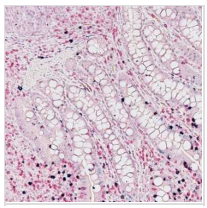

Oct 20, 2022

Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues

Jayne E Wiarda[1]

[1]National Animal Disease Center, ARS, USDA

dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

Jayne Wiarda

Jayne E Wiarda
National Animal Disease Center, ARS, USDA

ABSTRACT

A combined staining protocol for CD3e immunohistochemistry (IHC) and *TRDC* RNA *in-situ* hybridization (RNA-ISH) created for *in-situ* identification of porcine gamma delta T cells (*TRDC*$^+$) and alpha beta T cells (*TRDC*$^-$CD3e$^+$)

ATTACHMENTS

DualStain_PorcineTcells_CD3e_TRDC.pdf

DOI

dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

PROTOCOL CITATION

Jayne E Wiarda 2022. Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues. **protocols.io** https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

FUNDERS ACKNOWLEDGEMENT

USDA-ARS CRIS project
Grant ID: 5030-3200-225-00D

AFRI USDA-NIFA predoctoral grant
Grant ID: 2021-67011-35228

KEYWORDS

NP108, Porcine T cells, in-situ, in situ, IHC/ISH, ISH/IHC, in-situ hybridization, immunohistochemistry, CD3, CD3e, TRDC, RNAscope, Dual stain, in situ hybridization, pig, swine, Sus scrofa, porcine, T cell, T cells, gd T cell, gamma delta T cell, alpha beta T cell, ab T cell

CREATED

Sep 19, 2022

LAST MODIFIED

Oct 20, 2022

PROTOCOL INTEGER ID

70231

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

GUIDELINES

Assay Controls:

Here are a few controls you can use to ensure assay is working correctly:

- IHC controls:
  - Negative control (primary antibody only)
    - This slide receives 0.05% PBS-T in place of secondary antibody
  - Negative control (secondary antibody only)
    - This slide receives co-detection antibody diluent in place of diluted primary antibody
- RNA-ISH controls:
  - Positive control
    - This slide is incubated with PPIB probe
  - Negative control
    - This slide is incubated with DapB probe

Assay variations:

This assay can be used to detect other proteins or transcripts as well or for detection of protein/RNA in other species by adjusting the antibodies and probes used. Antibodies must be able to detect proteins following heat-induced target retrieval outlined in protocol.

This assay uses chromogens that cause the RNA signal to dominate the protein signal (i.e. green RNA signal covers up the lighter red protein signal when markers are co-expressed). Other chromogen combinations can be used to instead have the protein signal dominate the RNA signal. For example, Fast Red AP chromogen that comes with the RNAscope kit can be used to detect RNA signal, followed by protein detection with a darker DAB chromogen, such as DAB-Nickel (black), or other DAB chromogens in colors like blue or green.

Parameters for some steps (e.g. antibody incubations, protease incubations, target retrieval) may need to be further optimized for different tissues or targets.

MATERIALS TEXT

Equipment:

- Pipettes/pipette tips – volumes ranging between 2-1000 uL
- Drying oven (able to reach & hold 60°C)
- Fume hood
- Decloaking Chamber NxGen (Biocare Medical DC2012/DC2012-220V)
  - Can substitute with hot plate by using alternative target retrieval protocol; see Appendix B: Manual Target Retrieval from Advanced Cell Diagnostics [ACD] FFPE Sample Preparation and Pretreatment User Manual (Document No. 322452)
- Slide staining tray (e.g. Simport M920-2)
- HybEZ II Hybridization System with ACD EZ-Batch Slide System (ACD 321710/321720)
  - HybEZ oven (ACD 321710/321720)
  - Humidity control tray (ACD 310012)

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

- HybEZ Humidifying Paper (ACD 310025)
- EZ-Batch Wash Tray (ACD 321717)
- EZ-Batch Slide Holder (ACD 321716)

- 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)
- Bright field microscope

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)
- 10% NBF (3.7% formaldehyde; Cancer Diagnostics, Inc. 111)
- ImmEdge Hydrophobic Barrier Pen (Vector H-4000)
- RNAscope H2O2 & Protease Plus Reagents (ACD 322330)
  - Hydrogen Peroxide (ACD 322335)
  - Protease Plus (ACD 322331)
- RNA-Protein Co-Detection Ancillary Kit (ACD 323180)
  - Co-Detection Target Retrieval Reagents (ACD 323165/323166)
  - Co-Detection Antibody Diluent (ACD 33160)
  - Co-Detection Blocker (ACD 323170)
- RNAscope Wash Buffer Reagents (ACD 310091/320058)
- RNAscope 2.5 HD Detection Reagents – RED (ACD 322360)
  - AMP1 (ACD 322311)
  - AMP2 (ACD 322312)
  - AMP3 (ACD 322313)
  - AMP4 (ACD 322314)
  - AMP5 (ACD 322361)
  - AMP6 (ACD 322362)
- RNAscope probes, channel 1
  - *TRDC* (ACD 553141)
  - *PPIB* (ACD 428591)
  - *DapB* (ACD 310043)
- CD3e antibody (Dako A0452)
- Anti-rabbit horse radish peroxidase (HRP) polymer (Dako K4003)
- Green alkaline phosphatase (AP) chromogen (Enzo Life Sciences ENS-ACC130-0030)
- PermaRED HRP chromogen (Diagnostic BioSystems K075)
- Gill's Hematoxylin I (American Master Tech Scientific HXGHE1LT)
- VectaMount Permanent Mounting Medium (Vector H-5000)
- #1 thickness cover glass (Fisherbrand 12-545-F)

SAFETY WARNINGS

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

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

BEFORE STARTING

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 but not over-fixed as to over-fragment RNA. Tissues no thicker than 0.5 centimeters should be freshly harvested and placed into 10% neutral-buffered formalin (NBF; 3.7% formaldehyde) at a tissue:fixative volume no more than 1:20. Tissues should be fixed in 10% NBF for 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 baking slides:

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

- **Bake slides 30 min 60°C**

Optional stopping point: store slides in a dry place & use within 1 week

While slides bake:

- Prepare 0.05% PBS-T (can store at RT up to 1 month)
- Prepare 1X Co-Detection Target Retrieval solution by adding 1 bottle (70 mL) Co-Detection Target Retrieval Reagent (10X stock concentration) to 630 mL distilled water (can store at 4°C up to 1 month)

Deparaffinizing 25m

25m

2 Immediately before deparaffinizing:

- Add ~200 mL xylenes to each of two clearing agent dishes in a fume hood
- Add ~200 mL 100% ethanol to each of two staining dishes in a fume hood

- Submerge slide in fresh **xylenes 5 min RT**
- Submerge slide in fresh **xylenes 5 min RT**
- Submerge slides in fresh**100% ethanol 5 min RT**
- Submerge slides in fresh**100% ethanol 5 min RT**
- **Air dry slides ~5 min** or until completely dry

Optional stopping point: store slides in a dry place & use within 24 hours

While slides deparaffinize:

- Prepare decloaking chamber:
- ---------- Pour 500 mL distilled water into central chamber
- ---------- Pour 200 mL distilled water into left/right staining dishes
- ---------- Pour 200 mL prepared Co-Detection Target Retrieval solution into middle staining dish
- Turn off dry oven

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

Tissue Quenching

12m

3 Immediately before tissue quenching:

- Preheat the prepared decloaking chamber, programmed for 15 min at 95°C
- ---------- Chamber will take exactly 15 min to preheat, and there will be a 2 min window to add slides before chamber pressurizes & locks

- Unload slides from vertical slide rack and place on flat surface of bench top
- Incubate with **Hydrogen Peroxide 10 min RT**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues; let incubate on bench top
- 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**

While slides incubate with Hydrogen Peroxide:

- Discard deparaffinizing reagents
- Add ~200 mL distilled water to each of two staining dishes

Target Retrieval 16m

16m

4
- Leave slides in water at RT until decloaker is preheated (<5 min)
- Once decloaker has preheated, submerge slide rack in **preheated distilled water 10 sec** (left or right dishes in decloaker)
- Submerge slide rack in **preheated 1X Co-detection Target Retrieval solution 15 min 95°C**
- ---------- Once slides are places in center staining dish of decloaker, close the decloaker (make sure pressure valve is in place to hold pressure when replacing lid) & wait for alarm to go off in 15 min
- Release decloaker chamber pressure valve & open chamber
- Submerge slide rack in **preheated distilled water 10 sec** (left or right dishes in decloaker)
- Submerge slide rack in fresh **distilled water, dunking 3-5 times**
- Submerge slide rack infresh **distilled water, dunking 3-5 times**
- Submerge slide rack infresh **PBS-T, dunking 3-5 times**
- Leave slides in PBS-T

While slides incubate in 1X target retrieval solution:

- Discard tissue quenching reagents
- Add ~200 mL distilled water to each of two staining dishes
- Add ~200 mL PBS-T to one staining dish
- Prepare humidified slide staining tray by adding water to bottom & placing lid on top
- Add CD3e antibody to Co-Detection Antibody Diluent at a 1:50 dilution. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting.

Hydrophobic Barrier 10m

10m

- **Apply hydrophobic barrier** around each tissue

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

5 ▪ ---------- 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 slide flat in 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)

Primary Antibody 1h 7m

6 1h 7m
- Decant slides and again place flat in slide staining tray
- Incubate with **diluted primary antibody 60 min RT**; place lid back on slide staining tray during this time
- 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**

While slides are incubating with primary antibody:
- Discard target retrieval reagents
- Add ~200 mL PBS-T to each of three staining dishes
- Add ~200 mL 10% NBF to one staining dish in a fume hood

Antibody Cross-linking 37m

7 37m
- Submerge slide rack in **10% NBF 30 min RT**
- 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**

While slides are incubating with 10% NBF:
- Discard primary antibody reagents
- Add ~200 mL PBS-T to each of three staining dishes
- Prepare HybEZ Oven:
- ---------- Place humidifying paper within the humidity control tray & apply distilled water to fully wet paper
- ---------- Place humidifying tray into HybEZ oven and clamp down the gasket to seal
- ---------- Preheat oven to 40°C for at least 30 minutes before use

Protease

8 16m
- Transfer slides into EZ-Batch Slide Holder, taking care not to let tissues dry out
- Incubate with **Protease Plus 15 min 40°C**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **distilled water, dunking 3-5 times**
- Submerge slide holder in fresh **distilled water, dunking 3-5 times**

While slides are incubating with protease:

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

- Empty the slide staining tray used for primary antibody incubations & put away
- Discard antibody cross-linking reagents
- Add ~200 mL distilled water to each of two wash trays
- Preheat RNAscope probes to 40°C for 10 min before use; this can be done by placing them inside the HybEZ oven during protease incubation

Probe Hybridization 2h 5m

9 2h 5m

- Decant slides (without removing slides from holder)
- Incubate with appropriate RNAscope **probe 2 hours 40°C**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

While slides are incubating with probes:

- Discard protease reagents
- Prepare 1X wash buffer by adding 1 bottle (60 mL) Wash Buffer (10X stock concentration) to 3.94 L distilled water
- ---------- If 10X Wash Buffer solution has a precipitant formed, preheat bottle at 37°C for 1 hour before adding to distilled water
- ---------- Will have to prepare another batch of 1X wash buffer later in protocol, after first batch runs out. Alternatively, prepare both batches at once (120 mL 1X Wash Buffer + 7.88 L distilled water)
- ---------- Store at RT up to one month
- Add ~200 mL 1X wash buffer to each of two wash trays
- Place AMPs from RNAscope detection kit at RT for at least 30 min before use (should be RT when used)

RNA Signal Amplification 2h 45m

10 2h 45m

- Decant slides (without removing slides from holder)
- Incubate with **AMP1 30 min 40°C**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Decant slides (without removing slides from holder)
- Incubate with **AMP2 15 min 40°C**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Decant slides (without removing slides from holder)
- Incubate with **AMP3 30 min 40°C**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Decant slides (without removing slides from holder)
- Incubate with **AMP4 15 min 40°C**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Decant slides (without removing slides from holder)
- Incubate with **AMP5 30 min RT**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray left on bench top
- Remove slide holder from humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Decant slides (without removing slides from holder)
- Incubate with **AMP6 15 min RT**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray left on bench top
- Remove slide holder from humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

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

RNA Detection

11 25m

Immediately before RNA detection:
- Prepare diluted green AP chromogen by adding 80 uL HIGHDEF Green AP Chromogen per 920 uL HIGHDEF Green AP Substrate. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Store in the dark due to light sensitivity

- Decant slides (without removing slides from holder)
- Incubate with **diluted green AP chromogen 20 min RT**
- ---------- Pipette well to mix immediately before use; pipette appropriate volumes to completely cover tissues & transfer slide holder to humidifying tray left on bench top

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

- Remove slide holder from humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh**1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

While slides are incubating with green AP chromogen:
- Discard remaining AMP detection reagents
- Add ~200 mL 1X wash buffer to each of two wash trays

Tissue Blocking 20m

12 20m
- Decant slides (without removing slides from holder)
- Incubate with **Co-Detection Blocker 15 min 40°C**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray left on bench top
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

While slides are incubating with Co-Detection Blocker:
- Discard RNA detection reagents
- Add ~200 mL 1X wash buffer to each of two wash trays
- Add ~200 mL PBS-T to one wash tray

Secondary Antibody

13 35m
- Decant slides (without removing slides from holder)
- Incubate with **anti-rabbit HRP polymer 30 min RT**
- ---------- Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray on bench top
- Remove slide holder from humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

While slides are incubating with secondary antibody:
- Discard tissue blocking reagents
- Add ~200 mL 1X wash buffer to each of two wash trays
- Add ~200 mL PBS-T to one wash tray (if you only have two wash trays, complete this step after one PBS-T incubation after secondary antibody incubation)
- Turn off HybEZ oven

Protein Detection 11m

14 11m
Immediately before protein detection:
- Prepare diluted red horseradish peroxidase (HRP) chromogen by adding 1 drop PermaRed/HRP Chromogen per 1 mL PermaRed/HRP Substrate Buffer. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Store in the dark due to light sensitivity

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1

- Decant slides (without removing slides from holder)
- Incubate with **diluted red HRP chromogen 10 min RT**
- ---------- Pipette well to mix immediately before use; pipette appropriate volumes to completely cover tissues & transfer slide holder to humidifying tray left on bench top
- ---------- At ~3 and ~6 min into incubation, use a pipette to redistribute chromogen on each slide (this chromogen tends to sediment and settle on slides, causing uneven staining if it is not redistributed)
- Remove slide holder from humidifying tray, decant, & transfer slides into 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**
- Submerge slide rack in fresh **distilled water, dunking 3-5 times**

While slides are incubating with red HRP chromogen:
- Discard secondary antibody reagents
- Add ~200 mL distilled water to each of six staining dishes
- Add ~200 mL hematoxylin to one staining dish

Counterstaining 3m

15 3m
- Submerge slide rack in **hematoxylin 2 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**

Mounting 30m

16 30m
- Decant slides (without removing slides from rack)
- **Air dry slides ~20 min** or until completely dry
- **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 bright-field microscope; stores slides in the dark at RT

While slides are air drying:
- Discard protein detection and counterstaining reagents

Citation: Jayne E Wiarda Dual staining for gamma delta and alpha beta T cell detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues https://dx.doi.org/10.17504/protocols.io.q26g7yro1gwz/v1