

**United States Department of Agriculture  
Center for Veterinary Biologics  
Testing Protocol**

**SAM 113**

**Supplemental Assay Method for Titration of Parainfluenza 3 Neutralizing  
Antibody (Constant Virus - Varying Serum Method)**

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**Supplemental Assay Method for Titration of Parainfluenza 3 Neutralizing Antibody  
(Constant Virus - Varying Serum Method)**

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**Supplemental Assay Method for Titration of Parainfluenza 3 Neutralizing Antibody  
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**1. Introduction**

This Supplemental Assay Method (SAM) describes an *in vitro* assay method that determines the serum neutralizing (SN) antibody titer to Parainfluenza 3 virus (PI<sub>3</sub>V) in Test Sera as part of the potency requirements for veterinary vaccines. The assay uses Madin-Darby bovine kidney (MDBK) cells and inhibition of viral cytopathic effect (CPE) as an indicator of the specific SN activity.

**2. Materials**

**2.1 Equipment/instrumentation**

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

**2.1.1** Incubator, 36° ± 2°C, high humidity, 5% ± 1% CO<sub>2</sub>

**2.1.2** Water bath, 37° ± 1°C; 56° ± 1°C

**2.1.3** Pipettors, 50-µL and 500-µL and tips

**2.1.4** Vortex mixer

**2.1.5** Microscope, inverted light

**2.1.6** Multichannel pipettor, 50- to 300-µL x 8- or 12-channel

**2.1.7** Centrifuge and rotor

**2.2 Reagent/supplies**

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

**2.2.1** PI<sub>3</sub>V Positive Control

**2.2.2** MDBK cells free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR).

**2.2.3** Diluent Medium (National Veterinary Services Laboratories [NVSL] Media #20030)

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1. 9.61 g minimum essential medium with Earles salts without bicarbonate
2. 2.2 g sodium bicarbonate ( $\text{NaHCO}_3$ )
3. Dissolve with 900 mL deionized water (DW)
4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL of DW. Heat to  $60^\circ \pm 2^\circ\text{C}$  until dissolved. Add to **Section 2.2.4(3)** with constant mixing.
5. Q.S. to 1000 mL with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).
6. Sterilize through a 0.22- $\mu\text{m}$  filter.
7. Aseptically add:
  - a. 10 mL L-glutamine
  - b. 50  $\mu\text{g}/\text{mL}$  gentamicin sulfate
8. Store at  $4^\circ \pm 2^\circ\text{C}$ .

**2.2.4 Growth Medium**

1. 900 mL of Diluent Medium
2. Aseptically add 100 mL of gamma-irradiated fetal bovine serum (FBS).
3. Store at  $4^\circ \pm 2^\circ\text{C}$ .

**2.2.5 Maintenance Medium**

1. 980 mL of Diluent Medium
2. Aseptically add 20 mL of gamma-irradiated FBS.
3. Store at  $4^\circ \pm 2^\circ\text{C}$ .

**2.2.6 Alsever's Solution**

1. 20.5 g dextrose ( $\text{C}_6\text{H}_{12}\text{O}_6$ )
2. 8.0 g sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7\cdot 2\text{H}_2\text{O}$ )

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3. 4.2 g sodium chloride (NaCl)
4. 0.55 g citric acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>)
5. Q.S. to 100 mL with DW.
6. Filter through a 0.22- $\mu$ m filter.
7. Store at 4 $^{\circ}$  $\pm$  2 $^{\circ}$ C.

**2.2.7 Phosphate buffered saline 10X (PBS)**

1. 8.0 g NaCl
2. 0.2 g potassium chloride (KCl)
3. 0.2 g potassium phosphate, monobasic, anhydrous (KH<sub>2</sub>PO<sub>4</sub>)
4. 1.15 g sodium phosphate dibasic, anhydrous (Na<sub>2</sub>HPO<sub>4</sub>)
5. Q.S. to 1000 mL with DW, adjust the pH to 7.0-7.3 with 5N sodium hydroxide (NaOH) and autoclave at 15 psi, 121 $^{\circ}$  $\pm$  2 $^{\circ}$ C for 35  $\pm$  5 minutes.
6. Store at 4 $^{\circ}$  $\pm$  2 $^{\circ}$ C.

**2.2.8 1X PBS**

1. 100 mL 10X PBS
2. 900 mL DW
3. Store at 4 $^{\circ}$  $\pm$  2 $^{\circ}$ C

**2.2.9 Guinea pig red blood cells (RBCs) in an equal volume of Alsever's Solution**

**2.2.10 Trypsin Versene (TV) Solution**

1. 8.0 g NaCl
2. 0.40 g KCL
3. 0.58 g NaHCO<sub>3</sub>

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4. 0.50 g irradiated trypsin
5. 0.20 g versene or disodium salt ethylenediaminetetraacetic acid (EDTA)
6. 1.0 g dextrose
7. 0.4 mL 0.5% phenol red
8. Q.S. with DW to 1000 mL
9. pH to 7.3 with NaHCO<sub>3</sub>
10. Filter through a 0.22- $\mu$ m filter.
11. Store at  $-20^{\circ}\pm 4^{\circ}\text{C}$ .

2.2.11 Tissue culture plates, 96-well

2.2.12 Polystyrene tubes, 17 x 100-mm

2.2.13 Conical tube, 50-mL

2.2.14 Serological pipette, 10-mL

2.2.15 PI<sub>3</sub>V inactivated negative serum control (NSC)

2.2.16 PI<sub>3</sub>V inactivated positive serum control (PSC)

2.2.17 Plastic wash bottle, 500-mL

**3. Preparation for the test**

**3.1 Personnel qualifications/training**

Personnel must have training in cell culture technique and the propagation and maintenance of animal viruses. Personnel shall have an understanding of the immunological basis of SN assays and the principles of aseptic technique.

**3.2 Preparation of equipment/instrumentation**

3.2.1 On the day of test initiation, set a water bath at  $56^{\circ}\pm 2^{\circ}\text{C}$ .

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**3.2.2** On the day of test initiation, set a water bath at  $37^{\circ}\pm 1^{\circ}\text{C}$ .

**3.3 Preparation of reagents/control procedures**

**3.3.1 Preparation of MDBK Plate**

Cells are prepared from healthy, confluent MDBK cells that are maintained by passing every  $5 \pm 2$  days. Two days prior to test initiation, cells are removed from the growth containers by using TV Solution. Using a multichannel pipettor, add 200  $\mu\text{L}$ /well of  $10^{5.4}$  to  $10^{5.6}$  cells/mL cells suspended in Growth Medium into all wells of a 96-well cell culture plate. Prepare 1 MDBK plate for the controls and PI<sub>3</sub>V Positive Control Working Dilution. Each additional plate allows testing of 2 Test Serials either pre- or postvaccinations. These become the MDBK Test Plates. Incubate at  $36^{\circ}\pm 2^{\circ}\text{C}$  in a CO<sub>2</sub> incubator for 2 days  $\pm$  12 hours. Growth Medium is not changed unless excess acidity occurs or cells are not confluent in 48 hours.

**3.3.2 Preparation of the PI<sub>3</sub>V Positive Control Working Dilution**

**1.** On the day of test initiation a vial of PI<sub>3</sub>V Positive Control is rapidly thawed in a  $36^{\circ}\pm 2^{\circ}\text{C}$  water bath and diluted in Diluent Medium to contain 50 to 300 50% tissue culture infectious doses (TCID<sub>50</sub>) per 25  $\mu\text{L}$ . Prior testing of the virus is used to determine the dilution needed to obtain 50 to 300 TCID<sub>50</sub> for testing. This becomes the PI<sub>3</sub>V Working Dilution.

**2.** The PI<sub>3</sub>V Working Dilution is back titrated by preparing serial tenfold dilutions ( $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$ ) and allowing them to remain at room temperature ( $23^{\circ}\pm 2^{\circ}\text{C}$ ).

**a.** Place 4.5 mL of Diluent Medium into 3, 17 x 100-mm tubes labeled  $10^{-1}$ - $10^{-3}$  using a 10-mL serological pipette.

**b.** Using a 500- $\mu\text{L}$  pipettor, transfer 500 $\mu\text{L}$  of PI<sub>3</sub>V Working Dilution to the  $10^{-1}$  tube; mix by vortexing. Discard pipette tip.

**c.** Using a new pipette tip, transfer 500  $\mu\text{L}$  from the  $10^{-1}$  labeled tube to the  $10^{-2}$  tube; mix by vortexing. Discard pipette tip.

**c.** Repeat **Section 3.3.2(2.c)** to the remaining tube, transferring 500  $\mu\text{L}$  from the previous dilution.

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**3.3.3 Preparation of PSC and NSC**

On day of test initiation, serial twofold dilutions of inactivated PSC and NSC are made from the original whole sera in a 96-well tissue culture plate labeled as the Transfer Plate (**Appendix I**). The NSC final dilutions range from 1:2 to 1:16 (rows A - D). The range of the PSC twofold final dilutions will depend on its SN titer which has been previously determined.

1. With a multichannel pipettor, dispense 150  $\mu$ L of Diluent Medium/well into rows B through H.
2. With a 200- $\mu$ L pipettor, add 150  $\mu$ L of PSC to wells A11 and B11, and 150  $\mu$ L of NSC to wells A12 and B12 on the Transfer Plate. Use a new tip for each serum control.
3. With a multichannel pipettor, mix the samples by aspirating and expelling  $7 \pm 2$  times in the wells of row B11 and B12, then transfer 150  $\mu$ L of the mixture to the corresponding wells of row C. Discard the tips.
4. Using new pipettor tips, repeat **Section 3.3.3(3)** mixing row C and transferring to row D. Continue in this manner for the remaining wells in each column changing tips between dilutions until twofold serum dilutions are completed. Change tips, mix and discard 150  $\mu$ L from the highest dilution of both the PSC and NSC.

**3.3.4 Preparation of 0.5% RBC suspension for HAd test**

1. Upon receipt of the RBCs, transfer 20 mL of RBCs to a 50-mL conical tube.
2. Q.S. to 50 mL with Alsever's Solution.
3. Mix by inverting several times.
4. Centrifuge for  $15 \pm 5$  minutes at 400 x g (1500 rpm in the J6B centrifuge with a JS-4.0 rotor).
5. Remove supernatant and buffy coat by aspirating with a 10-mL serological pipette.
6. Repeat **Sections 3.3.4(2) through 3.3.4(4)** for a total of 3 washes.

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7. Pipette 500  $\mu$ L of packed RBCs to 100 mL of 1x PBS; mix by inverting for a 0.5% RBC suspension
8. Store at  $4^{\circ} \pm 2^{\circ}\text{C}$ ; use within one week of collection of RBCs.

**3.4 Preparation of the sample**

**3.4.1** On day of test initiation or prior to testing (inactivation of pre- and postserums maybe done at time of collection), serums are heat-inactivated at  $56^{\circ} \pm 2^{\circ}\text{C}$  for  $30 \pm 5$  minutes.

**3.4.2** On day of test initiation the Test Serums (pre- and/or postvaccination serums) are diluted in the Transfer Plate from 1:2 to 1:256 in rows A-H (**Appendix I**) of a 96-well tissue culture plate labeled as the Test Serum Transfer Plate as described for the PSC and NSC in **Sections 3.3.3(1)** through **3.3.3(4)**.

**4. Performance of the test**

**4.1** On day of test initiation with new pipette tips, dispense 150  $\mu$ L of the PI<sub>3</sub>V Working Dilution to each well of the Control and Test Serum Transfer Plate. Mix by gently tapping the sides each Transfer Plate. The addition of the PI<sub>3</sub>V Working Dilution to the wells results in an additional twofold serum dilution (final serum dilution).

**4.2** Incubate the Transfer Plates at  $36^{\circ} \pm 2^{\circ}\text{C}$  for  $60 \pm 10$  minutes in a CO<sub>2</sub> incubator.

**4.3** Aseptically decant Growth Medium from the MDBK Test Plates into a suitable container.

**4.4** Using an 8 channels of an 8-channel or 12-channel multipipettor, inoculate 50  $\mu$ L/well of the virus-serum mixture into 5 wells for each dilution of the Test Serum into the MDBK Test Plate (see **Appendix II**). (All 8 dilutions of 1 Test Serum will be inoculated on cells at once.) Replace tips for each unique Test Serum column being tested. This becomes the PI<sub>3</sub>V Test Plate.

**4.5** Inoculate 5 wells/dilution with 50  $\mu$ L/well of both the PSC and NSC.

**4.6** Inoculate 5 wells/dilution with 25  $\mu$ L/well of the PI<sub>3</sub>V Working Dilution and Back Titrations.

**4.7** Maintain 5 or more wells as uninoculated cell culture controls per plate.

**4.8** Incubate the PI<sub>3</sub>V Test Plates at  $37^{\circ} \pm 1^{\circ}\text{C}$  in a CO<sub>2</sub> incubator for  $60 \pm 10$  minutes for adsorption.

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- 4.9** After incubation, add 200  $\mu$ L/well of Maintenance Medium to the PI<sub>3</sub>V Test Plates with a multichannel pipettor.
- 4.10** Incubate the PI<sub>3</sub>V Test Plates undisturbed at 37 $^{\circ}$  $\pm$  1 $^{\circ}$ C in a CO<sub>2</sub> incubator for 4 days  $\pm$  6 hours.
- 4.11** At the end of incubation, read the PI<sub>3</sub>V Test Plates at 100X magnification on an inverted light microscope and examine for CPE characterized by cell fusion.
- 4.11.1** Results of the Test Serum, PSC and NSC are recorded as the number of CPE negative (CPE -) wells versus total number of wells examined for each dilution.
- 4.11.2** Results of the PI<sub>3</sub>V Working Dilution and Back Titration are recorded as the number of CPE positive (CPE +) wells versus total number of wells examined for each dilution.
- 4.12** If CPE is not detected by microscopic examination, the PI<sub>3</sub>V Test Plates may be read by HAd as follows:
- 4.12.1** Decant the Maintenance Medium from the PI<sub>3</sub>V Test Plates into a suitable autoclavable container and rinse cells once with 1X PBS at room temperature using a plastic wash bottle or by immersion into a pan with 1X PBS at room temperature. Decant the 1X PBS immediately after filling.
- 4.12.2** Add 200  $\mu$ L of the 0.5% RBC Suspension to each well.
- 4.12.3** Incubate the plates for 15  $\pm$  5 minutes at room temperature.
- 4.12.4** Decant the 0.5% RBC suspension and wash the cell monolayer 3 times with 1X PBS as in **Section 4.12.1**.
- 4.12.5** Decant the final wash and examine the monolayers using an inverted light microscope at 100X magnification. Wells containing one or more RBC clusters adhering to the cell monolayer are considered to be positive for PI<sub>3</sub>V.
- 4.13** Calculate each endpoint of the Test Serum, PSC, and NSC using the Spearman-Kärber method as commonly modified by Finney. The endpoints of the Test Serum, PSC, and NSC are reported as SN titer which corresponds to the reciprocal of the highest serum dilution that neutralizes PI<sub>3</sub>V.

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Example:

1:2 dilution of Test Serum = 5 of 5 wells CPE negative  
1:4 dilution of Test Serum = 5 of 5 wells CPE negative  
1:8 dilution of Test Serum = 3 of 5 wells CPE negative  
1:16 dilution of Test Serum = 0 of 5 wells CPE negative

Titer =  $(X - d/2 + [d * S])$  where:

X =  $\text{Log}_{10}$  of lowest dilution (=0.3)  
d =  $\text{Log}_{10}$  of dilution factor (=0.3)  
S = Sum of proportion of CPE -  $(13/5=2.6)$   
Titer =  $(0.3 - 0.3/2 + [0.3 * 13/5]) = 0.93$   
antilog of 0.93 = 8.5

Titer of the Test Serum is 1:9

**4.14** By the same Spearman-Kärber method, calculate the endpoint of the PI<sub>3</sub>V Back Titration. The titer is expressed as  $\text{log}_{10}$  tissue culture infective doses fifty (TCID<sub>50</sub>) per 25  $\mu\text{L}$  dose.

Example:

$10^0$  dilution of the PI<sub>3</sub>V Back Titration = 5 of 5 wells CPE positive  
 $10^{-1}$  dilution of the PI<sub>3</sub>V Back Titration = 5 of 5 wells CPE positive  
 $10^{-2}$  dilution of the PI<sub>3</sub>V Back Titration = 3 of 5 wells CPE positive  
 $10^{-3}$  dilution of the PI<sub>3</sub>V Back Titration = 0 of 5 wells CPE positive

Titer =  $(X - d/2 + [d * S])$  where:

X =  $\text{Log}_{10}$  of lowest dilution (=0)  
d =  $\text{Log}_{10}$  of dilution factor (=1)  
S = Sum of proportion of CPE +  $(13/5=2.6)$

Titer =  $(0 - d/2 + [1 * 1.6]) = 2.1$

antilog of 2.1 = 125.9

Titer of the PI<sub>3</sub>V Back Titration is 126 TCID<sub>50</sub>/25  $\mu\text{L}$  dose in the test.

## **5. Interpretation of the test results**

**5.1** For a valid assay the following criteria must be met; otherwise the test is considered a **No Test** and is repeated without prejudice.

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**5.1.1** The NSC must have an SN titer of <1:2.

**5.1.2** The SN titer of the PSC should vary by no more than twofold from its mean titer as established from a minimum of 10 previously determined SN titrations.

**5.1.3** The uninoculated cell controls can not exhibit any CPE or cloudy media that would indicate contamination.

**5.1.4** The PI<sub>3</sub>V Back Titration titer must be between 50 to 300 TCID<sub>50</sub>/25 µL.

**5.2** For a **SATISFACTORY TEST**, the postvaccination titers shall meet the requirements as stated in an Animal and Plant Inspection Service (APHIS) filed Outline of Production (fourfold increase from the prevaccination titers).

**5.3** If postvaccination titers are less than the requirements in an APHIS filed Outline of Production, the sera will be retested (first retest).

**5.3.1** If the titers of the Test Serum from the first valid retest are less than the required titers in an APHIS filed outline of Production, the serial is **UNSATISFACTORY**.

**5.3.2** If the titers of the Test Serum from the first valid retest are greater than or equal to the titer in an APHIS filed Outline of Production, the serum will be retested (second retest).

**5.3.3** If the titers of the Test Serum from the second valid retest are greater than or equal to the titer in an APHIS filed Outline of Production, the serial is **SATISFACTORY**.

**5.3.4** If the titers of the Test Serum from the second valid retest are less than the titers in an APHIS filed Outline of Production, the serial is **UNSATISFACTORY**.

**6. Report of test results**

**6.1** Results are reported as SN titers.

**6.2** Record all test results on the test record.

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**7. References**

**7.1** Code of Federal Regulations, Title 9, Part 113.6, U.S. Government Printing Office, Washington, DC, 2007.

**7.2** Finney, DJ 1978. *Statistical method in biological assay*. Griffin, London. 3rd edition, pg 508.

**7.3** Cottral, GE, (Ed.) 1978, *Manual of standard methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY, pg. 731.

**8. Summary of Revisions**

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes impacting the test were made from the previous protocol, the following changes were made to the document:

- Joseph Hermann has been added as a Contact for this document.
- **1:** Primary bovine embryonic kidney cell cultures have been replaced by Madin-Darby bovine kidney-A cells.
- **2.2.3:** The description of antibiotics added to the Diluent Medium has been changed to reflect antibiotics used. Penicillin and streptomycin have been replaced by gentamicin sulfate, and amphotericin B has been eliminated from the formulation.
- **2.2.10:** The 24-well cell culture plates have been replaced by 96-well plates.

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Appendix I  
Dilution/Transfer Plate

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b> <b>1:2</b>	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	TS9	TS10	TS11	TS12
<b>B</b> <b>1:4</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>C</b> <b>1:8</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>D</b> <b>1:16</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>E</b> <b>1:32</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>F</b> <b>1:64</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>G</b> <b>1:128</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>H</b> <b>1:256</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

TS= Pre- or Post-Test Serum

Control/Transfer Plate

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b> <b>1:2</b>	PCS	NCS	TS									
<b>B</b> <b>1:4</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>C</b> <b>1:8</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>D</b> <b>1:16</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>E</b> <b>1:32</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>F</b> <b>1:64</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>G</b> <b>1:128</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<b>H</b> <b>1:256</b>	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

PCS = Positive Control Serum

NCS = Negative Control Serum

TS = Test Serum

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Appendix II  
 PI<sub>3</sub>V Test Plate

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b> <b>1:2</b>	TS1	TS1	TS1	TS1	TS1	CC	TS2	TS2	TS2	TS2	TS2	CC
<b>B</b> <b>1:4</b>	↓	↓	↓	↓	↓		↓	↓	↓	↓	↓	
<b>C</b> <b>1:8</b>	↓	↓	↓	↓	↓		↓	↓	↓	↓	↓	
<b>D</b> <b>1:16</b>	↓	↓	↓	↓	↓		↓	↓	↓	↓	↓	
<b>E</b> <b>1:32</b>	↓	↓	↓	↓	↓		↓	↓	↓	↓	↓	
<b>F</b> <b>1:64</b>	↓	↓	↓	↓	↓		↓	↓	↓	↓	↓	
<b>G</b> <b>1:128</b>	↓	↓	↓	↓	↓		↓	↓	↓	↓	↓	
<b>H</b> <b>1:256</b>	↓	↓	↓	↓	↓		↓	↓	↓	↓	↓	

TS= Test Serum

CC= Cell Control

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PI<sub>3</sub>V Control Test Plate

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b> <b>1:2</b>	PCS	PCS	PCS	PCS	PCS	CC	NCS	NCS	NCS	NCS	NCS	CC
<b>B</b> <b>1:4</b>												
<b>C</b> <b>1:8</b>												
<b>D</b> <b>1:16</b>												
<b>E</b> <b>1:32</b>							WD 10 <sup>0</sup> dil					
<b>F</b> <b>1:64</b>							WD 10 <sup>-1</sup> dil					
<b>G</b> <b>1:128</b>							WD 10 <sup>-2</sup> dil					
<b>H</b> <b>1:256</b>							WD 10 <sup>-3</sup> dil					

PCS = Positive Control Serum  
NCS = Negative Control Serum  
CC = Cell Control  
WD = PI<sub>3</sub>V Back Titration