

# ***In situ* detection of PRRSV in formalin-fixed, paraffin-embedded (FFPE) tissues**

An immunohistochemistry (IHC) staining protocol for *in situ* identification of porcine epidemic diarrhea virus (PEDV) in formalin-fixed, paraffin-embedded (FFPE) tissue.

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# Supporting Information

## Starting specimens

Starting samples = FFPE tissues cut to 4 micron thickness and adhered to positively-charged microscopy slides (e.g. SuperFrost Plus Slides; Fisher Scientific 12-550-15). It is crucial that tissues are adequately fixed to prevent tissue degradation. Tissues no thicker than 0.5 centimeters should be freshly harvested and placed into 10% neutral-buffered formalin (NBF) or 4% paraformaldehyde (PFA) at a ratio of at least 20 volumes fixative per one volume tissue. Fix tissues between 16-30 hours at room temperature (RT), followed by immediate transfer to 70% ethanol and processing into FFPE tissue blocks. Fixation times should be optimized for individual tissues and experiments.

## Equipment

- Pipettes/pipette tips
- Drying oven (able to reach & hold 60°C)
- Fume hood
- Slide staining tray (e.g. Simport M920-2)
- EZ-Batch Slide Holder (Advanced Cell Diagnostics 321716)
- EZ-Batch Wash Tray (Advanced Cell Diagnostics 321717)
- Tissue-Tek Vertical 24 slide rack (American Master Tech Scientific LWS2124)
- Tissue-Tek Staining Dishes (American Master Tech Scientific LWS20WH)
- Tissue-Tek Clearing Agent Dishes, xylene resistant (American Master Tech Scientific LWS20GR)
- Brightfield microscope and/or slide imaging platform

## Reagents/Supplies

\*\*\*For all reagents, refer to MSDS to determine appropriate precautions, personal protective equipment (PPE), and disposal methods before use\*\*\*

- Distilled water (obtained in-house)
- 0.05% PBS-Tween (PBS-T), pH 7.35 (made in-house)
- Xylenes (Macron Fine Chemicals 8668-16)
- 100% ethanol (Pharmco 111000200)
  - Dilute with distilled water to make 95%, 85%, and 70% concentrations
- Pro-Par Clearant (Anatech 510)
- Fixative
  - 10% NBF (Cancer Diagnostics, Inc. 111) or 4% PFA (Electron Microscopy Sciences 15713)
- ImmEdge Hydrophobic Barrier Pen (Vector H-4000)
- Ready to use Proteinase-K (Dako S302080-2/S302030-2)
- BLOXALL Endogenous Blocking Solution (Vector SP-6000)
- 1% bovine serum albumin (BSA) in PBS (made in-house)
- Mouse anti-PEDV antibody
  - Clone #2F7 (Immunology Consultants Laboratory MPEDV-5A-2F7, stock concentration 3.6mg/mL)
  - Clone #3D6 (Immunology Consultants Laboratory MPEDV-5A-3D6, stock concentration 5.4mg/mL)
  - Clone #5B9 (Immunology Consultants Laboratory MPEDV-5A-5B9, stock concentration 2.62mg/mL)
  - Clone #6H10 (Immunology Consultants Laboratory MPEDV-5A-6H10, stock concentration 1.95mg/mL)
  - Clone #7A12 (Immunology Consultants Laboratory MPEDV-5A-7A12, stock concentration 1.75mg/mL)
- ImmPRESS-VR Horse Anti-Mouse IgG Polymer Detection Kit, Peroxidase (Vector MP-6402-15)
  - Normal Horse Serum, 2.5%
  - ImmPRESS-VR Horse Anti-Mouse IgG Polymer Reagent
- ImmPACT DAB EqV Substrate Kit, Peroxidase (Vector SK-4103)
  - ImmPACT DAB EqV Reagent 1 (Chromogen)
  - ImmPACT DAB EqV Reagent 2 (Diluent)
- Gill's Hematoxylin I (American Master Tech Scientific HXGHE1LT)
- Refrax Mounting Medium (Anatech 711)
- #1 thickness cover glass (e.g. Fisherbrand 12-545-F)

## Assay-specific Information

- Primary antibodies were tested at dilutions listed in the table below in order to obtain concentrations of 100, 10, 1, 0.1, 0.01, and 0.001 ug/mL for testing based on provided undiluted stock concentrations

Antibody	Undiluted	100ug/ml	10ug/ml	1ug/ml	0.1ug/ml	0.01ug/ml	0.001ug/ml
MPEDV-5A-2F7	3.6mg/ml	1:36	1:360	1:3,600	1:36,000	1:360,000	1:3,600,000
MPEDV-5A-3D6	5.4mg/ml	1:54	1:540	1:5,400	1:54,000	1:540,000	1:5,400,000
MPEDV-5A-5B9	2.62mg/ml	1:26.2	1:262	1:2,620	1:26,200	1:262,000	1:2,620,000
MPEDV-5A-6H10	1.95mg/ml	1:19.5	1:195	1:1,950	1:19,500	1:195,000	1:1,950,000
MPEDV-5A-7A12	1.75mg/ml	1:17.5	1:175	1:1,750	1:17,500	1:175,000	1:1,750,000

## Assay Controls

- Controls will be required to ensure there is minimal/no cross-reactivity with off-target epitopes in the tissue and to ensure stains are specific to the intended antibody targets
- IHC controls:
  - Secondary only control
    - This slide receives only diluent in place of primary antibody. All other reagents are still applied to the slide.

## Assay Variations

- Parameters may need to be further optimized for different experiments, tissues, targets, or species.

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## Staining Protocol

Before starting the assay:

- Preheat a dry oven to 60°C
- Load slides for assay into vertical slide rack

## Baking

- **Bake slides 20 min 60°C**

While slides bake:

- Prepare 0.05% PBS-T

Immediately before deparaffinizing:

- Add ~200 mL xylenes to each of three clearing agent dishes in a fume hood
- Add ~200 mL 100% ethanol to each of two staining dishes in a fume hood
- Add ~200 mL 95% ethanol to a staining dish in a fume hood
- Add ~200 mL 85% ethanol to a staining dish in a fume hood
- Add ~200 mL 70% ethanol to a staining dish in a fume hood
- Add ~200 mL distilled water to a staining dish in a fume hood
- Add ~200 mL PBS-T to a staining dish

## Deparaffinizing & Rehydrating

- Submerge slide rack in fresh **xylenes 5 min RT**
- Submerge slide rack in fresh **xylenes 5 min RT**
- Submerge slide rack in fresh **xylenes 5 min RT**
- Submerge slides rack in fresh **100% ethanol 1 min RT**
- Submerge slides rack in fresh **100% ethanol 1 min RT**
- Submerge slides rack in fresh **95% ethanol 1 min RT**
- Submerge slides rack in fresh **85% ethanol 1 min RT**
- Submerge slides rack in fresh **70% ethanol 1 min RT**

- Submerge slides rack in fresh **distilled water 3 min RT**
- Submerge slides rack in fresh **PBS-T for transport**

While slides deparaffinize/rehydrate:

- Turn off dry oven
- Prepare humidified slide staining tray by adding water to bottom & placing lid on top

### Hydrophobic Barrier

- **Apply hydrophobic barrier** around each tissue
  - One by one, unload slides from vertical rack submerged in PBS-T. Dry off only the area around the tissue where a barrier will be drawn with a hydrophobic barrier pen. Keep tissue area wet the whole time. Draw barrier and place slides into the EZ-Batch slide holder placed inside the slide staining tray. Using a pipette, apply a small amount of PBS-T within the barrier (just enough to keep the tissue wet while drawing barriers on remaining slides). Slides will remain locked in the EZ-Batch slide holder throughout the protocol until being transferred back to a vertical rack for counterstaining.
- Leave slide holder in slide staining tray

### Proteinase Digestion

- Decant slide holder and again place flat in slide staining tray
- Incubate with **Dako Ready-to-use Proteinase K 3 min RT**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

While slides incubate with proteinase:

- Discard deparaffinizing & rehydrating reagents
- Add ~200 mL PBS-T to each of two staining dishes

### Tissue Quenching

- Decant slide holder and again place flat in slide staining tray
- Incubate with **BLOXALL Endogenous Blocking Solution 10 min RT**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

While slides incubate with enzyme block:

- Discard proteinase reagents
- Add ~200 mL PBS-T to each of two wash trays

### Protein Blocking

- Decant slide holder and again place flat in slide staining tray
- Incubate with **2.5% blocking serum 30 min RT**
  - Serum is a component of the secondary antibody reagent kit
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed

While slides incubate with serum:

- Discard tissue quenching reagents
- Prepare diluted primary antibody by adding antibody stock to 1% BSA in PBS at a dilution of 1:10,000. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Mix diluted antibody well before use.

### Primary Antibody

- Decant slide holder and again place flat in slide staining tray
  - Do not wash with PBS-T between decanting serum and applying secondary antibody
- Incubate with **diluted primary antibody 1 hour RT or 4°C overnight**
  - Refer to assay-specific information above to determine antibody incubation time
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

While slides incubate with primary antibody:

- Discard protein blocking reagents
- Add ~200 mL PBS-T to each of two wash trays

### Secondary Antibody

- Decant slide holder and again place flat in slide staining tray
- Incubate with **secondary antibody (HRP-conjugated polymer) 30 min RT**
  - Secondary antibody polymer is a component of the secondary antibody reagent kit
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

While slides incubate with secondary antibody:

- Discard primary antibody reagents
- Add ~200 mL PBS-T to each of two wash trays

Immediately before chromogen detection:

- Prepare diluted DAB chromogen by adding equal volumes of DAB Reagent 1 (Chromogen) and DAB Reagent 2 (Diluent) together. Total volume to use is dependent on tissue sizes. Make sure to mix reagents thoroughly. Store in the dark due to light sensitivity.

### Chromogenic Detection (DAB)

- Decant slide holder and again place flat in slide staining tray
- Incubate with **diluted DAB chromogen 7 min RT**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

While slides incubate with chromogen:

- Discard remaining secondary antibody reagents
- Add ~200 mL PBS-T to each of two wash trays
- Add ~200 mL hematoxylin to one staining dish
- Add ~200 mL distilled water to each of three staining dishes

## Counterstaining

- Transfer slides to vertical slide rack
  - Do quickly to avoid drying out slides or alternatively place vertical slide rack in a staining dish containing PBS-T and then transfer slides
- Submerge slide rack in **hematoxylin 1 min RT**
- Submerge slide rack in fresh **distilled water, dunking 3-5 times**
- Submerge slide rack in fresh **distilled water, dunking 3-5 times**
- Submerge slide rack in fresh **distilled water, dunking 3-5 times**
  - Water should no longer appear purple in the third water dish used

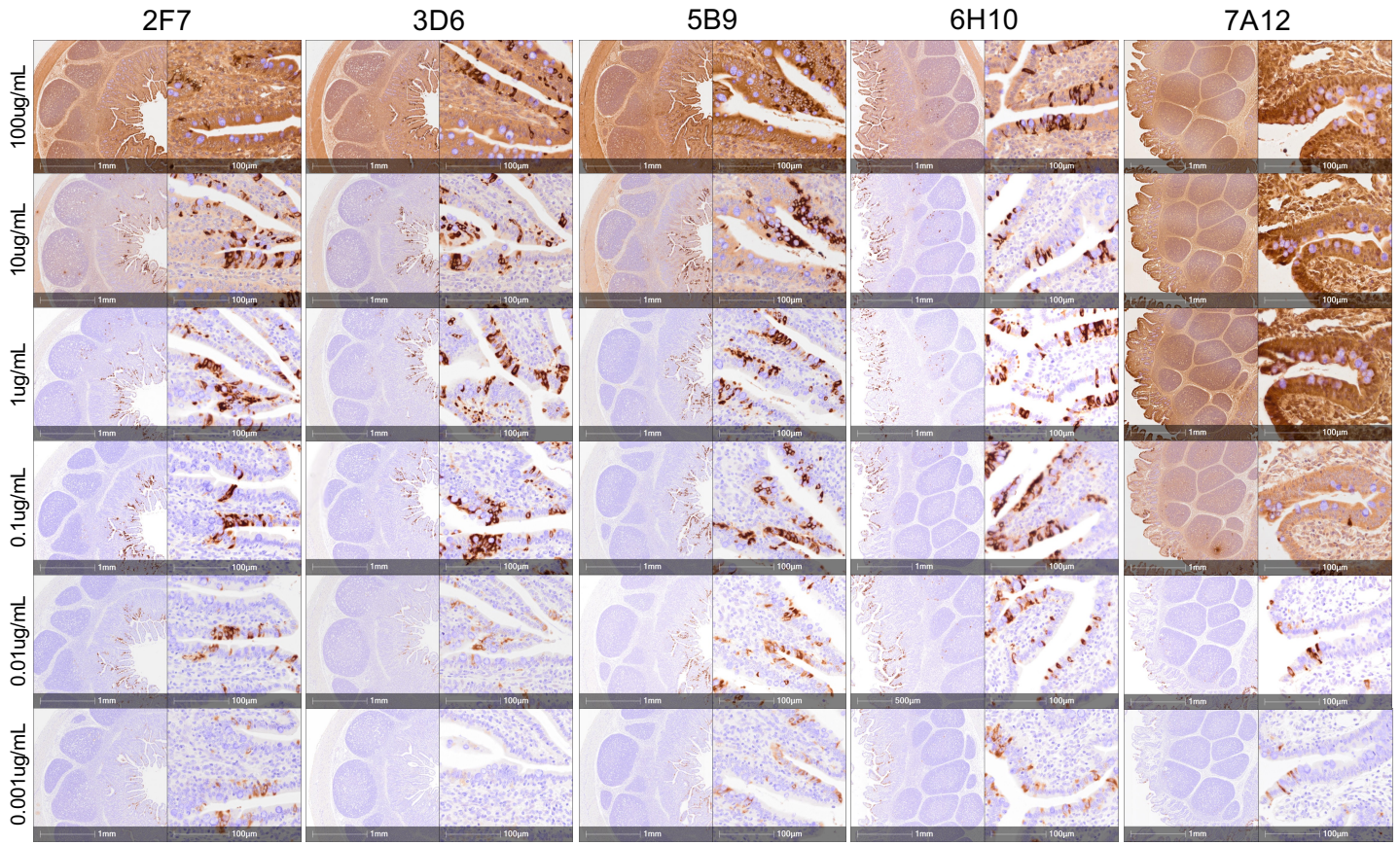
## Mounting

- Submerge slide rack in fresh **95% ethanol 1 min RT**
- Submerge slide rack in fresh **100% ethanol 1 min RT**
- Submerge slide rack in fresh **100% ethanol 1 min RT**
- Submerge slide rack in fresh **100% ethanol 1 min RT**
- Submerge slide rack in fresh **Pro-Par 5 min RT**
- Submerge slide rack in fresh **Pro-Par 5 min RT**
- Submerge slide rack in fresh **Pro-Par 5 min RT**
- **Mount slides** by adding 2-4 drops of mounting media to each slide, followed by application of a cover glass. Remove bubbles from tissue by applying pressure to cover glass
- Place slides flat in a dry, dark space to air dry at RT overnight
- Assess staining with a brightfield microscope

While slides are air drying:

- Discard chromogen detection, counterstaining, and slide mounting reagents
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## Results



IHC staining for PEDV nucleoprotein (brown) with five different antibody clones (columns) tested at concentrations of 100, 10, 1, 0.1, 0.01, and 0.001 ug/mL (rows) in FFPE tissue of ileum from a PEDV-infected nursery pig.

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## Contributions/Acknowledgements

- Staining protocol was developed by Dr. Jayne Wiarda
- Staining protocol was optimized and executed by Dr. Jayne Wiarda, Dr. Eraldo Zanella, and Colin Stoy
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