Virus Isolation

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2 Isolation of PRRSV

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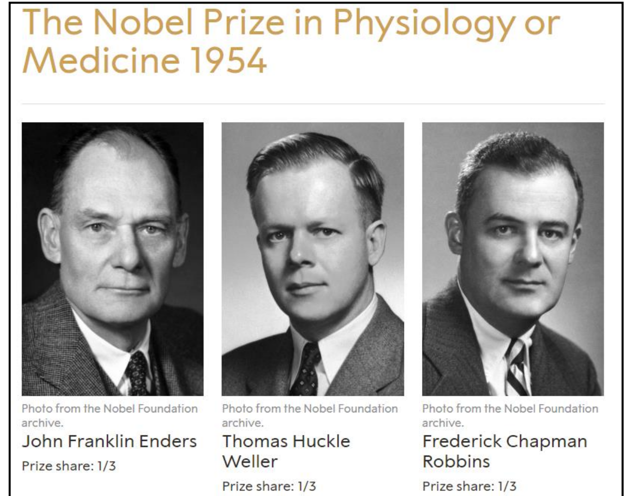
Virus isolation-Basics

- Virus isolation is a very specific method for diagnosis of viral infection and also generate a supply of virus for research purposes.

- Tools for virus isolation:
  - Lab animals (limited due to high cost and ethical reasons)
  - Embryonated Eggs (influenza A and some Avian viruses)
  - Cell Culture (monolayer/suspension)



In 1949, three scientists discovered the ability of poliomyelitis viruses to grow in cultures of various types of tissue. This great finding led the way to propagation of many other viruses in cultured cells

Virus isolation-Basics

-Virus isolation is a sensitive procedure when:

1) samples are collected and preserved in the right way (Infectious particles exist in the sample)
2) cultural conditions are optimal for the particular virus (Choose right cell line)

	Sample type(s)	Primary cell culture	Continuous cell lines
PRRSV	Buffy coat, serum, lung, lymph node, spleen, tonsils	PAM	MARC-145(adapted)
ASFV	Blood (EDTA), spleen, lymph node, tonsil, kidney	PBM, PAM	WSL
FMDV	Epithelium, OP liquid	Bovine Thyroid Cell, Pig/Calf Kidney cells	BHK-21, IB-RS-1
CSFV	Tonsil, spleen, kidney, lymph node, ileum	/	PK-15
SIV (Embryonated eggs)	Lung, nose swab	PK	MDCK

Summary of samples and cell culture types for important pig viruses

Virus isolation-Basics

1. Only isolating a virus from an animal does not necessarily implicate that virus is the causative agent of the disease we are investigating. (Info from Epidemiology investigation: Clinical sign, lesions, status of other animals in the population, inoculation of lab animals)

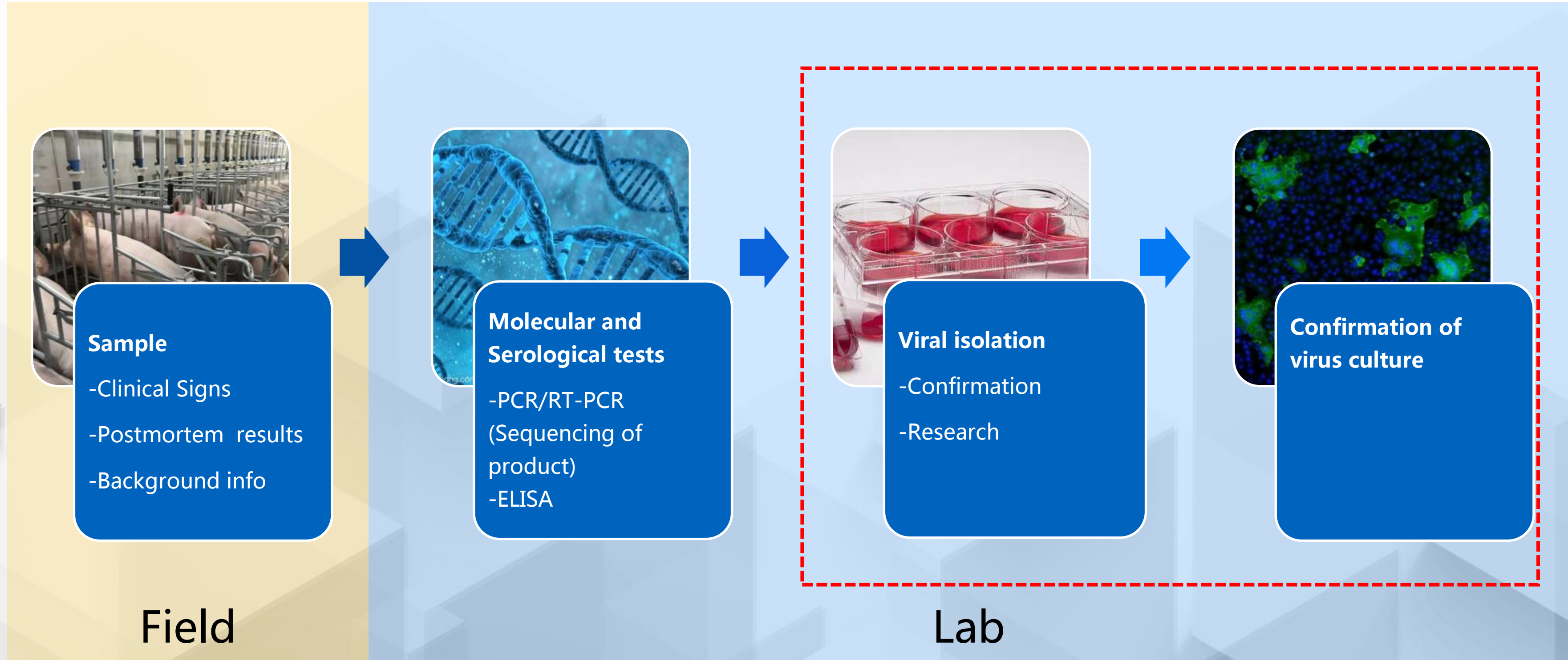
2. Biosafety: compare to diagnosis, isolation of some viruses may require higher level of biosafety. Check policies and regulations in your country.

-P2/BSL-2: molecular and serological diagnosis of most viral diseases (inactivation procedure may be required); isolation of PRRSV

-P3/BSL-3: Isolation of ASFV, FMDV, CSFV

Virus solation-PRRSV

Diagnosis of PRRSV-General Workflow

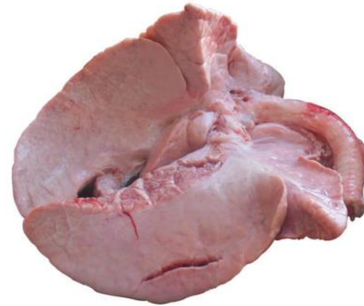


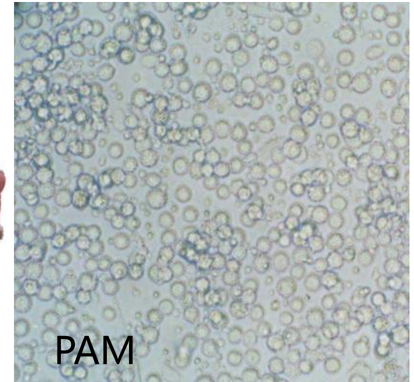
PRRSV isolation-Procedures

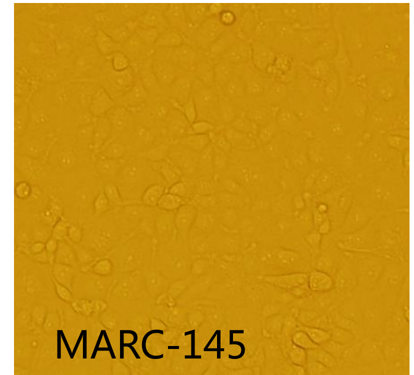
1. Cell preparation

   PAM(OIE manual), MARC-145

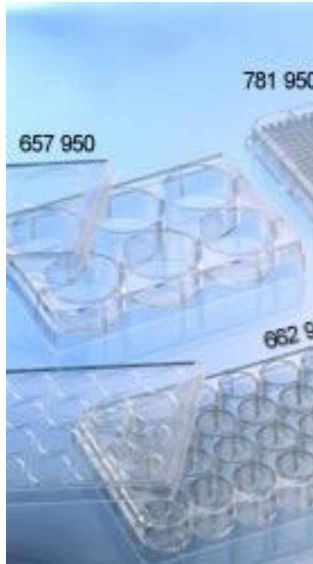
2. Sample preparation
3. Inoculation
4. Culture and passage of infected cells
5. Confirmatory tests







PRRSV isolation-Cell preparation



Flasks

Description	Growth area (cm^2)	Recommended working volume (mL)	Cell yield*
T-25	25	5-10	2.5×10^6
T-75	75	15-25	7.5×10^6
T-150	150	30-50	15.0×10^6
T-175	175	35-60	17.5×10^6
T-225	225	45-75	22.5×10^6

Cell Culture Dishes

Description	Growth area (cm^2)	Recommended working volume (mL)	Cell yield*
35	8	1-2	0.8×10^6
60	21	4-5	2.1×10^6
100	55	10-12	5.5×10^6
150	148	28-32	14.8×10^6

Multiwell Plates

Description	Growth area (cm^2)	Recommended working volume (mL)	Cell yield*
96-well	0.32	0.1-0.2	0.32×10^5
48-well	1.00	0.3-0.6	0.8×10^5
24-well	1.88	0.5-1.2	1.9×10^5
12-well	3.83	1.0-2.4	3.8×10^5
6-well	9.40	2.0-3.0	9.5×10^5

Roller Bottles

Description	Growth area (cm^2)	Recommended working volume (mL)	Cell yield*
Small	490	100-150	4.9×10^7
Standard	850	170-250	8.5×10^7
Pharmaceutical	1750	340-500	17.5×10^7

*Cell line dependent. Based upon a density of 1×10^5 cells/cm^2

Flasks: T25, T50, T75
Microtitre plates: 6/1

PRRSV isolation-Cell preparation

- **Preparation of MARC145 cell cultures**

1. T25 tissue culture flasks seeded with 1×10^5 MARC 145 cells/mL in growth media. DMEM with D-Glucose, L-glutamine, Phenol Red, Sodium Pyruvate. Add FBS (8%) and Penicillin (100U/ml) & Streptomycin (100U/ml)
2. Incubation: Flasks are placed in a humidified 37℃ incubator containing 3-5%CO_2. Observe daily. After 24-48 hours, the MARC145 cells will form about 90% confluent monolayer and are ready to be inoculated.

PRRSV isolation-Sample preparation

1. **Sample types and amount**: serum, ascitic fluid, 10% tissue suspension (lung, lymph nodes, spleen and tonsils), 0.5ml of prepared sample sample/serum/ascitic fluid is needed for one T25 flask of cells to be inoculated. Serum and ascitic fluid can be used directly.

2. **10% tissue suspension**: grinding 0.5 grams of tissue in 5 mL of PBS or DMEM containing antibiotics (pen. & strep.), using a mortar and pestle, or automatic homogenizer.

3. **Centrifuge** at 2500 g for 5 minutes to pellet solid matter. Decant the supernatant into a clean centrifuge tube for the next step.

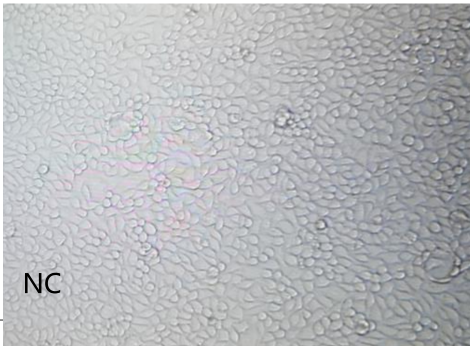
4. **Filtration (optional):** To avoid contamination, the samples can be filtrated by a 0.2μm filter.

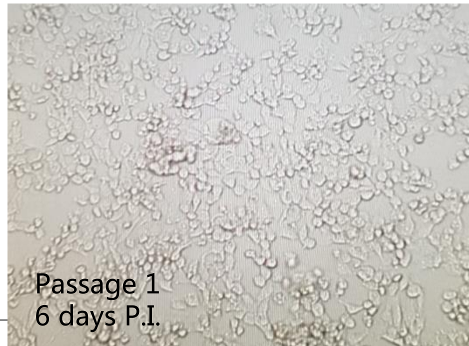
5. **Storage:** the supernatant can be stored at 4℃ shortly, ready for inoculation onto cell cultures.

PRRSV isolation-Inoculation and culture

Inoculation

1. **Add prepared sample to cell monolayer:** Pour cell media from a T25 flask with confluent Marc-145 cells. Do not remove media completely to avoid drying out. Add 0.5ml of serum/ascitic fluid/10% tissue suspension. (Negative control-0.5ml meida)
2. **Absorption:** Incubate inoculated cells in a humidified 37℃ incubator containing 5% CO_2 for 1 hours.
3. **Wash:** Discard media from the cells. Wash cell monolayer with PBS or DMEM containing antibiotics (Penicillin / Streptomycin, 10^4 IU/ml)
4. **Culture and Observation:** Add 5mL maintenance media (2% FBS) to each T25 flask. Incubate the cells for up to 7 days, check daily for CPE and/or cell death due to cell-toxicity. ‘Passage 1’cells.
5. **CPE or Cell toxicity? Passage infected cells (including control cells) for at least twice**. ‘Passage 3’cells





PRRSV isolation-Inoculation and culture

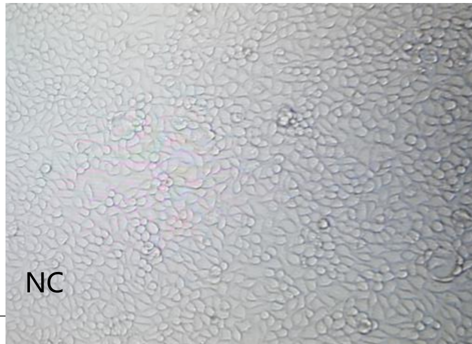
•**Passages of PRRSV in MARC145 cell**

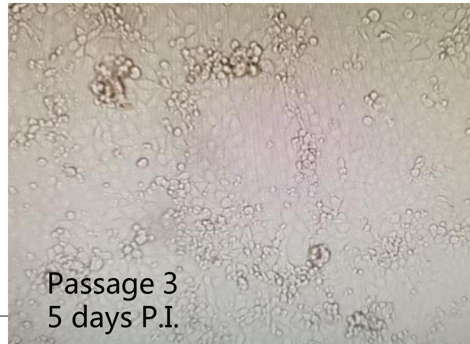
1. **Recovery of Tissue Culture Supernatant (TCSN):** Freeze /thaw the Passage 1 cells (and Negative control) at -80℃ for 3 times. Centrifuge and get supernatant.

2. Inoculate fresh MARC145 cells with TCSN: Remove cell media and add 0.5mL TCSN.

3. Absorption: Place test cells in a humidified 37℃ incubator containing 5% CO_2. After 1 hour, remove and discard the media from the cells. Add 5mL maintenance media.

4. Incubation: Incubate the passage 2 cells for 2-5 days, check daily for CPE.
5. Repeat step 1-4 to get Passage 3.

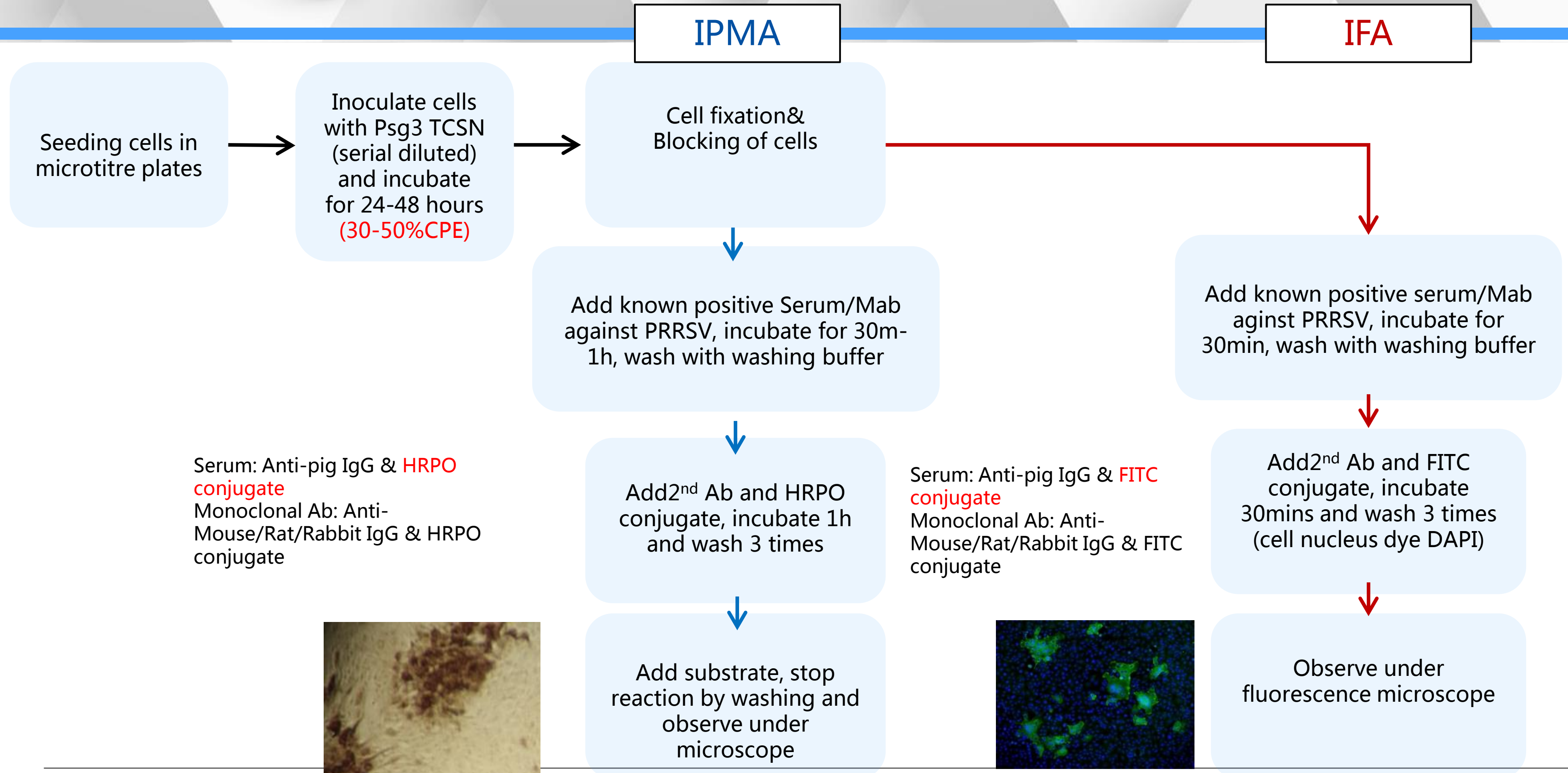




PRRSV isolation-Results Reading

Passage 1 and 2	Passage 3	Results interpretation	Confirmatory tests
No CPE	No CPE	Negative	IPMA/IFA
CPE	No CPE	Negative	
CPE	CPE	Suspected positive	IPMA/IFA
No CPE	CPE	Suspected positive	

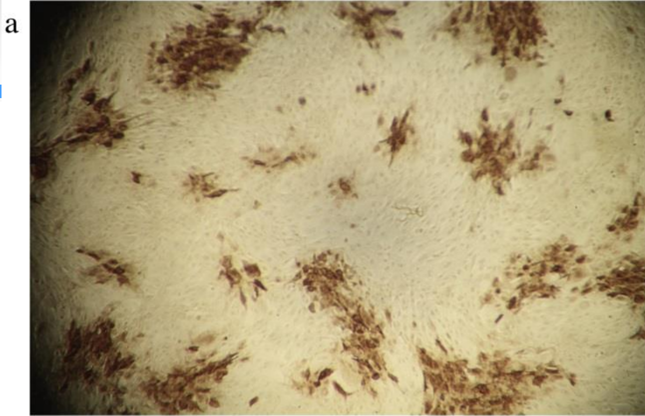
Confirmatory tests for PRRSV: Immunofluorescence assay(IFA) and Immunoperoxidase monolayer assay (IPMA)

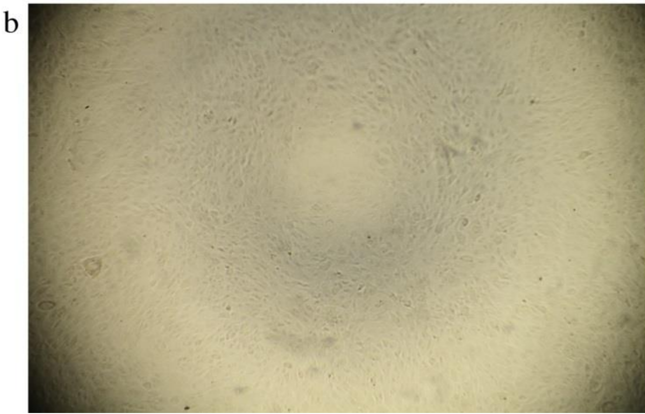


Applications of IPMA (OIE mannual)

	Cell sensitivity	Virus	Serum/Ab
Confirmation of virus culture	Yes	Unknown culture	Known (Positive sera or Mab)
Serological test for PRRSV Ab	Yes	Standard PRRSV with known titre	Unknown sera samples
Batch sensitivity test of PAM	Unknown	Standard PRRSV with known titre	Known (Positive sera or Mab)

IPMA





Examples of IPMA staining in a 96 well plate;
A: positive staining ; B: negative staining;
(Haegeman *et al.*, 2020)

Virus presence confirmed by Ag-Ab specific binding.

Next step: Determine virus titer

THANK YOU

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