STANDARD OPERATING PROCEDURE:

Detection of antibodies against vesicular and related viruses by the virus neutralisation test (VNT)

1. MATERIALS

1.1. Equipment

Bench centrifuge	Any make
Bottles	Sterile, plastic bijoux, universals, bottles or similar
Class 2 microbiological safety cabinet	Any make
Cryotubes	Approved make
Freezers	Any make kept between -30°C and -5°C and -50°C and -80°C.
Incubator	Any make kept at +35°C to +39°C.
Microplates	Nunc TC microwell, 96 well
Micro-plate sealers	Any make
Microscope	Any make, objectives as appropriate
Pipettes	Single and multiple channel Finnpipettes (or equivalent), Finnpipette multistepper and Eppendorf multipette.
Pipette Tips	Finntips or similar, some with aerosol filter plug and Eppendorf combitips
Reagent reservoirs	Reusable or disposable, any make
Refrigerator	Any make, kept between +1°C and +8°C
Water bath	Any make, for heat inactivation - kept between 54°C and 60°C

1.2. Reagents and consumables

Cells	IB-RS-2, at an approximate concentration of 1 $X10^6$ per ml
Diluent	Eagle`s MEM + HEPES (Sigma M-7278 or similar), add 2mls 1.0M NaOH and 1ml antibiotics
Disinfectant solutions	FAM (Evans) diluted 1/240 in water for FMDV and VSV and diluted 1/100 for SVD, made daily – or see current Disease Security regulations

Field antibiotics	Amphotericin B, Penicillin 10MU, Neomycin 25,000µg/ml, Polymixin B 100,000U/ml, Sterile Water
Glycerol	Any make, dispensed into aliquots and autoclaved
Isolates of the viruses	Foot-and-Mouth Disease Virus (FMDV) Swine Vesicular Disease Virus (SVDV), Vesicular Stomatitis Virus (VSV)
1M NaOH solution	Any make
Reference Sera (RS)	Convalescent or vaccinal serum (homologous or heterologous) against standard virus isolates can be used

NOTE: A new control is tested and validated before it is introduced into routine diagnostic testing. The control will be accepted only if the test passes the criteria as described in section 3.1.

NOTE: Future known positive VSV samples will be tested separately from diagnostic samples, to avoid contamination with positive samples.

2. PROCEDURE

Preparing Sera

2.1. Dilute the serum samples received. This is usually 1/4 making the first dilution on the plate 1/8. If only small amounts of serum are available a first well dilution of 1/16 is acceptable. If higher dilutions are used it will not be possible to ascertain negative results.

Testing new controls

2.2. A new virus control is titrated to obtain a titre and then tested with potential reference serum controls to obtain serum titres. It is not necessary for the serum control to be homologous to the virus control as long as it gives an acceptable titre *i.e.* ≥ $\log_{10}1.8$. Once a reference serum control has been selected three sets of test plates should be set up, (2 Virus Titration plates and 2 Reference Serum plates per set) *i.e.* if a virus had a titre of 4.5 one set would be set up at 4.5 and a further two sets, either side of this titre 4.4 and 4.6, 12 plates in total (see appendix 1b and 1c).

Virus Neutralisation Test (VNT) Procedure

NOTE: Appendix 1a highlights the different plate layouts that can be used:

Layout A - Full plate titration for positive or unknown samples

Layout B – Spot test to screen sera

Other layouts are acceptable.

NOTE: If the virus titre is known this assay can be performed as a single dose VNT. In this case the reference serum plates are still run using three virus doses; however, the test serum is only run against one virus dose.

2.3. Mark plates for including virus, test date and operators' initial (see Appendix 1): Colour code the test plates and bottles of diluent. Three groups of plates need to be set-up:

2.3.1. Test Plates – Appendix 1a

2.3.2. Virus Titration – Appendix 1b; two plates are required; however, controls only need to be added to the first plate

2.3.3. Reference Serum – Appendix 1c; two plates are required

2.4. Dispensing Diluent:

2.4.1. Reference Serum Plates - 50µl of diluent into every well

2.4.2. Virus titration Plate - 50µl of diluent to all wells in columns 1-9

2.4.3. In the first virus titration plate that contains the controls put 150µl into A10-12 and 100µl into C10-12. On the same plate put 150µl serum free media (if using) in B10-12 and 100µl into D10-12. A10-12 and B10-12 are diluent controls. C10-12 and D10-12 are for cell controls.

2.4.4. Test plates – for plate layout A add 50µl to rows B-H. For plate layout B add 25µl to rows B, D, F and H.

NOTE: Serum free media can be used if and when available.

2.5. On the two reference serum plates add 50µl of reference serum to every well in row H. Mix the contents of each well in row H and transfer 50µls from each well into row G. Mix as above then transfer 50µl into row F. Continue this procedure and discard 50µl from row A.

NOTE: Reference serum should be appropriately pre-diluted, inactivated and stored frozen, such that upon reading, the 50% endpoint occurs about halfway up the plate when 100 $TCID_{50}$ are used.

2.6. On the test plates add the pre-diluted test serum (2.1). For plate layout A add 50µl of sera to rows A and B. Mix the contents of each well in row B and transfer 50µl from each well into row C. Mix as above and then transfer into row D. Continue all the way down the plate and discard 50µl from the last row. For plate layout B add 50µl of the pre-diluted test sera to rows A, C, E and G and 25µl of sera to rows B, D, F and H.

2.7. Check the expected virus titre. If, for example, if the virus titre is $\log_{10}4.8$ ($10^{-4.8}$) subtract $\log_{10}2.0$ (100 TCID virus dose) to give the dilution ($\log_{10}2.8$). Together with a fourfold ($\log_{10}0.6$) step on either side, this will result in the three virus challenge dilutions: $\log_{10}2.2$, $\log_{10}2.8$ and $\log_{10}3.4$.

2.8. Calculate the volume required for each virus dilution. All 4 control plates need approximately 4mls. Test plates require approximately 5mls each.

2.9. Dilute the glycerinated virus by making a 10^{-1} step, unless the antigen is of such low titre that different volumes must be used. To ascertain other dilutions, use the "Dilution Factor Conversion Table" (see 10.2 -Appendix 2) and dispense the correct volumes of diluent into bottles.

2.10. The second and third virus dilutions ($\log_{10}2.8$ and $\log_{10}3.4$) are made by making two further fourfold steps. After the third virus dilution, make six fourfold steps to dilute the virus past its 50% endpoint.

2.11. Dispense 50µls of the highest virus dilution into every well of column 9 of both virus titration plates. Similarly, add 50µls of the next highest dilution to column 8 and so on until the third dilution, which should be dispensed into column 3. Also add this virus dilution to section A-H 9-12 of both reference serum plates then to any test sera as appropriate Likewise, add the second virus dilution

to column 2 of the virus titration plate and section A-H 5-8 in the reference serum plates and to any test sera. Finish by adding the first virus dilution to column 1 in both virus titration plates, then section A-H 1-4 of both reference serum plates and to the test sera if applicable, and then cover with a plastic lid.

2.12. Incubate plates for 1 hour +/-30 minutes, at room temperature.

2.13. Dispense 50µl of cell suspension to all wells used in the test, except for the A and B10-12 (if using serum free) on the virus titration plate. Agitate the cell suspension occasionally to prevent sedimentation.

2.14. Cover the plates with micro-plate sealers, wipe with disinfectant and place in the incubator.

2.15. Read all plates in the following days microscopically.

2.16. For the virus control plates, wells showing any cytopathic effect are scored positive. Take final readings at any time when all of the acceptance criteria below are met. Ideally the virus titre should have achieved a steady maximum value, preferably after the minimum period of incubation of about 48 hours. However, readings taken at day 1 are acceptable if the control values are valid. SVD test plates should always be checked at day 1. Record the control values.

2.16.1. Virus Titration plates: add the number of wells which show CPE for both virus control plates together and enter into the "Sum" box for each dilution. For higher virus dilutions (lower concentration), add all totals less than 16 together to give as many full columns as possible, and accumulate the remainder in the next box along.

For example:

Log virus dilutions	2.2	2.8	3.4	4.0	4.6	5.2	5.8	6.4	7.0
CPE+ wells (sum of 2 plates)	16	16	16	16	14	8	5	0	0
Totals for calculation	16	16	16	16	16	11	0	0	0

2.16.2. Refer to the "calculation of virus control titres when diluted fourfold" (see 10.3- Appendix 3) to find the increment to be added to the last dilution in which all 16 wells showed CPE. In the case above this would be the $\log_{10}4.6$ dilution.

2.16.3. For the example above, add 0.71 to the virus dilution (4.6) at which all 16 wells showed CPE = 5.31 $TCID_{50}$.

2.16.4. To obtain the virus doses used for any given day's results, subtract the lowest virus dilution (2.2 from the virus titre (5.31) and record this figure (3.11) against 'Read 1 virus dose'. Repeat for the remaining two virus dilutions and record as appropriate. Thus, only the highest virus dilution giving a virus dose of 1.91 has given an acceptable virus dose (see 3.0).

2.17. For the reference serum control plates add the number of wells showing no CPE for both plates together enter into the 'Sum' box for each dilution (maximum in full column = 8/8 -v). For highest serum dilutions (lower concentration), add all totals less than 8 to give as many full columns as possible, and accumulate the remainder in the next box along.

For example:

Log reference serum dilutions	1.8	2.1	2.4	2.7	3.0	3.3	3.6	3.9
No CPE wells (sum of 2 plates)	8	8	8	8	6	3	1	0
Totals for calculation	8	8	8	8	8	2	0	0

2.17.1. Refer to the "calculation of reference serum control tires when diluted twofold" (see 10.4 - Appendix 4) to find the increment to be added to the last dilution in which all 16 wells showed no CPE. In the case above this would be the $\log_{10}3.0$ dilution

2.17.2. For the example above, add 0.23 to the reference serum dilution (3.0) at which the last full column where no CPE was observed (*i.e.* protected) = 3.23. Carry out this procedure for each virus dilution. Using the 3 virus doses and control serum titres calculate the serum titre at the $\log_{10}2$ dose of virus and record.

2.18. Record the results. A "-"sign denotes a well not showing any CPE. Those showing any CPE are denoted by a "+" sign. Calculate each serum titre with reference to the table in Appendix 5 (see 10.5) and select the most appropriate set of readings with reference to the acceptance criteria below. If toxicity is observed for any test serum, record "T" in the boxes affected; "C" indicates contamination and "U" any test serum well which is unreadable.

2.19. Plates containing re-bleed samples are double checked by another competent operator. Record control data.

2.20. Without removing their seals, discard used VNT plates directly into double autoclave bags in an infectious tin.

3. RESULTS

3.1. Acceptance criteria

Tests are considered valid provided that:

a) The virus dose delivered (final titre minus Challenge Dilution) is within the range 1.5 to 2.5 $TCID_{50}$.

b) The reference serum titre is within $\log_{10}$ 0.3 of the running mean, at an acceptable virus dose (see above).

c) The cell sheet in the cell controls is intact and not showing CPE-like effects.

Results for individual test sera are considered acceptable provided that:

a) The virus dose delivered (see above) is within the range 1.5 to 2.5 $TCID_{50}$.

b) Contamination or toxicity does not prevent the test sera being accurately read.

3.2. Interpretation of results

3.2.1. Assuming the criteria for acceptance of results are met, test sera are considered negative if they have a titre of 1/11 or less. If, due to a small volume of test sera, the starting dilution is 1/16 rather than 1/8, then <16 is given as a negative result. Sera may also be passed as negative with such titres if the virus dose is too low, and/or if the reference serum titre is too high. This is based on the conditions having been biased in favour of neutralisation occurring. Sera with titres greater than

1/11 but less than or equal to 1/32 are considered doubtful and recorded as “inconclusive” (import/export animals must be re-bled and retested or a veterinary inspection must be carried out). Serum titres greater than or equal to 1/45 are considered positive.

4. TROUBLESHOOTING

4.1. Repeat the test if the cells in the cell controls are of such poor quality that they cannot readily distinguished from those where CPE is present. If cells are suspected to be causing test failures, then a different revival or passage should be requested. It may be necessary to request a re-bleed if test sera prove to be extremely toxic or show CPE-like effects. Latter effects can result from incorrect inactivation. If one of either the virus control or reference serum control plates is unreadable it is possible to calculate control titres with one plate using the Kärber formula:

Kärber Calculation:

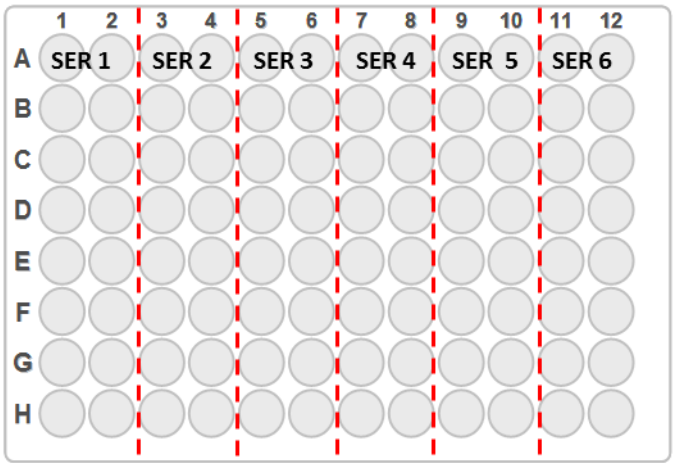
Virus Titre = L + d(S – 0.5) L = 1st dilution on the plate D = difference between log dilution steps (0.6) S = sum of wells showing CPE / total no. of wells per dilution 0.5 = constant	**Serum Titre = L + d(S – 0.5)** L = 1st dilution on the plate D = difference between log dilution steps (**0.3**) S = sum of wells showing **no** CPE / total no. of wells per dilution 0.5 = constant

5. APPENDIX/ APPENDICES

5.1. Appendix 1 – Plate Layouts

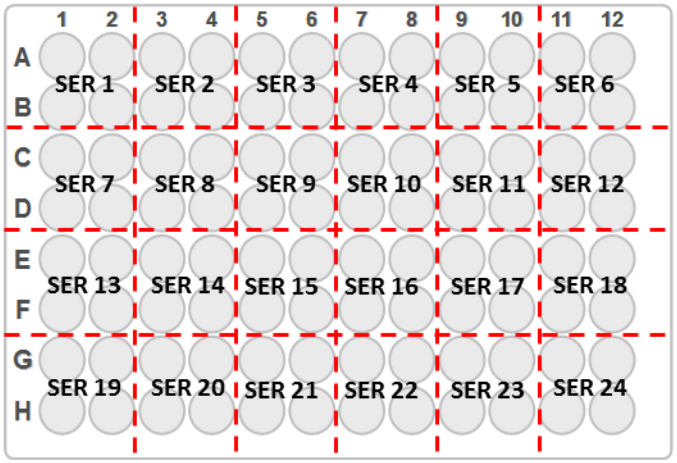
Appendix 1a – Plate layout for test samples

Layout A – full plate titration for positive or unknown samples



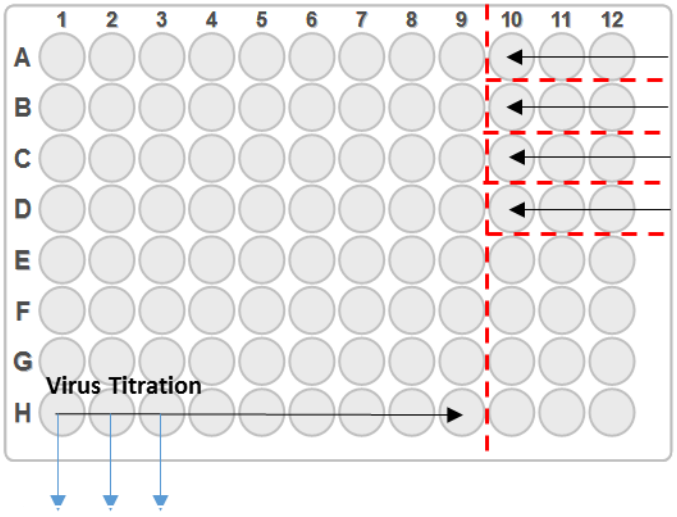
1. Add 50µl of diluent to rows B through H
2. Add 50µl of serum to rows A and B
3. Dilute from row B to H and discard last 50µl

Layout B - spot test to screen sera



1. Add 25µl of diluent to rows B, D, F and H
2. Add 50µl of test sera to rows A, C, E and G
3. Add 25µl of test sera to rows B, D, F and H

Appendix 1b – Virus Titrations



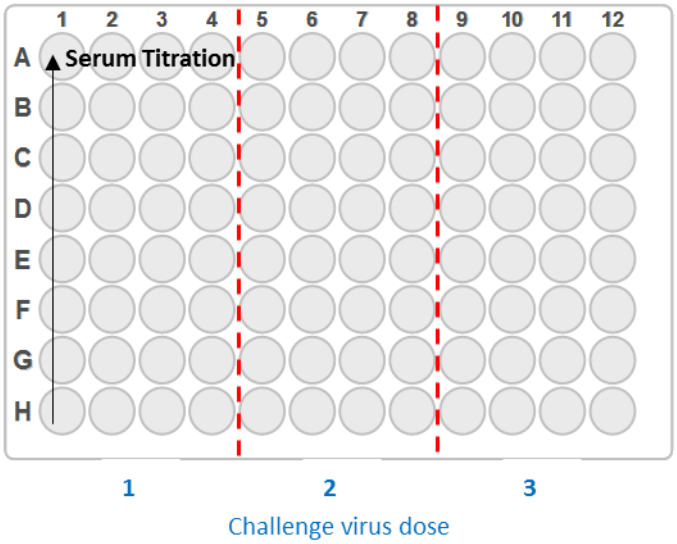
Diluent (medium) only

Serum Free Medium (If using)

Diluent and Cell control

Serum Free Medium and Cell Control (If using)

Appendix 1c – Reference Serum



5.2. **Appendix 2 – Dilution Factor Conversion table (logarithmic to arithmetic)**

p = mLs of previous dilution

x = mLs of diluent needed to make the next dilution

LOG STEP	ARITH-METIC p x	LOG STEP	ARITH-METIC p x
0.3	1 + 1	1.2	1 + 15
0.4	1 + 1.5	1.3	1 + 19
0.5	1 + 2.2	1.4	1 + 24
0.6	1 + 3	1.5	1 + 31
0.7	1 + 4	1.6	1 + 39
0.8	1 + 5.3	1.7	1 + 49
0.9	1 + 7	1.8	1 + 62
1.0	1 + 9	1.9	1 + 79
1.1	1 + 11.5	2.0	1 + 99

10.3 **Appendix 3 – Calculation of virus control titres when diluted fourfold ($log_{10}0.6$)**

WELLS WITH CPE	ADD TO LAST FULL COLUMN	WELLS WITH CPE	ADD TO LAST FULL COLUMN
0/16	0.30	8/16	0.60
1/16	0.34	9/16	0.64
2/16	0.38	10/16	0.68
3/16	0.41	11/16	0.71
4/16	0.45	12/16	0.75
5/16	0.49	13/16	0.79
6/16	0.53	14/16	0.83
7/16	0.56	15/16	0.86

10.4 **Appendix 4 – Calculation of reference serum control titres when diluted twofold ($log_{10}0.3$)**

WELLS PROTECTED	ADD TO LAST FULL COLUMN	WELLS PROTECTED	ADD TO LAST FULL COLUMN
0/8	0.15	5/8	0.34
1/8	0.19	6/8	0.38
2/8	0.23	7/8	0.41
3/8	0.26	8/8	0.45
4/8	0.30		

10.5 **Appendix 5 – Titres for sera tested in duplicate, based on numbers of protected well**

- = no CPE (neutralisation)

+ = CPE (no neutralisation)

8	16	32	64	128	256	512	1024	= reciprocal dilutions	
-	+	+	+	+	+	+	+	1/8 (arith)	0.9 (log_{10})
+	+	+	+	+	+	+	+		
-	+	+	+	+	+	+	+	1/11	1.05
-	+	+	+	+	+	+	+		
-	-	+	+	+	+	+	+	1/16	1.2
-	+	+	+	+	+	+	+		
-	-	+	+	+	+	+	+	1/22	1.35
-	-	+	+	+	+	+	+		
-	-	-	+	+	+	+	+	1/32	1.5
-	-	+	+	+	+	+	+		
-	-	-	+	+	+	+	+	1/45	1.65
-	-	-	+	+	+	+	+		
-	-	-	-	+	+	+	+	1/64	1.8
-	-	-	+	+	+	+	+		
-	-	-	-	+	+	+	+	1/90	1.95
-	-	-	-	+	+	+	+		
-	-	-	-	-	+	+	+	1/128	2.1
-	-	-	-	+	+	+	+		
-	-	-	-	-	+	+	+	1/178	2.25
-	-	-	-	-	+	+	+		
-	-	-	-	-	-	+	+	1/256	2.4
-	-	-	-	-	+	+	+		
-	-	-	-	-	-	+	+	1/355	2.55
-	-	-	-	-	-	+	+		
-	-	-	-	-	-	-	+	1/512	2.7
-	-	-	-	-	-	+	+		
-	-	-	-	-	-	-	+	1/708	2.85
-	-	-	-	-	-	-	+		
-	-	-	-	-	-	-	-	1/1024	3.0
-	-	-	-	-	-	-	+		
-	-	-	-	-	-	-	-	1/1413	3.15
-	-	-	-	-	-	-	-		