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Tri-plex staining for PAX5, CD4, and CSF1R detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues

Forked from a private protocol

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Jayne E Wiarda[1], Alyssa Ivy[1], Hannah Mazon[1], Colin Stoy[1], sage.becker[1], Crystal Loving[1]

[1]ARS-NADC

Crystal L Loving
ARS-NADC

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

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Abstract

A protocol for staining of protein (PAX5) and RNA (CD4, CSF1R) in pig tissues.

Guidelines

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 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) or 4% paraformaldehyde (PFA) at a ratio of at least 20 volumes fixative per one volume tissue. Tissues should be fixed 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.

Assay Controls:

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

o Negative control (primary antibody only)
This slide receives co-detection antibody diluent in place of diluted secondary antibody.
o Negative control (secondary antibody only)
This slide receives co-detection antibody diluent in place of diluted primary antibody.
o Batch control
If performing staining across multiple batches, include serial sections of one tissue in each batch that has positive staining for PAX5.

• RNA-ISH controls:

o Positive control
- This slide is incubated with PPIB probe.

o Negative control
- This slide is incubated with DapB probe.

Assay Variations:

Parameters for some steps (e.g. antigen retrieval, antibody incubations, protease plus incubation, fluorophore/opal incubations, some AMP incubations, counterstaining) may need to be further optimized for different tissues or targets. This protocol is likely applicable to tissues of other species. Consult ACD for optimization assistance.

Materials

Equipment:

• Pipettes/pipette tips – volumes ranging between 2-1000 µL
• Drying oven (able to reach & hold 60°C)
• Fume hood
• Decloaking Chamber NxGen (Biocare Medical DC2012/DC2012-220V)

Equipment

Decloaking Chamber™ NxGen NAME

NXGEN™ BRAND

DC2012 SKU

https://biocare.net/products/instrumentation/decloaking-chamber-nxgen/ LINK

Equipment

Decloaking Chamber™ NxGen NAME

NxGen™ BRAND

DC2012-220V SKU

https://biocare.net/products/instrumentation/decloaking-chamber-nxgen/ LINK

o 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)

Equipment

ACD HybEZ™ II Hybridization System (110v)With ACD EZ-Batch Slide System

NAME

Bio-Techne

BRAND

321710

SKU

https://acdbio.com/store/acd-hybeztm-ii-hybridization-system-110v-with-acd-ez-batch-slide-system.html

LINK

Equipment

ACD HybEZ™ II Hybridization System (220v)With ACD EZ-Batch Slide System

NAME

Bio-Techne

BRAND

321720

SKU

https://acdbio.com/store/acd-hybeztm-ii-hybridization-system-220v-with-acd-ez-batch-slide-system.html

LINK

o HybEZ oven (ACD 321710/321720)
o Humidity control tray (ACD 310012)

Equipment

ACD HybEZ Humidity Control Tray

NAME

Bio-Techne

BRAND

310012

SKU

https://acdbio.com/store/acd-hybez-humidity-control-tray.html

LINK

o HybEZ Humidifying Paper (ACD 310025)
o EZ-Batch Wash Tray (ACD 321717)

Equipment

ACD EZ-Batch Wash	NAME
Bio-Techne	BRAND
321717	SKU
https://acdbio.com/store/acd-ez-batch-wash-tray.html	LINK

o EZ-Batch Slide Holder (ACD 321716)

Equipment

ACD EZ-Batch Slide Holder	NAME
Bio-Techne	BRAND
321716	SKU
https://acdbio.com/store/acd-ez-batch-slide-holder.html	LINK

• Tissue-Tek Vertical 24 slide rack (American Master Tech Scientific LWS2124)

Equipment

TissueTek VERTICAL 24 SLIDE RACK/Ea	NAME
American Mastertech	BRAND
LWS2124	SKU
https://www.clinisciences.com/en/other-products-186/tissuetek-vertical-24-slide-rack-768002782.html	LINK

• Tissue-Tek Staining Dishes (American Master Tech Scientific LWS20WH)

Equipment

Staining Dish (White) w/lid /Each	NAME
American Mastertech	BRAND
LWS20WH	SKU
https://www.clinisciences.com/en/other-products-186/staining-dish-white-w-lid-each-768002781.html	LINK

• Tissue-Tek Clearing Agent Dishes, xylene resistant (American Master Tech Scientific LWS20GR)

• Confocal microscope

Reagents/Supplies:

Note

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

• Xylenes (Macron Fine Chemicals 8668-16)

Macron™ 8668-16 Xylenes, AR ACS Reagent Grade, 4L Poly Bottle **Capitol Scientific Catalog #**8666-16

• 100% ethanol (Pharmco 111000200)

• RNAscope H2O2 & Protease Plus Reagents (ACD 322330)

RNAscope® H202 & Protease Plus Reagents **Advanced Cell Diagnostics Catalog #**322330

o Hydrogen Peroxide (ACD 322335)

RNAscope® Hydrogen Peroxide **Advanced Cell Diagnostics Catalog #**322335

o Protease Plus (ACD 322331) RNAscope® Protease Plus **Advanced Cell Diagnostics Catalog #**322331

• Distilled water (obtained in-house)

• RNA-Protein Co-Detection Ancillary Kit (ACD 323180)

RNA-Protein Co-Detection Ancillary Kit **Advanced Cell Diagnostics Catalog #**323180

o Co-Detection Target Retrieval Reagents (ACD 323165/323166)

o Co-Detection Antibody Diluent (ACD 33160)

o Co-Detection Blocker (ACD 323170) (not applicable for fluorescence)

• 0.05% PBS-Tween (PBS-T), pH 7.35 (made in-house)

• ImmEdge Hydrophobic Barrier Pen (Vector H-4000)

ImmEdge® Hydrophobic Barrier PAP Pen (H-4000) **Vector Laboratories Catalog #**H-4000

• Anti-PAX5 monoclonal antibody (1H9), eBioscienceTM; rat IgG2a; stock concentration 500 ug/mL (Invitrogen 14-9918-82) PAX5 Monoclonal Antibody (1H9), eBioscience™ **Invitrogen Catalog #**14-9918-82

• 10% NBF (3.7% formaldehyde; Cancer Diagnostics, Inc. 111)

• RNAscope Probe, Channel 1 (interchangeable with other channel 1 probe to detect different transcript)

o Ss-CSF1R (ACD 1234601-C1)

RNAscope™ Probe- Ss-CSF1R-C1 **Advanced Cell Diagnostics Catalog #**1234601-C1

• RNAscope Probe, Channel 2 (interchangeable with other channel 2 probe to detect different transcript)

o CD4 (ACD 491891-C2) RNAscope™ Probe- Ss-CD4-C2 **Advanced Cell Diagnostics Catalog #**491891-C2

• RNAscope Wash Buffer Reagents (ACD 310091/320058)

RNAscope® Wash Buffer Reagents **Advanced Cell Diagnostics Catalog #**310091

• RNAscope Multiplex Fluorescent Detection Reagents v2 (ACD 323110)

RNAscope® Multiplex Fluorescent Detection Kit v2 **Advanced Cell Diagnostics Catalog #**323110

o Amp 1 (ACD 323101)

o Amp 2 (ACD 323102)

o Amp 3 (ACD 323103)

o HRP-C1 (ACD 323104)

o HRP-C2 (ACD 323105)

o HRP blocker (ACD 323107)

o DAPI (ACD 323108)

• TSA Vivid Fluorophore 520 (ACD 323271)

TSA Vivid Fluorophore 520 **Advanced Cell Diagnostics Catalog #**323271

• RNAscope Multiplex TSA Buffer (ACD 322809)
• Opal 650 (Akoya Biosciences FP1496001KT)

OPAL 650 REAGENT PACK **Akoya Biosciences Catalog #**FP1496001KT

• Protein Block – serum-free, ready-to-use (Dako X0909) Protein Block Serum free **Dako Catalog #**Ref. X0909

• Donkey anti-Rat IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (Thermo A-21209)

Anti-Rat **Invitrogen - Thermo Fisher Catalog #**A21209

• ProLong Gold Antifade reagent (Invitrogen P36930) Prolong Gold **Thermo Fisher Scientific Catalog #**P36930

• #1.5 thickness cover glass (Fisherbrand 12-545-F)

Fisherbrand™ Cover Glasses: Rectangles **Fisher Scientific Catalog #**12-545-F

Before start

• Preheat a dry oven to 60°C.
• Trim tissue on slides to smaller size if needed.
• Load slides for assay into vertical slide rack

Baking

30m

1 ▪ **Bake slides** 00:30:00 60 °C .

30m

Note

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

2 While slides bake:

2.1 Prepare 0.05% PBS-T (can store at Room temperature up to 1 month).

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

3 Immediately before deparaffinizing:

3.1 Add ~ 200 mL xylenes to each of two clearing agent dishes in a fume hood.

3.2 Add ~ 200 mL 100% ethanol to each of two staining dishes in a fume hood.

Deparaffinizing

40m

4 Submerge slides in fresh **xylenes** 00:05:00 Room temperature .

5m

Note

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

5 Submerge slides in fresh **xylenes** 00:05:00 Room temperature . 5m

6 Submerge slides in fresh **100% ethanol** 00:05:00 Room temperature . 5m

7 Submerge slides in fresh **100% ethanol** 00:05:00 Room temperature . 5m

8 **Air dry slides** ~ 00:05:00 or until completely dry. 5m

9 While slides deparaffinize:

Note

Turn off dry oven.

9.1 • Prepare decloaking chamber:

o Pour 500 mL distilled water into central chamber.

o Pour 200 mL distilled water into each left and right staining dishes.

o Pour 200 mL prepared 1X Co-Detection Target Retrieval solution into middle staining dish.

Note

Turn off dry oven.

9.2 Immediately before tissue quenching:

Note

Preheat the prepared decloaking chamber, programmed for 00:15:00 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.

Tissue Quenching

10m

10 Unload slides from vertical slide rack and place on flat surface of bench top.

11 Incubate with **Hydrogen Peroxide** 00:10:00 Room temperature .

10m

Note

Invert bottle immediately before use; apply drops to completely cover tissues; let incubate on bench top.

12 Decant slides and transfer to vertical slide rack.

13 Submerge slide rack in fresh **distilled water, dunking 3-5 times.**

14 Submerge slide rack in fresh **distilled water, dunking 3-5 times.**

15 While slides incubate with Hydrogen Peroxide:

15.1 Discard deparaffinizing reagents.

15.2 Add ~ 200 mL distilled water to each of two staining dishes.

Target Retrieval

35m 20s

16 Leave slides in water at Room temperature until decloaker is preheated (< 00:05:00). 5m

17 Once decloaker has preheated, submerge slide rack in **preheated distilled water** 00:00:10 (left dish in decloaker). 10s

18 Submerge slide rack in **preheated 1X Co-Detection Target Retrieval solution** 00:15:00 95 °C . 30m

o Once slides are placed in center staining dish of decloaker, close the decloaker & wait for alarm to go off in 00:15:00 .

> Note
>
> Make sure pressure valve is in place to hold pressure when replacing lid.

19 Release decloaker chamber pressure valve & open chamber.

19.1 Submerge slide rack in **preheated distilled water** 00:00:10 (right dish in decloaker). 10s

20 Submerge slide rack in fresh **distilled water, dunking 3-5 times.**

21 Submerge slide rack in fresh **distilled water, dunking 3-5 times.**

22 Submerge slide rack in fresh **PBS-T, dunking 3-5 times.**

23 Leave slides in PBS-T.

24 While slides incubate in 1X target retrieval solution:

24.1 Discard tissue quenching reagents.

24.2 Add ~ 200 mL distilled water to each of two staining dishes.

24.3 Add ~ 200 mL PBS-T to one staining dish.

24.4 Prepare humidified slide staining tray by adding water to bottom & placing lid on top.

24.5 Prepare diluted primary antibody by adding anti-PAX5 antibody to Co-Detection Antibody Diluent at a 1:100 dilution (if stock antibody concentration is 500 undetermined) at Room temperature . Total volume to use is dependent on tissue sizes.

> **Note**
>
> Make sure to mix reagents before pipetting.

Hydrophobic Barrier

25 **Apply hydrophobic barrier** around each tissue.

26 One by one, unload slides from vertical rack submerged in PBS-T.

27 Dry off only the area around the tissue where a barrier will be drawn with ImmEdge Hydrophobic Barrier Pen.

Note

Keep tissue area wet the whole time.

28 Draw barrier and place slide flat in the slide staining tray.

29 Using a pipette, apply a small amount of PBS-T within the barrier.

Note

Just enough to keep the tissue wet while drawing barriers on remaining slides.

Primary Antibody

6m 10s

30 Decant slides and again place flat in slide staining tray.

31 Incubate with **diluted primary antibody** Overnight at 4 °C .

10s

Note

Pipette well to mix immediately before use; pipette appropriate volumes to completely cover tissues and let incubate in slide staining tray with lid closed.

32 While slides are incubating with primary antibody:

32.1 Discard target retrieval and primary antibody reagents.

33 The next day:

33.1 Remove slides from slide staining tray, decant, and transfer to vertical slide rack.

33.2 Submerge slide rack in fresh **PBS-T** 00:02:00 Room temperature . 2m

33.3 Submerge slide rack in fresh **PBS-T** 00:02:00 Room temperature . 2m

33.4 Submerge slide rack in fresh **PBS-T** 00:02:00 Room temperature . 2m

34 While slides are sitting in PBS-T:

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

34.2 Add ~ 200 mL 10% NBF to one staining dish in a fume hood.

Antibody cross-linking 1h 6m

35 Submerge slide rack in **10% NBF** 00:30:00 Room temperature . 30m

36 Submerge slide rack in fresh **PBS-T** 00:02:00 Room temperature . 2m

37 Submerge slide rack in fresh **PBS-T** 00:02:00 Room temperature . 2m

38 Submerge slide rack in fresh **PBS-T** 00:02:00 Room temperature . 2m

39 While slides are incubating with 10% NBF:

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

40 Prepare HybEZ Oven:

40.1 Place humidifying paper within the humidity control tray & apply distilled water to fully wet paper.

40.2 Place humidifying tray into HybEZ oven and clamp down the gasket to seal.

40.3 Preheat oven to 40 °C for at least 00:30:00 before use. 30m

Protease 25m

41 Transfer slides into EZ-Batch Slide Holder, taking care not to let tissues dry out.

42 Incubate with **Protease Plus** 00:15:00 40 °C . 15m

> **Note**
>
> Invert bottle immediately before use; apply drops or pipette appropriate volumes to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

43 Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder).

44 Submerge slide holder in fresh **distilled water, dunking 3-5 times.**

45 Submerge slide holder in fresh **distilled water, dunking 3-5 times.**

46 While slides are incubating with protease:

46.1 Empty the slide staining tray used for primary antibody incubation & put away.

46.2 Discard antibody cross-linking reagents.

46.3 Add ~ 200 mL distilled water to each of two wash trays.

46.4 Preheat RNAscope probes to 40 °C for 00:10:00 before use. This can be done by placing them inside the HybEZ oven during protease incubation. 10m

46.5 Once preheated, add CD4-C2 probe to CSF1R-C1 probe at a dilution of 1:50. Total volume to use is dependent on tissue sizes.

> **Note**
>
> Make sure to mix reagents before pipetting. Place probes back at 4 °C.

Probe Hybridization 3h 34m

47 Decant slides (without removing slides from holder).

48 Incubate with appropriate RNAscope **probe cocktail** 02:00:00 40 °C. 2h

> **Note**
>
> Invert bottle or pipette well to mix immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

49 Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder).

50 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature. 2m

51 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

52 While slides are incubating with probes:

52.1 Discard protease reagents.

52.2 Prepare 1X wash buffer by adding 1 bottle (60 mL) Wash Buffer (10X stock concentration) to 2.94 L distilled water.

53 If 10X Wash Buffer solution has a precipitant formed, preheat bottle at 37 °C for 01:00:00 before adding to distilled water. 1h

54 Will have to prepare another batch of 1X wash buffer later in protocol, after first batch runs out.

55 Alternatively, prepare both batches at once (120 mL 1X Wash Buffer + 5.88 L distilled water).

> **Note**
>
> Store at Room temperature up to one month.

56
- Add ~ 200 mL 1X wash buffer to each of two wash trays.
- Place AMPs from RNAscope Multiplex Fluorescent Detection kit at Room temperature for at least 00:30:00 before use (should be Room temperature when used).

30m

RNA Detection 3h 51m

57 Decant slides (without removing slides from holder).

58 Incubate with **AMP1** 00:30:00 40 °C . 30m

Note

Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

59 Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder).

60 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature 2m

61 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

62 Decant slides (without removing slides from holder).

63 Incubate with **AMP2** 00:30:00 40 °C . 30m

Note

Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

64 Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder).

65 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

66 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

67 Decant slides (without removing slides from holder).

68 Incubate with **AMP3** 00:15:00 40 °C . 15m

Note

Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

69 Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder).

70 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

71 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

72 Decant slides (without removing slides from holder).

73 Incubate with **HRP-C1** 00:15:00 40 °C . 15m

Note

Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

74 Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder).

74.1 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

74.2 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature 2m

75 Immediately before TSA Vivid Fluorophore 520 incubation:

Prepare diluted fluorophore by diluting TSA Vivid Fluorophore 520 into Multiplex TSA Buffer at a dilution of 1:1000 at Room temperature. Total volume to use is dependent on tissue sizes.

Note

- Make sure to mix reagents before pipetting.
- Store in the dark due to light sensitivity.

76 Decant slides (without removing slides from holder).

77 Incubate with **diluted TSA Vivid Fluorophore 520** 00:30:00 40 °C. 30m

Note

Pipette well to mix immediately before use; pipette appropriate volumes to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

78 Remove slide holder from humidifying tray & decant (without removing slides from holder).

79 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature. 2m

80 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature. 2m

81 Incubate with **HRP blocker** 00:15:00 40 °C. 15m

Note

Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

82 Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder).

83 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

84 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

85 Decant slides (without removing slides from holder).

86 Incubate with **HRP-C2** 00:15:00 40 °C . 15m

Note

Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

87 Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder).

88 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

89 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature 2m

90 Immediately before Opal 650 incubation:

- Prepare diluted Opal fluorophore by diluting Opal 650 into Multiplex TSA Buffer at a dilution of 1:1000 at RT. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting.

Note

Store in the dark due to light sensitivity.

91 Decant slides (without removing slides from holder).

92 Incubate with **diluted Opal 650** 00:30:00 40 °C . 30m

Note

Pipette well to mix immediately before use; pipette appropriate volumes to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

93 Remove slide holder from humidifying tray & decant (without removing slides from holder).

94 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

95 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

96 Incubate with **HRP blocker** 00:15:00 40 °C . 15m

Note

Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven.

97 Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder).

98 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

99 Submerge slide holder in fresh **1X wash buffer** 00:02:00 Room temperature . 2m

100 During each incubation:

100.1 Discard reagents from previous incubation step.

100.2 Add ~ 200 mL 1X wash buffer to each of two wash trays

Protein Blocking 24m

101 Decant slides (without removing slides from holder).

102 Incubate with Protein Block 00:20:00 Room temperature . 20m

> **Note**
>
> Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray on bench top.

103 Remove slide holder from humidifying tray & decant (without removing slides from holder).

104 Submerge slide holder in fresh PBS-T 00:02:00 Room temperature . 2m

105 Submerge slide holder in fresh PBS-T 00:02:00 Room temperature . 2m

106 While slides are incubating with protein block:

106.1 Discard RNA detection reagents.

106.2 Add ~ 200 mL PBS-T to each of two wash trays.

> **Note**
>
> Turn off HybEZ oven.

107 Immediately before protein detection:

Prepare diluted secondary antibody by adding Alexa FluorTM 594 donkey anti-rat IgG to Co-Detection Antibody Diluent at a dilution of 1:500 at Room temperature . Total volume to use is dependent on tissue sizes.

> **Note**
>
> Make sure to mix reagents before pipetting. Store in the dark due to light sensitivity.

Protein Detection 1h 4m

108 Decant slides (without removing slides from holder).

109 Incubate with diluted secondary antibody 01:00:00 Room temperature . 1h

Note

Pipette well to mix immediately before use; pipette appropriate volumes to completely cover tissues & transfer slide holder to humidifying tray on bench top.

110 Remove slide holder from humidifying tray & decant (without removing slides from holder).

111 Submerge slide holder in fresh PBS-T 00:02:00 Room temperature . 2m

112 Submerge slide holder in fresh PBS-T 00:02:00 Room temperature 2m

113 While slides are incubating with secondary antibody:

113.1 Discard protein blocking reagents.

113.2 Add ~ 200 mL PBS-T to each of two wash trays

Nuclei Staining and Cover Slipping 30m 30s

114 One at a time, remove slides from slide holder and:

114.1 Apply DAPI 00:00:30 Room temperature . 30s

114.2 Decant slide to remove DAPI.

114.3 Mount slides by adding 2-4 drops of ProLong Gold antifade mounting media to each slide, followed by application of a #1.5 cover glass.

Note

Remove bubbles from tissue by applying pressure to cover glass.

115 Place slides flat in a dry, dark space to air dry 00:30:00 Room temperature . 30m

116 Store at 4 °C and image within two weeks.