



Standard operating procedure for the detection of peste des petits ruminants virus by real-time qPCR (differential RT-PCR for vaccine strain and wild strain)

1 Sample processing

Samples for PPR virus isolation: tissue; nasal and oral swabs

1.1 Equipment and Materials

Freezer, centrifuge, homogenator, Eppendorff tubes of 1.5 ml, nitrile gloves, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl), sterile tips (1-10 µl, 1-200 µl and 100-1000 µl), and Lyser beads.

1.2 Reagents

Phosphate buffer saline (PBS 1×) pH 7.2,

1.3 Methodology

Tissue samples:

a. Prepare a 1/10 homogenate of the tissue in sterile PBS (100 mg tissue per 1 ml PBS) using a homogenizer (Roche MagNA Lyser or equivalent).

b. Centrifuge at 1,000g (2000 rpm) for 5 minutes.

c. Transfer the supernatant to a new tube.

Swabs: directly squeezed or extracted with 1 mL PBS (pH 7.2)

2 Viral RNA extraction

2.1 sample preparation

Sample preparation is performed based on the sample processing procedure 1.3. 200µl of the sample is used for the further detection.

2.2 Equipment and Materials

Nucleic acid extractor, centrifuge, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl) and sterile tips (1-10 µl, 1-200 µl and 100-1000 µl).

2.3 Reagents

Viral DNA/RNA extraction kit: Magnetic beads pre-filled DNA/RNA extraction kits (Gefine, China)

2.4 Methodology

Viral RNA was manually extracted with High Pure viral Nucleic Acid kit (Roche) or automatically extracted by nucleic acid extraction system (Tianlong Science and Technology, Xi'an, China) according to the product instructions.

The extracted RNA was eluted in 100 µl of nuclease-free water for direct use or stored at -20°C.

3 Real-time RT-PCR

3.1 Materials and reagents

Nuclease-free water; Pipettes (1-10 µl, 10-100 µl and 10-200 µl) and sterile tips (1-10 µl, and 1-200 µl); 5 × Quantitative PCR reaction master mix (Q621, Vazyme); TaqMan probe and Primer mix;

PPRV primers and Probe at a concentration of 10 pmol/µl. Store<-20°C in aliquots. Expiry date: 1 year.

Forward primer sequence: 5'-AACGTGTCCTCAGTGTTTACCRT-3'

Reverse primer sequence: 5'-ATCTCGAAGACTCTTAAAAATGGCC-3'

Probe for **wild strain** sequence:



5'- VIC-TGCCAGGGATCCGAGCACCGA-BHQ1-3'

Probe for **all strain** sequence:

5'- FAM-CGGAAGAACATATACYGCTCTGGAGATCCG-BHQ1-3'

R+: positive control for the reaction

R-: negative control for the reaction

3.2 Methodology

3.2.1 Master mix preparation

RT-qPCR reaction mixtures are prepared in a sterile 1.5 ml micro centrifuge tube as described below. At least two extra samples mixtures are involved besides the samples to be tested (including controls).

Pipetting step	Reagents	volume
1	H ₂ O	10.2 µl
2	5 × reaction mix	4 µl
3	forward primer	0.8 µl
4	reverse primer	0.8 µl
5	FAM-probe	0.4 µl
6	VIC-probe	0.4 µl
7	ROX (dye II)	0.4 µl
Total volume		17µl

Add 17 µl of the Real-time PCR master mix to the 0.2 ml optical PCR tubes. Add 3µl of RNA template to each PCR tube. Include R+, R- for each PCR run. After addition of the template, cover the lid of reaction tube and spin down the PCR tube/plate.

Place all PCR tubes to real-time thermocycler. Run the program detailed below.

3.2.2 Real-time PCR program

Step	Temperature	Time	No. cycles
1	53°C	15 min	1
2	95°C	5 min	1
3	95°C	15 sec	40
	60°C	45 sec	

FAM fluorescence signal was collected at 60°C in each cycle.

VIC fluorescence signal was collected at 60°C in each cycle.

3.3 Interpretation of results

The procedure will be valid if R+ give a Ct value <35, and the R- do not show any Ct value.



For positive sample, a sigmoid-shaped amplification curve will be obtained, indicating the cycle number versus read fluorescence level, where the Ct value will be under 40. When the Ct values of both FAM fluorescence and VIC fluorescence channels in the tested sample are ≤ 37 and typical amplification curves are observed, the sample is judged as positive for the nucleic acid of wild-type PPRV. When only the FAM fluorescence channel shows a Ct value ≤ 37 with a typical amplification curve and the VIC channel does not, the sample is judged as positive for the vaccine strain.

For negative sample, the fluorescence level will be under the basal (background) fluorescence level and no Ct value is read-out by the equipment.



Standard operating procedure for the detection of peste des petits ruminants virus by real-time qPCR (universal)

1 Sample processing

Samples for PPR virus isolation: tissue; nasal and oral swabs

1.1 Equipment and Materials

Freezer, centrifuge, homogenator, Eppendorff tubes of 1.5 ml, nitrile gloves, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl), sterile tips (1-10 µl, 1-200 µl and 100-1000 µl), and Lyser beads.

1.2 Reagents

Phosphate buffer saline (PBS 1×) pH 7.2,

1.3 Methodology

Tissue samples:

a. Prepare a 1/10 homogenate of the tissue in sterile PBS (100 mg tissue per 1 ml PBS) using a homogenizer (Roche MagNA Lyser or equivalent).

b. Centrifuge at 1,000g (2000 rpm) for 5 minutes.

c. Transfer the supernatant to a new tube.

Swabs: directly squeezed or extracted with 1 mL PBS (pH 7.2)

2 Viral RNA extraction

2.1 sample preparation

Sample preparation is performed based on the sample processing procedure 1.3. 200µl of the sample is used for the further detection.

2.2 Equipment and Materials

Nucleic acid extractor, centrifuge, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl) and sterile tips (1-10 µl, 1-200 µl and 100-1000 µl).

2.3 Reagents

Viral DNA/RNA extraction kit: Magnetic beads pre-filled DNA/RNA extraction kits (Gefine, China)

2.4 Methodology

Viral RNA was manually extracted with High Pure viral Nucleic Acid kit (Roche) or automatically extracted by nucleic acid extraction system (Tianlong Science and Technology, Xi'an, China) according to the product instructions.

The extracted RNA was eluted in 100 µl of nuclease-free water for direct use or stored at -20°C.

3 Real-time RT-PCR

3.1 Materials and reagents

Nuclease-free water; Pipettes (1-10 µl, 10-100 µl and 10-200 µl) and sterile tips (1-10 µl, and 1-200 µl); 2 ×Quantitative PCR reaction master mix; TaqMan probe and Primer mix;

PPRV primers and Probe at a concentration of 10 pmol/µl. Store<-20°C in aliquots. Expiry date: 1 year.

Forward primer sequence: 5'-GAYTCCGAGTATGAGTATGAGGATG-3'

Reverse primer sequence: 5'-GCTATCATTATACTGGAMAGATGGCC-3'

Probe sequence: 5'- FAM-CGTGCAAGCATTGCYAARATCCATGA-BHQ1-3'.

R+: positive control for the reaction



R-: negative control for the reaction

3.2 Methodology

3.2.1 Master mix preparation

RT-qPCR reaction mixtures are prepared in a sterile 1.5 ml micro centrifuge tube as described below. At least two extra samples mixtures are involved besides the samples to be tested (including controls).

Pipetting step	Reagents	volume
1	H ₂ O	10.6 µl
2	5 × reaction mix	4 µl
3	forward primer	0.8 µl
4	reverse primer	0.8 µl
5	FAM-probe	0.4 µl
6	ROX (dye II)	0.4 µl
Total volume		17µl

Add 17 µl of the Real-time PCR master mix to the 0.2 ml optical PCR tubes. Add 3µl of RNA template to each PCR tube. Include R+, R-for each PCR run. After addition of the template, cover the lid of reaction tube and spin down the PCR tube/plate.

Place all PCR tubes to real-time thermocycler. Run the program detailed below.

3.2.2 Real-time PCR program

Step	Temperature	Time	No. cycles
1	53°C	15 min	1
2	95°C	5 min	1
3	95°C	15 sec	40
	60°C	45 sec	

FAM fluorescence signal was collected at 60°C in each cycle.

3.3 Interpretation of results

The procedure will be valid if R+ give a Ct value <35, and the R- do not show any Ct value.

For positive sample, a sigmoid-shaped amplification curve will be obtained, indicating the cycle number versus read fluorescence level, where the Ct value will be under 37.

For negative sample, the fluorescence level will be under the basal (background) fluorescence level and no Ct value is read-out by the equipment.



**Standard operating procedure for the detection of peste des petits ruminants virus
antibodies by blocking ELISA**

1. Introduction

To describe the use of blocking ELISA assay for the detection of antibodies against peste des petits ruminants virus.

2. Equipment and Materials

2.1 Equipment

96-well plate washer

96-well plate reader

Microcentrifuge for eppendorf tubes

Tubes shaker or vortex

Tube racks

Micropipettes of volumes 1-20 μ l, 20-200 μ l and 200-1000 μ l

-20°C or lower freezer

- 4°C fridge

2.2 Materials

Microcentrifuge tubes, sterile

Aerosol resistant tips

Disposable gloves

2.3 Reagents

1x Phosphate buffered saline (PBS) buffer, sterile

Antigen-coated plate

HRP-conjugated Mab (5 $\times$)

Positive serum

Negative serum

Diluent

Wash solution (20 $\times$)

Substrate solution

Stop solution

3. PROCEDURE

3.1 Sera preparation

1. Blood without anticoagulant is incubated for 1 hour at 37°C or overnight at 4°C for the separation of the coagulum.
2. Centrifuge at 780g (1500 rpm) for 10 minutes.
3. Transfer the supernatant into an Eppendorf tube.

3.2 Experimental preparation

All components of the kit must be brought to room temperature before use and mixed thoroughly prior to pipetting. The layout of PPRV negative control serum, PPRV positive control serum, diluent, and test samples on the antigen-coated plate is shown in Table 1 (where N- represents negative control serum wells, P- represents positive control serum wells, Cm- represents diluent wells, and S1, S2, S3, S4 and remaining wells represent test sample wells).

Table 1



	1	2	3	4	5	6	7	8	9	10	11	12
N												
B	N											
C	P											
D	P											
E	Cm											
F	Cm											
G	S1											
H	S2											

3.3 Wash solution (1×) preparation

Before use, take the 20× wash solution from the kit, equilibrate it to room temperature, and shake it to dissolve any precipitate. Alternatively, heat it in a 37°C water bath for 5 to 10 minutes while shaking to dissolve the precipitate. Once fully dissolved, dilute the 20× wash solution 20-fold with pure water or deionized water (e.g., to prepare 100 ml of working-concentration wash solution, add 5 ml of 20× wash solution to 95 ml of pure or deionized water). Mix thoroughly to obtain the working-concentration wash solution.

3.2 ELISA

1. Add 50 µl of positive serum to well A1 and B1 of the antigen-coated plate.
2. Add 50 µl of negative serum to well C1 and D1 of the antigen-coated plate.
3. Add 50 µl of diluent to well E1 and F1 of the antigen-coated plate.
4. Add 25 µl of diluent to test sample wells of the antigen-coated plate.
5. Add 25 µl of sera to test sample wells with diluent.
6. Gently mix the samples in the wells (avoid spillage) and incubate at 37°C for 60 minutes.
7. Discard the liquid from the antigen-coated plate wells.
8. Add 300 µl of wash solution (1×) to each well and wash 4 times. Avoid drying the wells during washing. After the final wash, pat the antigen-coated plate on absorbent paper.
9. Add 50 µl of HRP-conjugated Mab to each well. Incubate at 37°C for 30 minutes.
10. Repeat step 7 to step 8.
11. Add 50 µl of substrate solution to each well and incubate at room temperature in the dark for 15 minutes.
12. Add 50 µl of stop solution to each well.
13. Measure the OD values at a wavelength of 450 nm using a 96-well plate reader.

4 Result Interpretation

1. The average OD value of the PPRV negative control serum is referred to as OD_{NC}. The average OD value of the PPRV positive control serum is referred to as OD_{PC}. The average OD value of the HRP-conjugated Mab (blank) is referred to as OD_{cm}. The OD value of the test sample is called OD_s.

2. Calculation of CUT OFF value:

The blocking rate of the positive control serum is denoted as PB_{PC}. $PB_{PC} = 100 - OD_{PC} / OD_{cm} \times 100$

The blocking rate of the negative control serum is denoted as PB_{NC}. $PB_{NC} = 100 - OD_{NC} / OD_{cm} \times 100$



The blocking rate of the test sample is denoted as PB_S . $PB_S = 100 - OD_S / OD_{cm} \times 100$

3. Test validity conditions: The assay is considered valid if $PB_{NC} < 40$ and $PB_{PC} > 60$. If these criteria are not met, the test should be repeated.

4. Interpretation

If $PB_S > 50$, the sample is considered as positive, if $PB_S \leq 50$ the sample is considered as negative.

Troubleshooting

If any problems are experienced while following this protocol the immediate supervisor should be consulted in the first instance. If unavailable, advice from the Head of Group or any other suitably competent or experienced personnel should be sought.



Standard operating procedure for the detection of peste des petits ruminants virus by real-time PCR

1 Sample processing

Samples for PPR virus isolation: tissue; nasal and oral swabs

1.1 Equipment and Materials

Freezer, centrifuge, homogenator, Eppendorff tubes of 1.5 ml, nitrile gloves, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl), sterile tips (1-10 µl, 1-200 µl and 100-1000 µl), and Lyser beads.

1.2 Reagents

Phosphate buffer saline (PBS 1×) pH 7.2,

1.3 Methodology

Tissue samples:

a. Prepare a 1/10 homogenate of the tissue in sterile PBS (100 mg tissue per 1 ml PBS) using a homogenizer (Roche MagNA Lyser or equivalent).

b. Centrifuge at 1,000g (2000 rpm) for 5 minutes.

c. Transfer the supernatant to a new tube.

Swabs: directly squeezed or extracted with 1 mL PBS (pH 7.2)

2 Viral RNA extraction

2.1 Sample preparation

Sample preparation is performed based on the sample processing procedure 1.3. 200µl of the sample is used for the further detection.

2.2 Equipment and Materials

Nucleic acid extractor, centrifuge, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl) and sterile tips (1-10 µl, 1-200 µl and 100-1000 µl).

2.3 Reagents

Viral DNA/RNA extraction kit: Magnetic beads pre-filled DNA/RNA extraction kits (Gefine, China)

2.4 Methodology

Viral RNA was manually extracted with High Pure viral Nucleic Acid kit (Roche) or automatically extracted by nucleic acid extraction system (Tianlong Science and Technology, Xi'an, China) according to the product instructions.

The extracted RNA was eluted in 100 µl of nuclease-free water for direct use or stored at -20°C.

3 RT-PCR

3.1 Materials and reagents

Nuclease-free water; Pipettes (1-10 µl, 10-100 µl and 10-200 µl) and sterile tips (1-10 µl, and 1-200 µl); 2 × RT-PCR reaction master mix (P614, Vazyme); Primer mix.

PPRV primers at a concentration of 10 pmol/µl. Store<-20°C in aliquots. Expiry date: 1 year.

Forward primer sequence for N gene: NP3 5'-GTCTCGGAAATCGCCTCACAGACT-3'

Reverse primer sequence for N gene: NP4 5'-GGGTCTCGAAGGCTAGGCCCGAATA-3'

Forward primer sequence for F gene: F1b 5'-AGTACAAAAGATTGCTGATCACAGT-3'

Reverse primer sequence for F gene: F2d 5'-ATCTCGAAGACTCTTAAAAATGGCC-3'



R+: positive control for the reaction

R-: negative control for the reaction

3.2 Methodology

3.2.1 Master mix preparation

RT-PCR reaction mixtures are prepared in a sterile 1.5 ml micro centrifuge tube as described below. At least two extra samples mixtures are involved besides the samples to be tested (including controls).

Pipetting step	Reagents	volume
1	H ₂ O	7.5 µl
2	2 × reaction mix	12.5 µl
3	Forward primer	1 µl
4	Reverse primer	1 µl
Total volume		22µl

Add 22 µl of the PCR master mix to the 0.2 ml optical PCR tubes. Add 3µl of RNA template to each PCR tube. Include R+, R- for each PCR run. After addition of the template, cover the lid of reaction tube and spin down the PCR tube/plate.

Place all PCR tubes to real-time thermocycler. Run the program detailed below.

3.2.2 PCR program

Step	Temperature	Time	No. cycles
1	50°C	30 min	1
2	94°C	3 min	1
3	94°C	20 sec	40
	56°C	30 sec	
	72°C	35 sec	
4	72°C	5 min	1

3.3 Nucleic acid electrophoresis

The resulting PCR products were analyzed by electrophoresis on an 1.5% agarose gel under 110 voltage for 40 minutes. The results of PCR products are observed under UV light.

3.3 Interpretation of results

The procedure will be valid if R+ give amplification products with right size (N: 351bp, F: 448bp), and the R- do not show products.

For positive samples, amplification products with right size (N: 351bp, F: 448bp) are collected.

For negative samples, no amplification products were collected.



Standard operating procedure for the detection of peste des petits ruminants virus by droplet digital PCR (ddPCR)

1 Sample processing

Samples for PPR virus isolation: tissue; nasal and oral swabs

1.1 Equipment and Materials

Freezer, centrifuge, homogenator, Eppendorff tubes of 1.5 ml, nitrile gloves, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl), sterile tips (1-10 µl, 1-200 µl and 100-1000 µl), and Lyser beads.

1.2 Reagents

Phosphate buffer saline (PBS 1×) pH 7.2,

1.3 Methodology

Tissue samples:

a. Prepare a 1/10 homogenate of the tissue in sterile PBS (100 mg tissue per 1 ml PBS) using a homogenizer (Roche MagNA Lyser or equivalent).

b. Centrifuge at 1,000g (2000 rpm) for 5 minutes.

c. Transfer the supernatant to a new tube.

Swabs: directly squeezed or extracted with 1 mL PBS (pH 7.2)

2 Viral RNA extraction

2.1 sample preparation

Sample preparation is performed based on the sample processing procedure 1.3. 200µl of the sample is used for the further detection.

2.2 Equipment and Materials

Nucleic acid extractor, centrifuge, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl) and sterile tips (1-10 µl, 1-200 µl and 100-1000 µl).

2.3 Reagents

Viral DNA/RNA extraction kit: Magnetic beads pre-filled DNA/RNA extraction kits (Gefine, China)

2.4 Methodology

Viral RNA was manually extracted with High Pure viral Nucleic Acid kit (Roche) or automatically extracted by nucleic acid extraction system (Tianlong Science and Technology, Xi'an, China) according to the product instructions.

The extracted RNA was eluted in 100 µl of nuclease-free water for direct use or stored at -20°C.

3 ddPCR

3.1 Materials and reagents

Nuclease-free water; Pipettes (1-10 µl, 10-100 µl and 10-200 µl) and sterile tips (1-10 µl, and 1-200 µl); 2×One step RT-dPCR Master Mix (ROX) (Sniper, China); Enzyme Mix (Sniper, China), Primer and Probe.

PPRV primers at a concentration of 10 pmol/µl. Store<-20°C in aliquots. Expiry date: 1 year.

Forward primer sequence: 5'-GAYTCCGAGTATGAGTATGAGGATG-3'

Reverse primer sequence: 5'-GCTATCATTATACTGGAMAGATGGCC-3'

Probe sequence: 5'- FAM-CGTGCAAGCATTGCYAARATCCATGA-BHQ1-3'.



3.2 Methodology

3.2.1 Master mix preparation

ddPCR reaction mixtures are prepared in a sterile 1.5 ml micro centrifuge tube as described below. At least two extra samples mixtures are involved besides the samples to be tested (including controls).

Expiry date: 1 year.

Pipetting step	Reagents	volume
1	2×One step RT-dPCR Master Mix (Rox)	11μL
2	Enzyme Mix	2μL
3	Forward primer (10μM)	1μL
4	Reverse primer (10μM)	1μL
5	Probe (10μM)	0.5μL
7	RNase-free water	4.5μL
Total volume		20μl

Add 20 μl of the PCR master mix to the 0.2 ml optical PCR tubes. Add 2μl of RNA template to each PCR tube, include R+, R-, for each PCR run. After addition of the template, cover the lid of reaction tube and spin down the PCR tube/plate.

Place all PCR tubes to ddPCR machine. Run the program detailed below.

3.2.2 PCR program

Step	Temperature	Time	No. cycles
1	50°C	20 min	1
2	95°C	3 min	1
3	95°C	30 sec	45
	55°C	30 sec	
Droplet identification channel: ROX			

3.3 Interpretation of results

The positive control detected the corresponding copy number, and the negative control detected no copy number, the test was valid. According to the instrument's detection results, the samples were determined to be negative or positive, and the copy numbers of positive samples were obtained at the same time.



**Standard operating procedure for the virus isolation of peste des petits ruminants
virus in cell culture**

1. Introduction

1.1 Purpose: To describe the virus isolation technique to culture the peste des petits ruminants virus (PPRV) in Vero dog-slam cells.

1.2 Background: PPRV may be isolated in primary Chlorocebus kidney/lung cells and some cell lines (Vero, B95a). Unfortunately, PPRV isolation using such cells is not always successful on first passage and may require multiple blind passages. Recently, derivatives of cell lines (Vero, CV1) expressing the morbillivirus receptor, the signaling lymphocyte activation molecule (SLAM or CD150), have been developed that enable isolation of field viruses from pathological specimens in less than 1 week, without requirement for blind passages. PPR can be confirmed by virus isolation. Cultures are examined for cytopathic effect (CPE). The identity of the virus can be confirmed by virus neutralization or other methods.

2. Equipment and Materials

2.1 Equipment

Class II microbiological safety cabinet of any make

Centrifuge (rotor SLA 1500)

Phase Contrast Inverted cell Culture Microscope

Thermo Scientific Heracell CO₂ incubator or similar

Tube racks

Micropipettes of volumes 1-10 µl, 20-200 µl and 100-1000µl

Ice bucket with ice

-20°C or lower freezer

-70°C or lower freezer

- 4°C fridge

2.2 Materials

Filter MINISART 0.45 micras.

Plastic pipettes for volume of 1-25 ml

Sterile forceps

Cell 6-well plate

Microcentrifuge tubes, sterile

Micropipette

Disposable gloves

2.3 Reagents

Vero Dog-Slam Cells

1x Phosphate buffered saline (PBS) buffer, sterile

Dulbecco's Modified Eagle Medium (DMEM)

FETAL BOVINE SERUM (FBS)

0.25% TRYPSIN Solution

Antibiotics (penicillin 10000 units/ml and streptomycin 10mg/ml)

Growth media (DMEM with 10% FBS and 1% antibiotics)



Maintenance media (DMEM with 2% FBS and 1% antibiotics)

3. PROCEDURE

3.1 Sample preparation

3.1.1 Swab samples

1. Swabs samples were collected in a tube containing 1 ml sampling media.
2. Transfer the swabs sample contained tubes in a safety cabinet.
3. Open the tube carefully. Squeeze the swab. Transfer the liquid to a 1.5 ml Eppendorf tube.
4. Centrifuge at 3000 r.p.m at 4°C for 10 min.
5. Recover the supernatant and put in a filter of 0.45 micras.
6. Add antibiotics into the filtered supernatant to a final concentration of 1%.

3.1.2 Tissue samples

1. Transfer the tissue in a safety cabinet. Using sterile forceps and scissors cut the tissue to approximate 1 g (about 1 cm³) into a sterile mortar.
2. Chop the tissue finely into small pieces using sterile scissors and transfer it into a microcentrifuge tube prefilled with ceramic beads.
3. Add about 1 ml of sterile PBS and a small amount of sterile sand to the mortar and ground to a paste with a sterile pestle.
4. Transfer the mix into a sterile tube and clarify in a bench centrifuge at 3000r.p.m at 4°C for 10 min.
5. Recover the supernatant and put in a filter of 0.45 micras.
6. Add antibiotics into the filtered supernatant to a final concentration of 1%.

3.2 Virus isolation

1. For each sample, use one monolayer of cells grown in growth media in a 6-well plate, and reserve one additional wells as controls.
2. Remove the growth media of Vero dog-slam cells. Wash the monolayer with sterile PBS one time.
3. Add 200 µl supernatant from treated samples and 1ml 2% FBS DMEM into one well of 6 well cell culture plate. Incubate the plate at 37°C with 5% CO₂ for 1 hour, shaking gently every 20 minutes.
4. Add maintenance media to the final volume of 2ml.
5. Incubate 6 well cell culture plate at 37°C with 5% CO₂ for 3-7 days. Check every day for the appearance of cytopathic effects (CPE).

4. Results

The CPE produced by PPRV can develop within 5 days. It consists of cell rounding and aggregation culminating in syncytia formation in Chlorocebus kidney cells and cell lines expressing SLAM.

In unmodified Vero cells, it is sometimes difficult to see the syncytia. If they exist, they are very small. However, small syncytia are always seen in infected Vero cells stained with haematoxylin and eosin. Syncytia are recognised by a circular arrangement of nuclei giving a 'clock face' appearance. Cover-slip cultures may show CPE earlier than day 5. Some cells may contain intracytoplasmic and intranuclear inclusions, others may be vacuolated. Similar cellular changes may be seen in stained histopathological sections of infected tissues. After 5-6 days, blind passages should always be carried out as CPE may take time to appear.



5. Troubleshooting

If any problems are experienced while following this protocol the immediate supervisor should be consulted in the first instance. If unavailable, advice from the Head of Group or any other suitably competent or experienced personnel should be sought.



Standard operating procedure for the detection of peste des petits ruminants virus by multienzyme isothermal rapid amplification (MIRA)

1 Sample processing

Samples for PPR virus isolation: tissue; nasal and oral swabs

1.1 Equipment and Materials

Freezer, centrifuge, homogenator, Eppendorff tubes of 1.5 ml, nitrile gloves, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl), sterile tips (1-10 µl, 1-200 µl and 100-1000 µl), and Lyser beads.

1.2 Reagents

Phosphate buffer saline (PBS 1×) pH 7.2,

1.3 Methodology

Tissue samples:

a. Prepare a 1/10 homogenate of the tissue in sterile PBS (100 mg tissue per 1 ml PBS) using a homogenizer (Roche MagNA Lyser or equivalent).

b. Centrifuge at 1,000g (2000 rpm) for 5 minutes.

c. Transfer the supernatant to a new tube.

Swabs: directly squeezed or extracted with 1 mL PBS (pH 7.2)

2 Viral RNA extraction

2.1 sample preparation

Sample preparation is performed based on the sample processing procedure 1.3. 200µl of the sample is used for the further detection.

2.2 Equipment and Materials

Nucleic acid extractor, centrifuge, pipettes (1-10 µl, 10-100 µl, 10-200 µl and 200-1000 µl) and sterile tips (1-10 µl, 1-200 µl and 100-1000 µl).

2.3 Reagents

Viral DNA/RNA extraction kit: Magnetic beads pre-filled DNA/RNA extraction kits (Gefine, China)

2.4 Methodology

Viral RNA was manually extracted with High Pure viral Nucleic Acid kit (Roche) or automatically extracted by nucleic acid extraction system (Tianlong Science and Technology, Xi'an, China) according to the product instructions.

The extracted RNA was eluted in 100 µl of nuclease-free water for direct use or stored at -20°C.

3 MIRA

3.1 Materials and reagents

MIRA commercial kits, Primer and Probe.

PPRV primers and probes at a concentration of 10 pmol/µl. Store<-20°C in aliquots. Expiry date: 1 year.

Fluorescence

Forward primer sequence: 5'-TCATGGATCTTGGCAATGCTTGCACG-3'

Reverse primer sequence: 5'-CTGTGCCAGAGTCAAGATGGGAGTC-3'

Probe sequence: 5'-GTTRACACAAGAATCTGGTACCATAGCA-FAM-dt-CA-THF-



BHQ1-dt-GACTCAGCCTAAAGARAA-3'-C3-spacer.

3.2 Methodology

3.2.1 Master mix preparation

MIRA reaction mixtures are prepared in a sterile 1.5 ml micro centrifuge tube as described below. At least two extra samples mixtures are involved besides the samples to be tested (including controls).

Expiry date: 1 year.

Pipetting step	Reagents	volume
1	Buffer A	29.4μL
2	Forward primer (10μM)	2μL
3	Reverse primer (10μM)	2μL
4	Probe (10μM)	0.6μL
5	RNase-free water	8.5μL
6	Template	5μL
7	Buffer B (added last)	2.5μL
Total volume		50μl

After adding Buffer B, immediately cap the tube. Mix by inverting the tube 8–10 times (do not vortex, to avoid enzyme inactivation). Immediately after mixing, place the reaction tube into the constant-temperature fluorescence detector. For LFD, Dilute the amplification product 1:20 with RNase-free ddH₂O (e.g., 5 μL of product + 95 μL of water). Apply 80 μL of the diluted solution to the sample well of the test strip. Observe and record the color development result of the test strip within 5 minutes.

3.3 Interpretation of results

After the amplification reaction is completed in the constant-temperature fluorescence detector, observe the fluorescence curve and the final fluorescence signal reading. Positive result: A significant exponential fluorescence increase is observed, with the amplification curve showing a typical S-shaped or rising pattern. The final fluorescence value exceeds the preset threshold. This indicates that the target nucleic acid has been successfully amplified and is present in the sample. Negative result: No significant fluorescence increase is observed throughout the reaction. The amplification curve remains flat or shows only a slight baseline drift, and the final fluorescence value remains below the threshold. This indicates that no target nucleic acid is detected in the sample. Invalid result: If the positive control fails to show an amplification curve, or if the negative control shows an amplification curve, the test is



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considered invalid and shall be repeated.