STANDARD OPERATING PROCEDURE:

Two-Dimensional Virus Neutralisation Test (VNT) for Strain Differentiation

1. MATERIALS AND EQUIPMENT

1.1. Equipment:

Absorbent towels/tissues	Disposable or cloth, lint-free and non-abrasive
Reagent reservoirs	Reusable or disposable, any make
Disinfectant reservoir	Appropriate size(s) for use
Incubator	Range 35ºC to 39ºC, any make
Fridge	Range 1ºC to 8ºC, any make
Freezers	Range -30°C to -5°C, and -50ºC to -80ºC, any make
Plastic-ware	Sterile plastic universals and bijoux, bottles or similar
Transmitter	Comark or similar
Pipettes	Single and multi-channel Finnpipettes (or equivalent), Finnpipette multi-stepper and Eppendorf multipette
Pipette Tips	Finntips or similar, some with aerosol filter plug and Eppendorf combitips
Micro-plates	Nunc TC microwell 96 well (Fisher Scientific)
Plate sealers	Adhesive plastic 'BIS', or similar
Water bath	For heat inactivation, kept between 54°C to 60°C, any make
Microscope	Any make, objectives as appropriate

1.2. Reagents:

Foot-and-mouth disease virus*	Homologous to the BVS reference sera (vaccine strain) and any type specific heterologous field isolate required for comparison.
Bovine Vaccinal Serum (BVS) Reference serum*****	Ideally, BVS reference serum is derived from at least 5 cattle vaccinated with a monovalent, single vaccine dose.
IB-RS-2 cells	A cell suspension containing approximately 1 x 10^6 cells per ml.
Media	Eagle's MEM + HEPES (Sigma Cat no. M7278 or similar), add 2mls 1M NaOH and 1ml field antibiotics to 500mls pH is 7.4 – 7.6.
1M NaOH solution	Any make

Field Antibiotics	
Citric acid crystals (disinfectant)	Riedel-de Haen 27102 or similar 0.2 – 0.8% w/v solution in tap water with a small amount of detergent.
Detergent	Hederol or similar
FAM (disinfectant)	Evans Vanodine International - Dilute FAM 30 in tap water to form a 1x solution 1:240 or 2x solution 1:120
Amido Black Stain (ABSt)	Add 0.2% w/v citric acid crystals to physiological saline, then add 0.2% w/v Amido Black powder
0.85% saline	
Phosphate buffered saline	

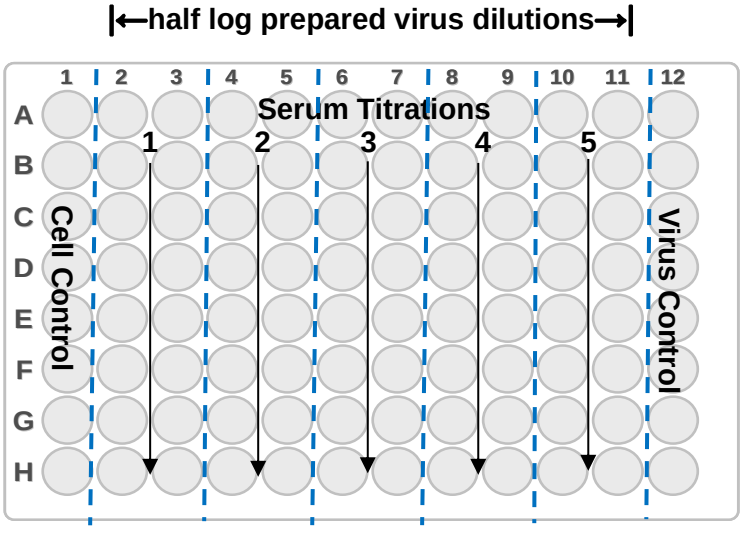
2. PROCEDURE

Historically, field isolates do not produce high yields of virus particles when grown in IB-RS-2 tissue culture and rarely reach a concentration of more than $10^{5.0}$, unless serial passaging is taken to length. To save time, the titration step can be omitted, providing acceptable cytopathic effect (CPE) of 80-100% is observed in tissue culture ideally within 24 hrs. In the following example, the pre-determined virus titre (*e.g.* $10^{4.5}$) is either ascertained by analysis or is assumed, and the half log dilution series for this example is $10^{-1.5}$ to $10^{-3.5}$, which ensures the theoretical 2 log dose ($10^{-2.5}$) is midway (*i.e.* in the middle of the plate).

2.1. Prepare plates as shown below:

Two plates are needed for each virus analysis: virus neutralisation (VN) plate and a virus titration (VT) plate. An additional virus titration plate is required for a control virus titration. This is utilised in every test in order to help analyse the test conditions (especially cell sensitivity and operator technique).

Virus Neutralisation Plate Layout:

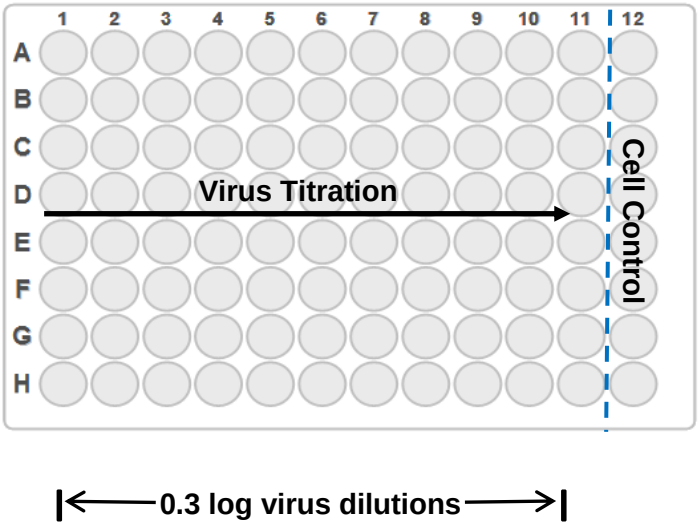


2.1.1. Add 100µl/well of media to all wells in column 1 (cell control) and 50µl/well to all wells in columns 2-12.

2.1.2. Add 50µl/well of BVS reference sera to the top well rows A2-A11. If neat serum is used, the initial serum dilution will be ½.

2.1.3. Serial-dilute 50µl serum 'down' the plate (*i.e.* A-H) using a multichannel pipette, ensuring to mix well throughout. Discard 50µl from the last row.

2.2. Virus Titration Plate Layout:



2.2.1. Add 50µl/well of media to columns 2-12. Leave column 1 empty.

2.3. Virus dilution series

2.3.1. Using the example detailed in section **5.** as a guideline, set out as many appropriate containers as needed for each virus to be tested, including the BVS reference virus, and label accordingly.

2.3.2. Aliquot the required volume of media into appropriate containers. The dilution series on the plates are in $10^{-0.5}$ dilution steps. Table 1 below gives an example using the previous theoretical field virus titre. These volumes are sufficient to test one field virus against three BVS reference sera.

Table 1 – Example on an initial virus titre of $10^{-4.5}$ (1/32000)

Volume of media	Volume of Virus	Log dilution	Arithmetic dilution	Expected virus dose
6.2 ml	0.2 ml	$10^{-1.5}$	1/32	$10^{-3.0}$ (1/1000)
4.4 ml	2 ml	10^{-2}	1/100	$10^{-2.5}$ (1/320)

4.4 ml	2 ml	$10^{-2.5}$	1/320	$10^{-2.0}$ (1/100)
4.4 ml	2 ml	10^{-3}	1/1000	$10^{-1.5}$ (1/32)
4.4 ml	2 ml	$10^{-3.5}$	1/3200	$10^{-1.0}$ (1/10)

2.3.3. Carry out the virus dilutions in the containers. For the example above (Table 1), in a MSC transfer 0.2 ml stock virus suspension into the first ($10^{-1.5}$) dilution bottle, mix well, and discard tip. Using a new tip, remove 2 ml of this dilution and transfer into the second (10^{-2}) bottle, mix, and again discard the tip. Remove 2 ml from this bottle and transfer to the third ($10^{-2.5}$). Continue transferring 2 ml of the previous virus dilution to the end of the series. Repeat for all the test viruses. Adjust the above volumes if a different dilution series is needed.

2.4. Set-up the VN and VT plates

2.4.1. For the VN plate add 50µl/well of the appropriate virus dilution to each well in paired columns. For the example above add the $10^{-3.5}$ dilution in columns 10, 11 and 12 (virus control), $10^{-3.0}$ in columns 8 and 9 and so on. Repeat for each VN plate.

2.4.2. For the VT plate add 100µl/well of chosen virus dilution (*e.g.* $10^{-3.5}$) to each well in column 1 (in the example above this is taken from the 5th dilution bottle) and dilute two-fold (0.3log) 50µl across the plate from column 1 to column 11, Discard the last 50µl.

2.4.3. For the VT plate overlay every well with 50µl of media.

2.4.4. Stack all plates and cover. Leave for 1 hour (+/- 15 minutes) in the cabinet at room temperature (22 ± 3 ºC).

Alternative method, mix 50% media with 50% IB-RS-2 cell suspension in a sterile container, then add 100µl of the media/cell mixture to all wells on all virus titration plates. Seal each of the virus titration plate.

2.4.5. After incubation (step 5.5.4.) add 50µl of IBRS2 cells to every well on all the plates. Seal each plate and incubate for approximately 72 hours at 35-39°C.

Note: *If following the "alternative method", add 50µl of IBRS2 cells to the virus neutralisation plates only.*

2.5. Stain plates:

2.5.1. Prepare a suitably sized wash container with ≥0.8% citric acid in unsterile PBS with a dash of detergent. Also prepare a discard container with ≥0.2% citric acid concentration/water/detergent disinfectant, and place in cabinet.

2.5.2. Treating each plate in a turn, remove the plate sealer and place into the discard container. Submerge the plate in the citric acid/PBS wash and agitate gently, ensuring the whole plate is disinfected. Once all plates have been submerged, leave to disinfect for a minimum of 30 minutes.

2.5.3. Remove the plate(s) from the PBS container and discard the disinfectant from the plate back into the container, tap each plate on an absorbent paper to expel any excess solution.

Note: *At this point the plates can be removed from the MSC cabinet.*

2.5.4. Fill the stain reservoir with Amido black stain (ABSt) and dispense 50µl of stain into every well on all test plates. Dispense excess stain back into the stain bottle. Leave the plates for at least 30 minutes - up to 1 hour (after the last plate has been stained) at room temperature (22 ± 3 ºC).

Note: *In case of bubbles forming in some of the plate wells when adding ABst, dispense another 50µl of stain into every well in that column*.

2.5.5. Transfer the stained plates to the sink and wash each plate under cold running water.

3. RESULTS / OUTPUTS

3.1. Virus titre:

3.1.1. Each dilution step has a maximum of eight wells where the cells can form into a monolayer. If each individual well contains sufficient infectious virus particles, cell death will occur and no cell monolayer will form. Count all wells which show signs of CPE (count all wells without ABSt stain). Virus titre* is calculated by the formula in SAU-FORM-20, or as follows if we assume column 1, 2 and 3 exhibit 100% CPE and column 7-11 column have no CPE:

Column 3	8 wells	exhibiting 100% CPE at virus log. dilution $10^{-4.1}$
Column 4	7 wells	exhibiting 100% CPE at virus log. dilution $10^{-4.4}$
Column 5	4 wells	exhibiting 100% CPE at virus log. dilution $10^{-4.7}$
Column 6	1 well	exhibiting 100% CPE at virus log. dilution $10^{-5.0}$

$$\frac{\text{Total number of wells exhibiting 100\% CPE}}{\text{Number of wells per dilution}} = \frac{20}{8} = 2.5$$

Subtract 0.5 (correction factor) = 2.0
Multiply by 0.3 (dilution interval) = 0.60

Add the highest dilution step with 100% CPE in all wells ($10^{-4.1}$) = $10^{-4.7}$
The virus titre is expressed as **$10^{4.7}$ tcid50 per 50µl of virus stock**

3.1.2. From this result, the log dilutions used in the neutralisation plate can be extrapolated. For example, the 'strongest' dilution of virus used was $10^{-1.5}$, which would be determined as 4.7 log (virus titre) minus 1.5 (virus dilution factor), resulting in the log dose of 3.2 (*i.e.* 1585 tissue culture infective

units). Similarly the rest of the dilution steps used in the neutralisation plate can be calculated.

3.2. Antibody titre:

3.2.1. Each serum dilution step has a maximum of two wells where the cells can form into a monolayer. If the dilution step contains sufficient antibody levels, infectious virus particles will be neutralised, and a cell monolayer will form. Antibody titre is calculated by the presence of these cell sheets and all wells are counted (count all wells with ABSt stain).

3.2.2. In order to calculate this, the antibody titre for each virus dilution must be obtained. Again, the titre is at the point where 50% of the cell monolayers remain. If the example from above is used the initial dilution was 1/2 (or 0.3 log.) after the addition of neat sera. However, this was again diluted 1/2 by the addition of 50µl of the virus dilution, making the actual serum dilution 1/4 (or 0.6 log.) See Table 2 below.

Table 2

Complete well(s)	Neat serum log titre*****	1/2 serum log titre	1/4 serum log titre	1/8 serum log titre	1/16 serum log titre
1	0.60	0.90	1.20	1.50	1.80
2	0.78	1.08	1.38	1.68	1.98
3	0.90	1.20	1.50	1.80	2.10
4	1.08	1.38	1.68	1.98	2.28
5	1.20	1.50	1.80	2.10	2.40
6	1.38	1.68	1.98	2.28	2.58
7	1.50	1.80	2.10	2.40	2.70
8	1.68	1.98	2.28	2.58	2.88
9	1.80	2.10	2.40	2.70	3.00
10	1.98	2.28	2.58	2.88	3.18
11	2.10	2.40	2.70	3.00	3.31
12	2.28	2.58	2.88	3.18	3.49
13	2.40	2.70	3.00	3.31	3.60
14	2.58	2.88	3.18	3.49	3.78
15	2.70	3.00	3.31	3.60	3.90
16	2.88	3.18	3.49	3.78	4.08

***** Serum placed on the plate.

3.2.3. The 2log antibody titres are calculated from regression data as the $\log_{10}$ reciprocal antibody dilution required for 50% neutralisation of 100 tissue culture infective units of virus. This is carried out for all neutralisation titres.

Note:

- *Example 2log calculation formula in Microsoft Excel: 2log = TREND(serum titre array, virus dose array, 2)*

3.2.4. **r_1 values:**

Calculate the 'r_1' value using the following formula if arithmetic antibody titres are used:

$$r_1 = \frac{\text{heterologous serum titre (field isolate)}}{\text{homologous serum titre (vaccine strain}}$$

$$\text{e.g. } r_1 = \frac{110}{200} = 0.55$$

If using log antibody titres value:

$$r_1 = \text{antilog of (heterologous titre} - \text{homologous titre)}$$

$$\text{e.g. } r_1 = \text{heterlogous titre } (2.04) - \text{homologous titre } (2.3) = -0.26$$
$$r_1 = \text{antilog of} - 0.26 = 0.55$$

3.3. Acceptance criteria: Tests are considered valid provided that:

3.3.1. The cell sheet in each cell control well must be intact and not showing viral CPE-like effects (wells with ABSt stain). If the cell sheet is not intact (wells without ABSt stain).the virus plate should fail unless the reason for its occurrence can be justified.

3.3.2. The virus control on the neutralisation plate must show viral CPE-like effect (wells without ABSt stain). If the cell sheet is intact (wells with ABSt stain).the neutralisation plate should fail unless it can be justified.

3.3.3. There must be a clear end-point to all virus titres and neutralisation titres. If there is not a clear end-point the plate(s) must fail.

3.3.4. All homologous and control virus titres must be within ± 0.3log of the running mean. The test can be accepted if the homologous virus titre has failed but the neutralisation titre has passed; however, this is at the discretion of an experienced operator.

3.3.5. All homologous neutralisation titres must be within ± 0.3log of the running mean.

3.3.6. The R^2 value on the neutralisation plates must be greater than or equal to 0.85 (85%). If it is less than 85% the plate fails.

3.3.7. The 2 log virus dose is (ideally) within the middle three on the plate. If the 2 log virus dose is not on the plate then the plate fails.

3.3.8. Each test must be repeated and the heterologous neutralisation titre of the two tests must be within a two-fold log of each other.

3.4. Interpretation of results:

3.4.1. $\mathbf{r_1} \geq 0.3$ - Suggests that there is a close antigenic relationship between the field isolate and vaccine strain tested. A potent vaccine containing the vaccine strain is likely to confer protection.

3.4.2. $\mathbf{r_1} < 0.3$ - Suggests that the field isolate is antigenically different from the vaccine strain and that the vaccine is unlikely to protect.

3.4.3. No regression (NR) = no neutralisation at a virus dose of $100TCID_{50}$ for the field virus was observed.

3.4.4. At least two valid results are required for each test. The mean of r_1 values for each of these tests will be reported.

4. MAINTENANCE & MONITORING

4.1. Some viruses may form small 'plaques' and the cell sheet may appear intact. If in doubt, examine microscopically to determine if the plaque is viral or not. Generally speaking, the edges of a plaque will be ragged, disrupted and showing cells with classic CPE if it is caused by virus. If the edge is connected, this may indicate a physical 'hole or tear' in the sheet, and therefore classed as non-viral. Any viral plaques present in an otherwise healthy cell sheet, are recorded as a '+'.

4.2. When the last aliquot of pre-diluted BVS reference serum is taken from storage, it is necessary to make a fresh stock dilution batch and record appropriately. Make a maximum of 200 ml of BVS stock dilution and store at -80^{o}C in aliquots of 25ml. Test the new batch of BVS in parallel with the previous batch (three tests). If similar results (example within a two-fold) are obtained an experienced operator can accept the BVS.

4.3. Compare the running mean of the homologous neutralisation titres three times over a twelve-month period, to ensure the neutralisation titre has remained stable and not fluctuated by more than ± 0.3.

5. TROUBLESHOOTING

5.1. If the cells used in the test do not demonstrate a suitable level of susceptibility to viral replication as shown by the inclusion of a control virus titration plate, the test must be repeated. However, as this is a comparative study of field isolates, the relationship between the vaccine virus and the field virus will still be constant, and the results can be discussed with the line manager.

5.2. If the serum used is of abnormal toxicity, incorrect neutralisation titres may be obtained. This may be due to incorrect serum inactivation. Heat inactivate the serum again and repeat the test in parallel with a new aliquot of diluted BVS.

5.3. Test failure maybe due to alkaline BVS serum. Consider discarding alkaline serum (decision to be

made by an experienced operator).

6. APPENDIX / APPENDICES

N/A

7. GLOSSARY OF TERMS

N/A