ≤64	negative
The titre of a sample ≥90	positive
The titre of a sample in the range of 65-90	inconclusive

c. Rebleeds

Refer to the current version of OIE manual for test criteria.

NOTE: Positive and inconclusive samples should be confirmed by VNT.

3.4. **Spot test**

3.4.1. Accept the assay if the results meet the following criteria

a. Strong positives should give >90% inhibition

b. Weak positives should give 50-90% inhibition

c. The average of the negatives must give <50% inhibition

NOTE: The weak positive may not result in the predicted reactivity. Test results may still be accepted when the weak positive exceeds 90% or is lower than 50% inhibition on the condition that all other controls pass and at the discretion of the line manager or an experienced competent operator.

3.4.2. Interpretation of spot results

3.4.2.1. Sera are considered positive if both wells show ≥ 50% inhibition *i.e.* have a titre of >1/90. Sera with only one or neither well showing 50% inhibition are negative.

NOTE: Positive sera should be titrated by ELISA or confirmed by VNT.

4. TROUBLESHOOTING

4.1. For optimal performance, equilibrate all reagents needed to room temperature before use.

4.2. It is useful to compare OD readings with the actual intensity of colour development in the wells to detect any discrepancies between the OD reading recorded and the colour intensities observed visually in case the plates haven't been properly positioned in the plate reader. If this is the case step 2.21 to 2.24 should be re-performed. High OD readings from wells which have obviously been badly washed should be discarded.

4.3. If the virus stock contains glycerol, remove the glycerinated FMDV stocks from the freezer and allow equilibrating to room temperature for 5-10 min prior to making the dilution series. As the glycerol is very viscous, care must be taken to ensure that the stock was well mixed before pipetting and that the pipette tip used to transfer the glycerinated antigen is filled with the correct volume and properly rinsed with the diluent buffer to which it is being added and that all of the antigen is dispersed in the diluent.

4.4. It is useful to keep a sample of the conjugate and the substrate used in a test until the end of that particular test. If a colour development problem occurs *e.g*. no, slow or rapid colour development (out of the range as described above) then the reagents can be checked as follows:

4.4.1. Pipette 100µl of substrate/chromogen solution used for the test (H_2O_2 + OPD solution at the correct concentration) into a well of a microtitre plate or into a tube. Add 50µl of conjugate as prepared for the test. Colour should develop within 30 seconds.

4.4.2. If still no colour develops: Prepare a new substrate/chromogen solution. Repeat the step 2.2.1 but use new substrate/chromogen solution.

4.4.3. If still no colour develops: Prepare a new conjugate solution and repeat 2.2.1 using the "old" and "new" substrate/chromogen solution.

4.4.4. If all the above tests fail to give satisfactory results it may be necessary to purchase new conjugate.

4.5. If the colour which develops is uneven (*i.e.* there is a large variation between duplicates of individual samples), more care is needed with procedures including pipetting, mixing or washing. Keep plates covered during incubation or storage to avoid uneven evaporation of fluid.

4.6. If the test results fail to meet set specifications check that equipment such as fridge/freezers is functioning properly. Refer to the records in the relevant log books. Refer to the test tracking sheet to identify if there have been any operator errors, *e.g*. that the appropriate batch/type of plate and reagents have been used, and that the reagents were properly diluted *etc*. Repeat the test using the same reagents, pipettes, working dilutions. If the repeat test is satisfactory it must be assumed that the original failure was due to either human error or an intermittent equipment failure.

4.7. If the repeat test is not satisfactory, it should be repeated using fresh aliquots of all the reagents. If this repeat test is satisfactory then it must be assumed that one of the reagents has lost activity. The individual faulty reagent may be identified using the systematic approach of replacing one reagent at a time with a fresh aliquot. Once identified, the suspect reagent should be discarded. Alternatively, if all reagents are held in small aliquots, the aliquots used for the original test should be discarded.

4.8. If any equipment subsequently fails a calibration check, test results may be accepted if they meet the criteria described above**.**

4.9. The working concentrations need to be optimized before a new virus/antigen or trapping/detecting antibody or conjugate is introduced into the test. When new control sera are introduced, they need to be tested several times until a good range of titres is obtained. The running mean may then be accepted as early results may be unrepresentative.

5. APPENDIX/ APPENDICES

5.1. LPBE Plate layout:

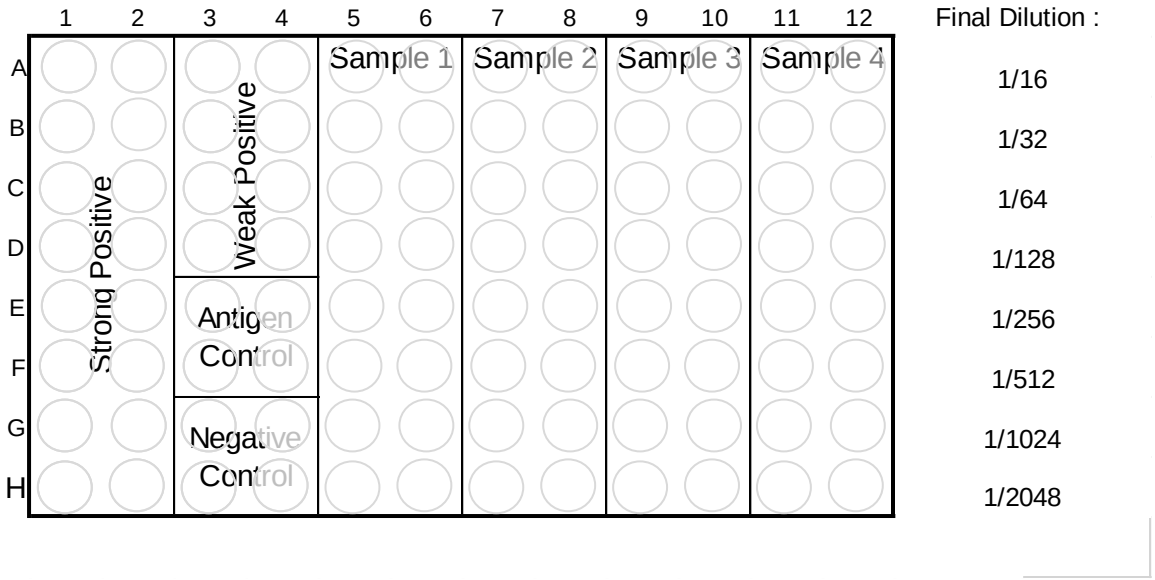
Plate Layout A – Spot Test (40 samples per plate)

	1 2	3 4	5 6	7 8	9 10	11 12	Final Dilution:
A	Strong Positive	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	1/90
B		Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	1/90
C	Weak Positive	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	1/90
D		Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	1/90
E	Negative	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	1/90
F		Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	1/90
G	Antigen Control	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	1/90
H		Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	1/90

Plate Layout B – Half-plate titration (8 samples per plate)

	1 2	3 4	5 6	7 8	9 10	11 12	Final Dilution:
A	Strong Positive	Weak Positive	Sample 1	Sample 3	Sample5	Sample 7	1/16
B							1/32
C							1/64
D							1/128
E		Antigen Control	Sample 2	Sample 4	Sample 6	Sample 8	1/16
F							1/32
G		Negative Control					1/64
H							1/128

Plate Layout C – Full-plate titration (4 samples per plate)



5.2- Karber titres for the serum tested in duplicate, based on numbers of positive* wells per serum dilution

Dilution of serum	**Serum (antibody) Titres****			
	One well/dilution positive		**2 wells/dilution positive**	
	Arithmetic	**Logarithm**	**Arithmetic**	**Logarithm**
1/16	**16**	**1.20**	**22**	**1.34**
1/32	**32**	**1.50**	**45**	**1.65**
1/64	**64**	**1.80**	**90**	**1.95**
1/128	**128**	**2.10**	**181**	**2.26**
1/256	**256**	**2.40**	**362**	**2.56**
1/512	**512**	**2.70**	**724**	**2.86**
1/1024	**1024**	**3.00**	**1448**	**3.16**
1/2048	**2048**	**3.30**	**2896**	**3.46**
1/4096	**4096**	**3.60**	**5792**	**3.76**
1/8192	**8192**	**3.90**	**11584**	**4.06**
1/16384	**16384**	**4.20**	**23168**	**4.36**
1/32768	**32768**	**4.50**	**46336**	**4.67**

* If the PI of a well is equal to or greater than the 50% it is considered to be positive.

**The serum titre is defined as the reciprocal of the final dilution at which half the wells show 50% inhibition or more.