The Test for 50% Tissue Culture Infective Dose of PRRSV

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- **50% Tissue Culture Infective Dose ($TCID_{50}$) is the measure of infectious virus titer. This endpoint dilution assay quantifies the amount of virus required to kill 50% of infected hosts or to produce a cytopathic effect (CPE) in 50% of inoculated tissue culture cells. This assay may be more common in clinical research applications where the lethal dose of virus must be determined or if the virus does not form plaques. When used in the context of tissue culture, host cells are plated and serial dilutions of the virus are added. After incubation, the percentage of cell death (i.e. infected cells) is manually observed and recorded for each virus dilution, and results are used to mathematically calculate a $TCID_{50}$ result. Reed-Muench method is commonly used to calculate $TCID_{50.}$ The Reed–Muench method is a simple method for determining 50% endpoints in experimental biology , that is, the concentration of a test substance that produces an effect of interest in half of the test units.**

Contents

- **Materials**
- **Reed-Muench method (TCID50)**
- **Notes**

Materials

A. Cell cultures and reagents

- **Growth medium-MARC145 cells (GM)**
- DMEM (Dulbecco's Modified Eagle Medium)(with D-Glucose, L-glutamine, Phenol Red, Sodium Pyruvate)
- 8%FBS（Fetal bovine serum）
- 100 units/mL Penicillin
- 100μg/mL Streptomycin
- **Maintenance medium-MARC145 cells ((MM)**
- DMEM
- 2%FBS（Fetal bovine serum）
- 100 units/mL Penicillin
- 100μg/mL Streptomycin
- Virus: HP-PRRSV attenuated live vaccine

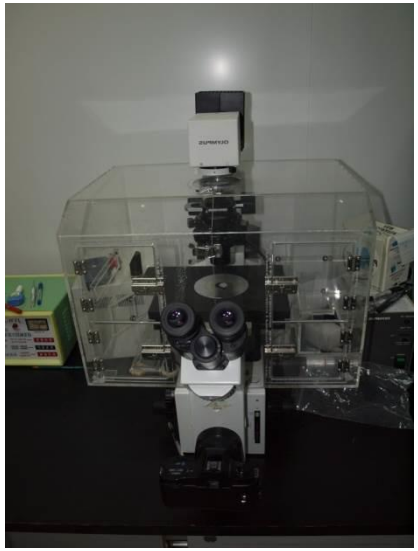
B. Supplies

- Cell culture flasks
- Cell culture microplates
- Sterile capped tubes.
- Assorted sterile pipettes and pipetting device including multi-channel pipette.
- Containers for discarding cultures.
- Cryogenic vials
- Centrifuge tubes
- Gloves
- Liquid waste container
- Pens/markers
- Cell record book

C. Equipment

- ✓ Class II biological safety cabinet.
- ✓ Water baths, 37℃ and 56℃.
- ✓ Incubator, 37℃, 5% CO2.
- ✓ Inverted microscope or standard microscope for the observation of cells.
- ✓ Freezer, - 70℃ (for long term virus storage) or 4℃/- 20℃(for serum storage)
- ✓ Low speed , bench top centrifuge preferably with refrigeration.
- ✓ Liquid nitrogen for cell storage.
- ✓ Automated cell counter

Carbon dioxide (CO2) incubator



Inverted microscope

Biological safety cabinet

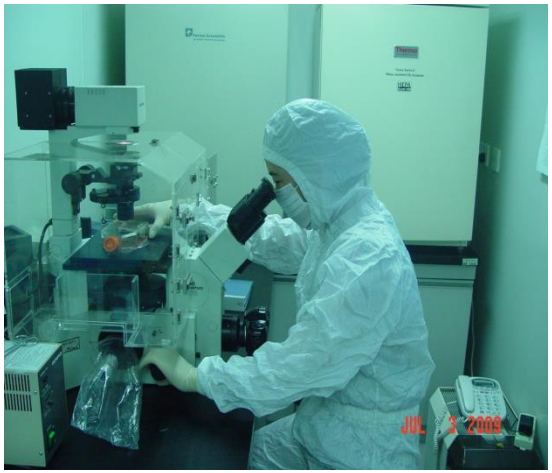
$TCID_{50}$ PROCEDURE

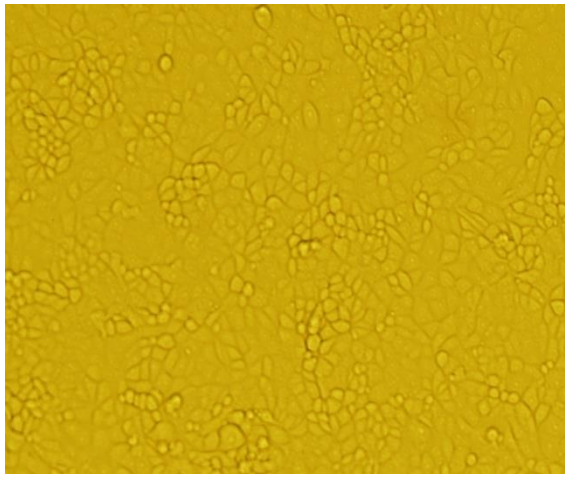
- ✓ 1. Preparation of infected cells
- ✓ 2. PRRSV dilution
- ✓ 3. PRRSV diluent distribution
- ✓ 4. Reading and $TCID_{50}$ calculation

PROCEDURE

1. Preparation of infected cells

- 1.96 well microplates are seeded with MARC145 cells (1.0×10^4cells /100μl/well) and incubated in a humidified 37℃ environment containing 5% CO_2.
- 2.After 24 hours, MARC145 cells have formed a 100% confluent monolayer, prepare to infect cells.





PROCEDURE

2.Dilution virus

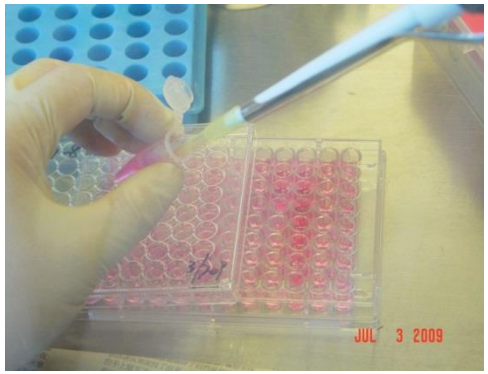
10 sterile 1.5millilitre(mL) centrifuge tubes were placed in centrifuge tube rack and dispense 900µl serum-free DMEM cell culture medium to each tube, and marked number 1-10. Add 100 µl PRRSV to the number 1(1/10 dilution), shake the tube and transfer 100 µl from number 1 to number 2(1/100 dilution), shake the tube and transfer 100 µl from number 2 to number 3(1/100 0dilution)….. shake the tube and transfer 100 µl from number 7 to number 8(1/100 000000 dilution). Discard 100 µl from the last dilution row. The number 9 is negative control and the number 10 is positive control. (table 1).

No. centrifuge tube	1	2	3	4	5	···8
PRRSV（µl）	100	100	100	100	100	..100
Serum-free cell culture medium（µl）	900	900	900	900	900	..900
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	.10^{-8}

PROCEDURE

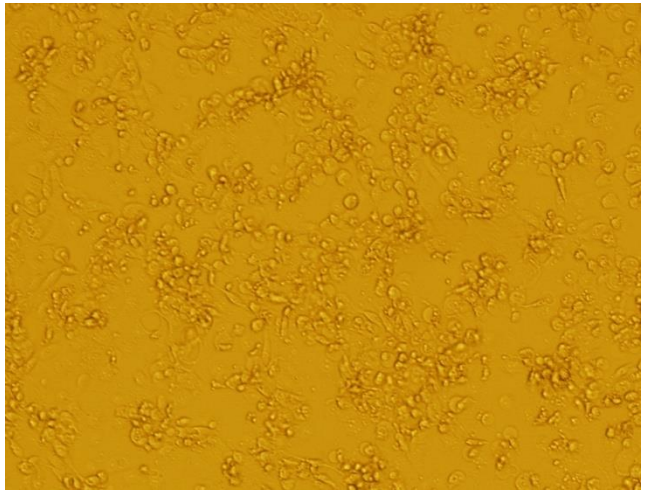
3. PRRSV diluent distribution

- 1. Remove medium from 96 well microplate and add 100μL of each PRRSV dilution with 6 duplications (table 2).
- 2. Incubate the plates for 1 hours in a humidified 37℃ incubator containing 5% CO2.
- 3. Add 100μl of 4% FBS supplemented DMEM to each well without discarding PRRSV dilution.
- 4. Incubate the plates in a humidified 37℃ incubator containing 5% CO2 for 3-5 days and observe daily for the CPE under inverted microscope and record results.

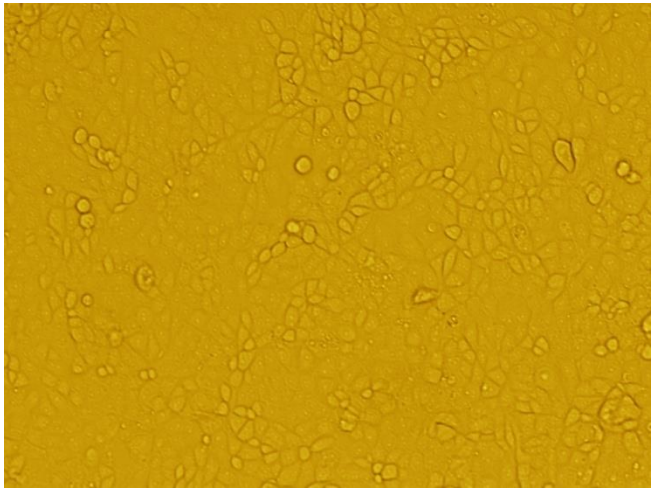


A\1	2	3	4	5	6	7	8	9	10	11	12
B	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Negative Control	Positive Control 对照	
C	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	
D	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	
E	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	
F	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	
G	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	100 μL	
H											

PROCEDURE



CPE



No CPE

- **Incubate for 2–5 days and observe daily for a CPE**

PROCEDURE

4. Reading and $TCID_{50}$ Calculation

- Calculation of $TCID_{50}$/mL of virus suspension using Reed-Muench method.
- **Formulae**
- $TCID_{50} = 10^{\text{log total dilution above 50\% - (I} \times \text{log h)}}$
- I (or Proportional) = (% of wells infected at dilution above 50% - 50%) /

(% of wells infected at dilution above 50% - % of wells infected at dilution below50%)

- I: Interpolated value of the 50% endpoint (also known as the proportional distance)
- h = dilution factorI

PROCEDURE

Observe CPE well

Example:

	1	2	3	4	5	6	7	8	9	10	11	12
A		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}				
B		+	+	+	+	+						
C		+	+	+	+							
D		+	+	+	+	+	+					
E		+	+	+	+	+						
F		+	+	+								
G		+	+	+	+	+						
H												

$TCID_{50}$ Calculation

Dilution of Inoculum	Check CPE well		Accumulated numbers		Total (A+B)	Percentage infected（%） A/(A+B) × 100%
	Number of wells infected (CPE +)	Number of wells not infected (CPE-)	Infected (CPE+) (A)	Not infected (CPE-)(B)		
10^{-3}	6	0	16	0	16	100（16/16）
10^{-4}	5	1	10	1	11	90.9（10/11）
10^{-5}	4	2	5	3	8	62.5（5/8）
10^{-6}	1	5	1	8	9	11.1（1/9）
10^{-7}	0	6	0	14	14	0（0/14）
…	…	…	…	…	…	…

$TCID_{50}$ Calculation

- I=（62.5-50）/（62.5-11.1）=0.24
- h=10
- Since each well was inoculated with 0.1 ml of each virus dilution, the $TCID_{50}$ is expressed as $TCID_{50}$/0.1 ml.
- $TCID_{50} = 10^{\text{log total dilution above 50\% - (I} \times \text{ log h)}} = 10^{-5 - (0.24 \times 1)} = 10^{-5.24}$ / 0.1 ml
- 1 mL of the virus suspension will contain ten times the reciprocal of the calculated dilution.
- Therefore infectivity titre of virus suspension in $TCID_{50}/mL = 10 \times 10^{5.24} = 10^{6.24}$

Note

- (a) Avoid the digestion cell uneven dispersion and affect the test result.
- (b) Using serum-free DMEM cell culture medium dilution virus should be at room temperature or below, avoid the temperature is too high. Dilution the virus and the operation are as far as possible fast .
- (c) Diluting virus sample should replace tip, at the same time to ensure that the tip installed firmly.
- (d) Add the diluting virus, the tip not move into the liquid.
- (e) Avoid the 96 well microplates edge effect, the edge well do not add the samples.

THANK YOU FOR YOUR ATTENTION!