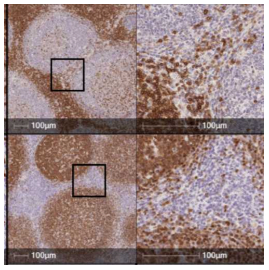
Dec 19, 2024

Single-plex brightfield detection of CD3e, IBA-1, Pax5, Ki-67, and pan-cytokeratin type I/II in formalin-fixed, paraffin-embedded tonsil tissues of pigs, cattle, and white-tailed deer



Forked from a private protocol

DOI
https://dx.doi.org/10.17504/protocols.io.14egn9zrpl5d/v1

Jayne Wiarda[1], Eraldo L Zanella[1], Crystal Loving[2], Mitchell Palmer[1]

[1]National Animal Disease Center, ARS, USDA; [2]ARS-NADC

Jayne Wiarda

Jayne E Wiarda
National Animal Disease Center, ARS, USDA

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.14egn9zrpl5d/v1

Protocol Citation: Jayne Wiarda, Eraldo L Zanella, Crystal Loving, Mitchell Palmer 2024. Single-plex brightfield detection of CD3e, IBA-1, Pax5, Ki-67, and pan-cytokeratin type I/II in formalin-fixed, paraffin-embedded tonsil tissues of pigs, cattle, and white-tailed deer. **protocols.io** https://dx.doi.org/10.17504/protocols.io.14egn9zrpl5d/v1

Protocol status: Working
We use this protocol and it's working

Created: November 25, 2024

Last Modified: December 19, 2024

Protocol Integer ID: 112727

Keywords: deer detection of cd3e, palatine tonsil tissues from pig, palatine tonsil tissue, embedded tonsil tissue, tonsil tissues of pig, plex brightfield detection of cd3e, tailed deer detection, macrophage, epithelial cell, tailed deer, plex brightfield detection, cell, dendritic cell

Funders Acknowledgements:
USDA-ARS
Grant ID: CRIS #5030-32000-230-000-D

Disclaimer

All opinions expressed in this paper are the authors' and do not necessarily reflect the policies and views of USDA, ARS, DOE, or ORAU/ORISE. Mention of trade names or products is for information purposes only and does not imply endorsement by the USDA. USDA is an equal opportunity employer and provider.

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 formalin-fixed, paraffin-embedded (FFPE) palatine tonsil tissues from pigs, cattle, and white-tailed deer.

Attachments

PDF

Wiarda_SingleplexIHC...

2.4MB

Guidelines

Assay-specific Information

· Reagents for protein blocking, primary antibody, and secondary antibody 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.

	Protein block	Primary antibody	Secondary antibody
	2.5% horse serum	Rabbit anti-CD3e	Horse anti-rabbit IgG-HRP polymer
	2.5% horse serum	Rabbit anti-IBA-1	Horse anti-rabbit IgG-HRP polymer
	2.5% horse serum	Goat anti-IBA-1	Horse anti-goat IgG-HRP polymer
	2.5% goat serum	Rat anti-Pax5	Goat anti-rat IgG-HRP polymer
	2.5% horse serum	Mouse anti-Ki-67	Horse anti-mouse IgG-HRP polymer
	2.5% horse serum	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.

	A	B	C	D	E
	Target	**Primary antibody**	**Pig**	**Cow**	**Deer**
	CD3e (T cells)	Rabbit anti-CD3e (stock concentration 400 ug/mL)	0.4 ug/mL (1:1,000∗) overnight	0.4 ug/mL (1:1,000∗) overnight	0.4 ug/mL (1:1,000∗) overnight 4C

	A	B	C	D	E
			4C	4C	
	IBA-1 (macrophages/dendritic cells)	Rabbit anti-IBA-1 (stock concentration 500 ug/mL)	0.063 ug/mL (1:8,000*) overnight 4C	0.05 ug/mL (1:10,000*) overnight 4C	0.05 ug/mL (1:10,000*) overnight 4C
	IBA-1 (macrophages/dendritic cells)	Goat anti-IBA-1 (stock concentration 600 ug/mL)	0.3 ug/mL (1:2,000*) 1 hour RT	0.6 ug/mL (1:1,000*) 1 hour RT	0.3 ug/mL (1:2,000*) 1 hour RT
	Pax5 (B cells)	Rat anti-Pax5 (stock concentration 500 ug/mL)	0.05 ug/mL (1:10,000*) overnight 4C	0.05 ug/mL (1:10,000*) overnight 4C	0.05 ug/mL (1:10,000*) overnight 4C
	Ki-67 (proliferating cells)	Mouse anti-Ki-67 (stock concentration 250 ug/mL)	0.025 ug/mL (1:10,000*) overnight 4C	0.125 ug/mL (1:2,000*) 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	0.05 ug/mL (1:4,000*) 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

·

IHC controls:

o 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.

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)**
- **EZ-Batch Slide Holder (Advanced CellDiagnostics 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)**
- **5.5 Quart Digital Steamer (Hamilton Beach 37530Z)**
- **Brightfield microscope and/or slide imaging platform**

Reagents/Supplies

- Distilled water (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 8668-16)
- 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)
- 1X Sodium Citrate Antigen Retrieval Solution, pH 6.0 (made in-house)
  - 10X sodium citrate solution: Dissolve 29.4 g sodium citrate dihydrate in 950 mL distilled water. Add water to total volume of 1L after solid has dissolved. Store at 4C up to one year.
  - 1X sodium citrate solution: 100 mL 10X sodium citrate solution + 900 mL distilled water. Adjust pH to 6.0. Store at 4C up to three months.
- BLOXALL Endogenous Blocking Solution (Vector SP-6000)

- 1% bovine serum albumin (BSA) in PBS (made in-house)
- 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

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

20m

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

1.1

2
- **Bake slides 20 min 60°C**

20m

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

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

Deparaffinizing & Rehydrating

25m

3
- 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**

25m

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 steamer:
- o Fill the bottom reservoir of steamer to the 'Fill' line with tap water

- 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
- Pour 200 mL prepared 1X Sodium Citrate Buffer, pH 6.0 into staining dish and place in the steamer
- Preheat the prepared steamer, programmed for 1 hour
- Perform this step ~25-30 minutes before use so that retrieval solution can adequately heat to ~105-110°C

Heat-Induced Epitope Retrieval (HIER) 20m

4 20m

- Leave slide rack in PBS-T at RT until steamer is preheated
- Once steamer has preheated, submerge slide rack in **preheated 1X Sodium Citrate Buffer 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 **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

4.1 While slides incubate with sodium citrate buffer:

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

Hydrophobic Barrier 20m

5 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). 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

Tissue Quenching 15m

6 15m

- 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**

6.1 While slides incubate with enzyme block:
- o Discard HIER reagents
- o Double check steamer is turned off
- o Add ~200 mL PBS-T to each of two wash trays

Protein Blocking 30m

7 30m
- Decant slide holder and again place flat in slide staining tray
- Incubate with **2.5% blocking serum 30 min RT**
  - o Refer to assay-specific information above to determine which serum to use
  - o Serum is a component of the secondary antibody reagent kit
  - o Apply to completely cover tissues; let incubate in slide staining tray with lid closed

7.1 While slides incubate with serum:
- o Discard tissue quenching reagents
- o Prepare diluted primary antibody by adding antibody stock to 1% BSA in PBS. 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.

Primary Antibody 18h

8 1h 5m
- Decant slide holder and again place flat in slide staining tray
  - o Do not wash with PBS-T between decanting serum and applying secondary antibody
- Incubate with diluted **primary antibody 1 hour RT or 4C overnight**
  - o Refer to assay-specific information above to determine antibody incubation time
  - 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**

8.1 While slides incubate with primary antibody:
- o Discard protein blocking reagents
- o Add ~200 mL PBS-T to each of two wash trays

Secondary Antibody 35m

9 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
  - 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**

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

9.2 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) 12m

10 12m

- 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**

10.1 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 2m

11 2m

- 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

45m

12 45m

- 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

12.1 While slides are air drying:
- Discard chromogen detection, counterstaining, and slide mounting reagents

Protocol references

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
· We thank Judith Stasko and Adrienne Shircliff for slide sectioning and imaging
· We thank Drs. Mitchell Palmer and Crystal Loving for providing archived tissue specimens