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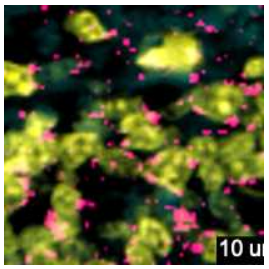
Co-detection of RNA and protein (CD3, IBA-1, Pax5, Ki-67, or pan-cytokeratin type I/II) in formalin-fixed, paraffin-embedded tonsil tissues of pigs, cattle, and white-tailed deer



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We use this protocol and it's working

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Abstract

Detection of CD3e (T cells), IBA-1 (macrophages/dendritic cells), Pax5 (B cells), Ki-67 (proliferating cells), or pan-cytokeratin type I/II (epithelial cells) in conjunction with an RNA marker (housekeeping gene, *UBC*, used herein) in formalin-fixed, paraffin-embedded (FFPE) palatine tonsil tissues from pigs, cattle, and white-tailed deer.

Attachments

PDF

DualRNAProteinStain_...

3.6MB

Guidelines

Assay-specific Information

· Reagents for primary and secondary antibodies are specific to the target being detected. The general scheme of these reagents used to detect each specific target are listed in the table below.

Primary antibody	Secondary antibody
Rabbit anti-CD3ε	Horse anti-rabbit IgG-HRP polymer
Rabbit anti-IBA-1	Horse anti-rabbit IgG-HRP polymer
Goat anti-IBA-1	Horse anti-goat IgG-HRP polymer
Rat anti-Pax5	Goat anti-rat IgG-HRP polymer
Mouse anti-Ki-67	Horse anti-mouse IgG-HRP polymer
Mouse anti-pan-cytokeratin type I/II	Horse anti-mouse IgG-HRP polymer

· Primary antibodies are used at species-specific dilutions and incubation times listed in the table below.

Target	Primary antibody	Pig	Cow	Deer
CD3ε (T cells)	Rabbit anti-CD3ε (stock concentration 400 ug/mL)	2 ug/mL (1:200*) overnight 4C	0.4 ug/mL (1:1,000*) overnight 4C	4 ug/mL (1:100*) overnight 4C
IBA-1 (macrophages/dendritic cells)	Rabbit anti-IBA-1 (stock concentration 500 ug/mL)	0.25 ug/mL (1:2,000*) overnight 4C	0.25 ug/mL (1:2,000*) overnight 4C	0.25 ug/mL (1:2,000*) overnight 4C
IBA-1 (macrophages/dendritic cells)	Goat anti-IBA-1 (stock concentration 600 ug/mL)	3 ug/mL (1:200*) 1 hour RT	6 ug/mL (1:100*) 1 hour RT	6 ug/mL (1:100*) 1 hour RT
Pax5 (B cells)	Rat anti-Pax5 (stock concentration 500 ug/mL)	0.5 ug/mL (1:1,000*) overnight 4C	5 ug/mL (1:100*) overnight 4C	5 ug/mL (1:100*) overnight 4C
Ki-67 (proliferating cells)	Mouse anti-Ki-67 (stock concentration 250 ug/mL)	0.025 ug/mL (1:10,000*) overnight 4C	1.25 ug/mL (1:200*) overnight 4C	10 ug/mL (1:25*) overnight 4C
Cytokeratin type I/II (epithelial cells)	Mouse anti-pan-cytokeratin type I/II (stock concentration 200 ug/mL)	0.067 ug/mL (1:3,000*) overnight 4C	0.2 ug/mL (1:1,000*) overnight 4C	0.05 ug/mL (1:4,000*) overnight 4C

*Dilution factors will need to be adjusted if stock antibody concentrations differ from those listed in the table

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

· To create a negative control slide, antibody diluent should be applied in place of primary antibody, and the DapB RNAscope probe should be applied to slides. All other reagents are still applied to the slide as normal.

Assay Variations

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

Materials

Equipment

- **Pipettes/pipette tips**
- **Dry weigh scale (0.1 gram increments required)**
- **Weigh boats**
- **Reagent weighing spatulas**
- **pH meter**
- **Graduated cylinders for liquid measurements (1 mL increments required)**
- **Glassware/plasticware for liquid reagents**
- **Drying oven (able to reach & hold 60°C)**
- **Fume hood**
- **Slide staining tray (e.g. Simport M920-2)**
- **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)**
- **HybEZ II Hybridization System with EZ-Batch Slide System (Advanced Cell Diagnostics 321710/321720)**
  - **HybEZ oven (Advanced Cell Diagnostics 321710/321720)**
  - **Humidity control tray (Advanced Cell Diagnostics 310012)**
  - **HybEZ Humidifying Paper (Advanced Cell Diagnostics 310025)**
  - **EZ-Batch Wash Tray (Advanced Cell Diagnostics 321717)**
  - **EZ-Batch Slide Holder (Advanced Cell Diagnostics 321716)**
- **5.5 Quart Digital Steamer (Hamilton Beach 37530Z)**
- **Confocal/fluorescent microscope and/or slide imaging platform**

Reagents/Supplies

- Distilled waterc(obtained in-house)
- Phosphate-buffered saline (PBS), pH 7.2-7.4 (made in-house)
- 0.05% PBS-Tween (PBS-T), pH 7.35 (made in-house)
- Xylenes (Macron Fine Chemicals 8668-06)
- 100% ethanol (Pharmco 111000200)
- Fixative
  - 10% NBF (Cancer Diagnostics, Inc. 111) or 4% PFA (Electron Microscopy Sciences 15713)
- ImmEdge Hydrophobic Barrier Pen (Vector H-4000)
- SSC buffer (ThermoFisher Scientific J60839.K3)
- RNAscope H2O2 & Protease Plus Reagents (Advanced Cell Diagnostics 322330/322381)
  - Hydrogen Peroxide (Advanced Cell Diagnostics 322335)

- Protease Plus (Advanced Cell Diagnostics 322337)
- RNA-Protein Co-Detection Ancillary Kit (Advanced Cell Diagnostics 323180)
  - Co-Detection Target Retrieval Reagents (Advanced Cell Diagnostics 322000)
  - Co-Detection Antibody Diluent (Advanced Cell Diagnostics 323160)
  - Co-Detection Blocker (Advanced Cell Diagnostics 323170)
- RNAscope Wash BufferReagents (Advanced Cell Diagnostics 310091/320058)
- RNAscope Multiplex Fluorescent Detection Reagents v2 (Advanced Cell Diagnostics 323110)
  - AMP 1 (Advanced Cell Diagnostics 323101)
  - AMP 2 (Advanced Cell Diagnostics 323102)
  - AMP 3 (Advanced Cell Diagnostics 323103)
  - HRP-C1 (Advanced Cell Diagnostics 323104)
  - HRP blocker (Advanced Cell Diagnostics 323107)
- RNAscope channel 1 probe (interchangeable with other RNAscope probes)
  - Housekeeping gene: H.s. *UBC* (Advanced Cell Diagnostics 310041)
  - Negative control: *DapB* (Advanced Cell Diagnostics 310043)
- Opal 570 fluorophore (Akoya OP001003)
- TSA 650 fluorophore (Advanced Cell Diagnostics 323273)
- Rabbit anti-CD3e polyclonal antibody; stock concentration 0.4 g/mL (Dako A0452)
  - Stock concentration can vary by antibody lot
- Rabbit anti-IBA-1 polyclonal antibody; stock concentration 500 ug/mL (Fujifilm Wako 019-19741)
  - Stock concentration can vary by antibody lot
- Goat anti-IBA-1 polyclonal antibody; stock concentration 600 ug/mL (Fujifilm Wako 011-27991)
  - Stock concentration can vary by antibody lot
- Mouse anti-Ki-67 monoclonal antibody, clone B56; stock concentration 250 ug/mL (BD Biosciences 550609)
- Mouse anti-cytokeratin type I/II clonal antibody, clones AE1/AE3; stock concentration 200 ug/mL (Novus Biologicals NBP2-29429)
- Rat anti-Pax5 monoclonal antibody, clone 1H9; stock concentration 500 ug/mL (Invitrogen 14-9918-82)
- Horse anti-rabbit IgG-HRP polymer (Vector MP-6401)
  - Horse Anti-Rabbit IgG Polymer Reagent
- Horse anti-goat IgG-HRP polymer (Vector MP-7405)
  - Horse Anti-Goat IgG Polymer Reagent
- Horse anti-mouse IgG-HRP polymer (Vector MP-6402)
  - Horse Anti-Mouse IgG Polymer Reagent
- Goat anti-rat IgG-HRP polymer (Vector MP-7404)
  - Goat Anti-Rat IgG Polymer Reagent
- ProLong Gold mounting media with DAPI (Invitrogen P36931)
- #1.5 thickness cover glass (e.g. Fisherbrand 2980-245)

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°C
- Load slides for assay into vertical slide rack

2 • **Bake slides 30 min 60°C** 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
- Submerge slide rack in fresh **xylenes 5 min RT**
- Submerge slide rack in fresh **xylenes 5 min RT**
- Submerge slide rack in fresh **100% ethanol 5 min RT**
- Submerge slide rack in fresh **100% ethanol 5 min RT**
- **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°C

Tissue Quenching 11m

4 11m
- Remove dried slides from vertical rack and place slides flat inside the slide staining tray
- Incubate with **Hydrogen Peroxide 10 min RT**
  - 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:
- Discard deparaffinizing & rehydrating reagents
- Add ~200 mL distilled water to each of two staining dishes

Heat-Induced Epitope Retrieval (HIER) 16m

5 16m
- Leave slide rack in water at RT until steamer is preheated
- Once steamer has preheated, submerge slide rack in **preheated 1X Co-detection Target Retrieval solution 15 min**
  - 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
- Prepare diluted primary antibody by adding antibody stock to co-detection antibody diluent. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Mix diluted antibody well before use. Refer to assay-specific information above to determine the appropriate antibody dilution to use.

Hydrophobic Barrier 20m

6 20m
- **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).

- Leave slide holder in slide staining tray

Primary Antibody

18h

7

- Decant slide holder and again place flat in slide staining tray
- Incubate with **diluted primary antibody 1 hour RT or 4C 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**
- Submerge slide holder in fresh **PBS-T 2 min RT**

1h 6m

Optional stopping point #1

18h

8 While slides incubate with primary antibody:
- Prepare 5X SSC buffer by adding 15 mL 20X SSC buffer to 285 mL dH2O and placing into a wash tray
- Adjust additional preparations outlined during primary antibody incubation below to also accommodate needs/timeline

9

- Submerge slide holder in **5X SSC buffer overnight RT**
- 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**

18h

Antibody Crosslinking

36m

10 While slides incubate with primary antibody:
- Discard HIER reagents
- Add ~200 mL PBS-T to each of three wash trays
- Add ~200 mL 10% neutral-buffered formalin to one vertical slide staining dish 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.

11

- Decant slide holder and transfer to vertical slide staining rack for formalin fixation
- Submerge slide holder 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**

36m

- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

11.1 While slides incubate with formalin:
- o Discard primary antibody reagents
- o Add ~200 mL PBS-T to each of three staining dishes

Protease Digestion

16m

12 16m
- Transfer slides from vertical slide rack back into slide holder and place in humidifying tray taken from preheated HybEZ oven
- Incubate with **Protease Plus 15 min 40C**
  - o 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**

12.1 While slides incubate with protease:
- o Discard antibody crosslinking reagents
- o Add ~200 mL distilled water to each of two wash trays
- o Preheat RNAscope probe bottles to 40°C 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

13 2h 4m
- Decant slide holder and place in humidifying tray taken from preheated HybEZ oven
- Incubate with prewarmed, undiluted **RNAscope probe 2 hours 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**

Optional stopping point #2

18m

14 While slides incubate with probe:
- o Prepare 5X SSC buffer by adding 15 mL 20X SSC buffer to 285 mL dH2O and placing into a wash tray
- o Adjust additional preparations outlined during primary antibody incubation below to also accommodate needs/timeline

15 18m
- Submerge slide holder in **5X SSC buffer overnight RT**
- 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**

RNA Detection 2h 45m

16 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

17 2h 45m
- Decant slide holder and place in humidifying tray taken from preheated HybEZ oven
- Incubate with **AMP1 30 min 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 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**
  - 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**
  - 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**
  - 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**
  - 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**
  - 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**

17.1 Immediately before Opal incubation:
- 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.

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

17.3 While slides incubate with blocker:
- Discard remaining RNA detection reagents
- Add ~200 mL wash buffer to each of two wash trays

Secondary Antibody

35m

18 35m
- Decant slide holder and again place flat in slide staining tray
- Incubate with **secondary antibody (HRP-conjugated polymer) 30 min RT**
  - Refer to assay-specific information above to determine which secondary antibody to use
  - Secondary antibody polymer is a component of the secondary antibody reagent kit
  - Invert bottle immediately before use; 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**

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

18.2 Immediately before protein detection:
- o Prepare diluted TSA fluorophore by diluting TSA 650 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.

Protein Detection

1h 5m

19 1h 5m

- Decant slide holder and again place flat in slide staining tray
- Incubate with **diluted TSA 650 1 hour RT**
  - o Invert bottle immediately before use; apply to completely cover tissues; let incubate in humidifying tray placed within preheated HybEZ oven
- 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**

19.1 While slides incubate with TSA:
- o Discard secondary antibody reagents
- o Add ~200 mL PBS-T to each of two wash trays

Nuclei Staining and Coverslipping

1h

20 1h

- **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

20.1 While slides are air drying:
- o Discard protein detection reagents

Protocol references

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