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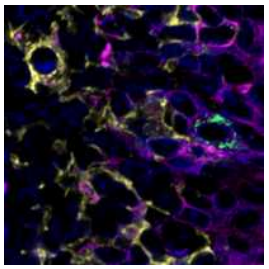
IBA-1 protein, cytokeratin type I/II protein, and Senecavirus RNA detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues



Forked from a private protocol

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We use this protocol and it's working

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Abstract

Detection of IBA-1 (macrophages/dendritic cells) and pan-cytokeratin type I/II (epithelial cells) in conjunction with Senecavirus A (SVA) viral capsid RNA in formalin-fixed, paraffin-embedded (FFPE) palatine tonsil tissue from a pig experimentally infected with SVA.

Attachments

PDF

DualStain_PorcineTis...

1.4MB

Materials

Equipment

- Pipettes/pipette tips – volumes ranging between 2-1000 uL
- Drying oven (able to reach & hold 60℃)
- Fume hood
- Slide staining tray (e.g. Simport M920-2)
- HybEZ II Hybridization System with EZ-Batch Slide System (Advanced Cell Diagnostics 321710/321720)
  - o HybEZ oven (Advanced Cell Diagnostics 321710/321720)
  - o Humidity control tray (Advanced Cell Diagnostics 310012)
  - o HybEZ Humidifying Paper (Advanced Cell Diagnostics 310025)
  - o EZ-Batch Wash Tray (Advanced Cell Diagnostics 321717)
  - o EZ-Batch Slide Holder (Advanced Cell Diagnostics 321716)
- Tissue-Tek Vertical 24 slide rack (Tissue Tech LWS2124)
- Tissue-Tek Staining Dishes (Tissue Tech LWS20WH)
- Tissue-Tek Clearing Agent Dishes, xylene resistant (Tissue Tech LWS20GR)
- 5.5 Quart Digital Steamer (Hamilton Beach 37530Z)
- Confocal/fluorescent microscope and/or slide imaging platform

Reagents/Supplies

- 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)
- 10% NBF (3.7% formaldehyde; Cancer Diagnostics, Inc. 111)
- ImmEdge Hydrophobic Barrier Pen (Vector H-4000)
- RNAscope H2O2 & Protease Plus Reagents (Advanced Cell Diagnostics 322330/322381)
  - o Hydrogen Peroxide (Advanced Cell Diagnostics 322335)
  - o Protease Plus (Advanced Cell Diagnostics 322337)
- RNA-Protein Co-Detection Ancillary Kit (Advanced Cell Diagnostics 323180)
  - o Co-Detection Target Retrieval Reagents (Advanced Cell Diagnostics 322000)
  - o Co-Detection Antibody Diluent (Advanced Cell Diagnostics 323160)
  - o Co-Detection Blocker (Advanced Cell Diagnostics 323170)
- RNAscope Wash Buffer Reagents (Advanced Cell Diagnostics 310091/320058)
- RNAscope Probes, Channel 1 (interchangeable with other channel 1 probes if desiring to target other transcripts)
  - o V-SVV-VP (Advanced Cell Diagnostics 450221)
    - Reactive to Senecavirus A nucleocapsid gene sequence
- RNAscope Multiplex Fluorescent Detection Reagents v2 (Advanced Cell Diagnostics 323110)
  - o AMP 1 (Advanced Cell Diagnostics 323101)
  - o AMP 2 (Advanced Cell Diagnostics 323102)
  - o AMP 3 (Advanced Cell Diagnostics 323103)
  - o HRP-C1 (Advanced Cell Diagnostics 323104)

- HRP blocker (Advanced Cell Diagnostics 323107)
- RNAscope Multiplex TSA Buffer (ACD 322809)
- Opal 570 (Akoya OP-001003)
- Mouse anti-cytokeratin type I/II clonal antibody, clones AE1/AE3; stock concentration 200 ug/mL (Novus Biologicals NBP2-29429)
- Goat anti-IBA-1 polyclonal antibody; stock concentration 600 ug/mL (Fujifilm Wako 011-27991)
  - Stock concentration can vary by antibody lot
- Donkey anti-goat IgG AF647 (Invitrogen A21447)
- Donkey anti-mouse IgG AF488 (Invitrogen A21202)
- ProLong Gold mounting media with DAPI (Invitrogen P36931)
- #1.5 thickness cover glass (e.g. Fisherbrand 2980-245)

Troubleshooting

Safety warnings

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

Before start

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.

Baking 30m

1 Before starting the assay:
- Preheat a dry oven to 60℃
- Load slides for assay into vertical slide rack

2 · **Bake slides 30 min 60℃** 30m

2.1 While slides bake:
o Prepare 0.05% PBS-T

2.2 Immediately before deparaffinizing:
o Add ~200 mL xylenes to each of two clearing agent dishes in a fume hood
o Add ~200 mL 100% ethanol to each of two staining dishes in a fume hood

Deparaffinizing & Rehydrating 25m

3 25m
o Submerge slide rack in fresh **xylenes 5 min RT**
o Submerge slide rack in fresh **xylenes 5 min RT**
o Submerge slide rack in fresh **100% ethanol 5 min RT**
o Submerge slide rack in fresh **100% ethanol 5 min RT**
o **Air dry slides ~5 min** or until completely dry

3.1 While slides deparaffinize/rehydrate:
o Turn off dry oven
o Prepare humidified slide staining tray by adding water to bottom & placing lid on top
o Prepare 1X Co-detection Target Retrieval solution by adding one bottle (70 mL) 10X co-detection target retrieval solution to 630 mL distilled water. 1X solution is good for up to one month stored at 4C.
o Prepare steamer:
  o Fill the bottom reservoir of steamer to the 'Fill' line with tap water
  o Assemble the steamer, using both tiers 1 and 2 of steam bowls. Do not place the divider between steam bowls, as an extra tall compartment is needed
    o Pour 200 mL prepared 1X Co-detection Target Retrieval solution into staining dish and place in the steamer
    o Preheat the prepared steamer, programmed for 1 hour
      o Perform this step ~25-30 minutes before use so that retrieval solution can adequately heat to ~105-110℃

Tissue Quenching 11m

4 · Remove dried slides from vertical rack and place slides flat inside the slide staining tray 11m
· Incubate with **Hydrogen Peroxide 10 min RT**
o Apply to completely cover tissues; let incubate in slide staining tray with lid closed
· Decant slides and transfer to vertical slide rack
· Submerge slide rack in fresh **distilled water, dunking 3-5 times**
· Submerge slide rack in fresh **distilled water, dunking 3-5 times**

4.1 While slides incubate with hydrogen peroxide:
o Discard deparaffinizing & rehydrating reagents
o Add ~200 mL distilled water to each of two staining dishes

Heat-Induced Epitope Retrieval (HIER) 16m

5 · Leave slide rack in water at RT until steamer is preheated 16m
· Once steamer has preheated, submerge slide rack in **preheated 1X Co-detection Target Retrieval solution 15 min**
o Slides should be maintained at ~105-110°C for the duration of target retrieval. To ensure temperature is not reduced, open steamer lid, load slides, and shut steamer lid as quickly as possible.
· Remove slide rack from steamer and turn off steamer
· Submerge slide rack in fresh **distilled water, dunking 3-5 times**
· Submerge slide rack in fresh **PBS-T, dunking 3-5 times**
· Leave slides submerged in PBS-T

5.1 While slides incubate with target retrieval solution:
· Discard tissue quenching reagents
· Add ~200 mL distilled water to one staining dish
· Add ~200 mL PBS-T to one staining dish
▪ Add antibodies for overnight incubation to Co-Detection Antibody Diluent at dilutions as follows: Cytokeratin 1:1000. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting.

Hydrophobic Barrier 25m

6 · **Apply hydrophobic barrier** around each tissue 25m
o 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).
· Leave slide holder in slide staining tray

Primary Antibody

19h 12m

7 18h

- Decant slide holder and again place flat in slide staining tray
- Incubate with **diluted cytokeratin antibody 4C overnight**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed

7.1 The next day:

- Add antibody for 1 hour incubation to Co-Detection Antibody Diluent at dilutions as follows: IBA-1 1:200. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting.

8 1h 12m

- 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**
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Decant slides and again place flat in slide staining tray
- Incubate with **diluted IBA-1 antibody 1 hour RT**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Remove slides from slide staining tray, decant, and transfer to vertical slide rack
- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

8.1 While slides are incubating with IBA-1 antibody:

- Discard HIER reagents
- Add ~200 mL PBS-T to each of three to each of three wash trays
- Add ~200 mL 10% neutral-buffered formalin to one vertical slide staining tray in a fume hood
- Prepare the HybEZ oven:
  - Place a humidifying paper inside the humidity control tray of the HybEZ oven. Turn the oven on and set to 40C to preheat with the humidifying tray inside the oven. Preheat the oven at least 30 minutes prior to use.

Antibody Crosslinking

36m

9 36m

- Decant slide holder and transfer to vertical slide staining rack for formalin fixation
- Submerge slide rack in fresh **10% neutral-buffered formalin 30 min RT**
- Decant slide rack and transfer to staining dishes for PBS-T washes
- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

9.1 While slides incubate with formalin:

- Discard primary antibody reagents

- Add ~200 mL PBS-T to each of three staining dishes

Protease Digestion

16m

10 16m

- Transfer slides from vertical rack back into slide holder and place in humidifying tray taken from preheated HybEZ oven
- Incubate with **Protease Plus 15 min 40C**
  - Apply to completely cover tissues; let incubate in humidifying tray placed within preheated HybEZ oven
- Remove slide holder from HybEZ oven, decant, and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **distilled water, dunking 3-5 times**
- Submerge slide holder in fresh **distilled water, dunking 3-5 times**

10.1 While slides incubate with protease:

- Discard antibody crosslinking reagents
- Add ~200 mL distilled water to each of two wash trays
- Preheat RNAscope probe bottle to 40℃ for 10 min before use by placing them inside the HybEZ oven during protease incubation. Total volume to use is dependent on tissue sizes.

Probe Hybridization

2h 4m

11 2h 4m

- Decant slide holder and place in humidifying tray taken from preheated HybEZ oven
- Incubate with prewarmed, undiluted **RNAscope Senecavirus probe 2 hours 40C**
  - Invert bottle immediately before use; apply to completely cover tissues; let incubate in humidifying tray placed within preheated HybEZ oven
- Remove slide holder from HybEZ oven, decant, and transfer to wash trays for buffer washes
- Submerge slide holder in fresh **1X WashBuffer 2 min RT**
- Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

11.1 While slides incubate with probe:

- Discard protease digestion reagents
- Prepare 1X Wash Buffer:
- Add two 10X bottles (2×60 mL) of wash buffer to 5.88 L of distilled water. 1X solution is stable at RT up to one month.
- Add ~200 mL wash buffer to each of two wash trays
- Place remaining RNAscope reagents at RT at least 30 min before use

RNA Detection

2h 45m

12 2h 45m

- Decant slide holder and place in humidifying tray taken from preheated HybEZ oven
- Incubate with **AMP1 30 min 40C**

o Invert bottle immediately before use; apply to completely cover tissues; let incubate in humidifying tray placed within preheated HybEZ oven

· Remove slide holder from HybEZ oven, decant, and transfer to wash trays for buffer washes

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Decant slide holder and place in humidifying tray taken from preheated HybEZ oven

· Incubate with **AMP2 30 min 40C**

o Invert bottle immediately before use; apply to completely cover tissues; let incubate in humidifying tray placed within preheated HybEZ oven

· Remove slide holder from HybEZ oven, decant, and transfer to wash trays for buffer washes

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Decant slide holder and place in humidifying tray taken from preheated HybEZ oven

· Incubate with **AMP3 15 min 40C**

o Invert bottle immediately before use; apply to completely cover tissues; let incubate in humidifying tray placed within preheated HybEZ oven

· Remove slide holder from HybEZ oven, decant, and transfer to wash trays for buffer washes

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Decant slide holder and place in humidifying tray taken from preheated HybEZ oven

· Incubate with **HRP-C1 15 min 40C**

o Invert bottle immediately before use; apply to completely cover tissues; let incubate in humidifying tray placed within preheated HybEZ oven

· Remove slide holder from HybEZ oven, decant, and transfer to wash trays for buffer washes

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Decant slide holder and place in humidifying tray taken from preheated HybEZ oven

· Incubate with **diluted Opal 570 30 min 40C**

o Invert bottle immediately before use; apply to completely cover tissues; let incubate in humidifying tray placed within preheated HybEZ oven

· Remove slide holder from HybEZ oven, decant, and transfer to wash trays for buffer washes

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

· Decant slide holder and place in humidifying tray taken from preheated HybEZ oven

· Incubate with **HRP blocker 15 min 40C**

o Invert bottle immediately before use; apply to completely cover tissues; let incubate in humidifying tray placed within preheated HybEZ oven

· Remove slide holder from HybEZ oven, decant, and transfer to wash trays for buffer washes
· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**
· Submerge slide holder in fresh **1X Wash Buffer 2 min RT**

12.1 Immediately before Opal incubation:
o Prepare diluted Opal fluorophore by diluting Opal 570 into Multiplex TSA Buffer at a dilution of 1:750. Total volume to use is dependent on tissue sizes. Make sure to mix reagents thoroughly. Store in the dark due to light sensitivity.

12.2 During each incubation:
- Discard reagents from previous incubation step
- Add ~200 mL 1X wash buffer to each of two wash trays

12.3 While slides incubate with HRP blocker:
o Discard remaining RNA detection reagents
o Add ~200 mL wash buffer to each of two wash trays
o Prepare diluted secondary antibody by diluting into Co-detection Antibody Diluent as follows: donkey anti-goat AF647 1:250, donkey anti-mouse AF488 1:100. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Store in the dark due to light sensitivity.

Secondary Antibody/Protein Detection

1h 5m

13 1h 5m
· Decant slide holder and again place flat in slide staining tray
· Incubate with **diluted secondary antibody cocktail 1 hour RT**
o 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**

13.1 While slides incubate with secondary antibody:
o Discard HRP blocker reagents
o Add ~200 mL PBS-T to each of two wash trays
o Turn off HybEZ oven

Nuclei Staining and Coverslipping

50m

14 · **Mount slides** by adding 2-4 drops of ProLong Gold mounting media containing DAPI to each slide, followed by application of a #1.5 thickness cover glass. Remove bubbles from tissue by applying pressure to cover glass.
· Place slides flat in a dry, dark space to **air dry 30 min RT**
· **Transfer to 4C** and image with a fluorescent or confocal microscope within two weeks

14.1 While slides are air drying:
- Discard secondary antibody/protein detection reagents

Protocol references

Staining protocol was developed by Dr. Jayne Wiarda
Staining protocol was optimized and executed by Dr. Jayne Wiarda and Colin Stoy
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