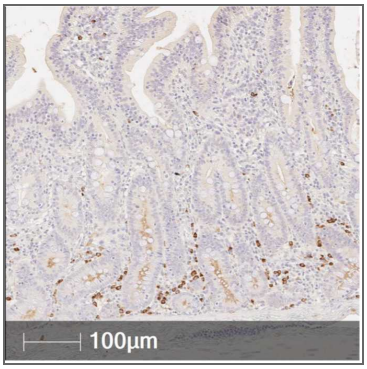

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Protocol status: Working
We use this protocol and it's working

Created: Aug 11, 2023

In situ immunoglobulin A (IgA) detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues

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ABSTRACT

An immunohistochemistry (IHC) staining protocol for *in situ* identification of IgA in pig tissue.

ATTACHMENTS

IHC_IgA_Pig_ManualAssay.pdf

GUIDELINES

Assay Controls:

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

- IHC controls:

o Negative control (primary antibody only)

§ This slide receives 0.05% PBS-T in place of secondary antibody

o Negative control (secondary antibody only)

§ This slide receives 1% BSA in PBS 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 Salmonella

Assay variations:

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

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PROTOCOL integer ID: 86384

MATERIALS

Equipment:

- Pipettes/pipette tips – volumes ranging between 2-1000 uL
- Drying oven (able to reach & hold 60°C)
- Fume hood
- Slide staining tray (e.g. Simport M920-2)
- 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)
- 1% bovine serum albumin (BSA) in PBS (made in-house)
- Xylenes (Macron Fine Chemicals 8668-16)
- 100% ethanol (Pharmco 111000200)

o Dilute with distilled water to make 95%, 85%, and 70% concentrations

- Pro-Par Clearant (Anatech 510)
- Fixative

o 10% NBF (Cancer Diagnostics, Inc. 111)

- ImmEdge Hydrophobic Barrier Pen (Vector H-4000)
- Proteinase K, ready to use (Dako S3020)
- Dual Endogenous Enzyme Block (Dako S2003)
- Protein Block (Dako X0909)
- Goat anti-porcine IgA Heavy Chain Secondary Antibody (Novus Biologicals NB724)

o Stock concentration 1 mg/mL

- ImmPRESS Reagent Kit Peroxidase Anti-Goat Ig (Vector MP-7405)
- Liquid DAB+ (Dako K346811-2)

o DAB+ Substrate Buffer

o DAB+ Chromogen

- Gill's Hematoxylin I (American Master Tech Scientific HXGHE1LT)
- Refrax Mounting Medium (Anatech 711)
- #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

BEFORE START INSTRUCTIONS

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) 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.

Baking

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

Baking
- **Bake slides 20 min 60°C**

While slides bake:
- Prepare 0.05% PBS-T (can store at RT up to 1 month)

Deparaffinizing & Rehydrating

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

Deparaffinizing & Rehydrating

- Submerge slide in fresh **xylenes 5 min RT**
- Submerge slide in fresh **xylenes 5 min RT**
- Submerge slide in fresh **xylenes 5 min RT**
- Submerge slides in fresh **100% ethanol 1 min RT**
- Submerge slides in fresh **100% ethanol 1 min RT**
- Submerge slides in fresh **95% ethanol 1 min RT**
- Submerge slides in fresh **85% ethanol 1 min RT**
- Submerge slides in fresh **70% ethanol 1 min RT**
- Submerge slides in fresh **distilled water 3 min RT**
- Submerge slides in fresh **PBS-T for transport**

While slides deparaffinize/rehydrate:

- Turn off dry oven
- Prepare humidified slide staining tray by adding water to bottom & placing lid on top
- Add ~200 mL PBS-T to each of two staining dishes

Hydrophobic Barrier

3 **Hydrophobic Barrier**

- **Apply hydrophobic barrier** around each tissue

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 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)

- Leave slides in slide staining tray

Protease Digestion

4 **Protease Digestion**

- Decant slides and again place flat in slide staining tray
- Incubate with **Proteinase K 3 min RT**

o ----------Invert bottle immediately before use; apply drops 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 **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

Tissue Quenching

5 **Tissue Quenching**

- Decant slides and again place flat in slide staining tray
- Incubate with **Dual Endogenous Enzyme Block 10 min RT**

o ----------Invert bottle immediately before use; apply drops 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 **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

While slides incubate with enzyme block:

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

Protein Blocking

6 **Protein Blocking**

- Decant slides and again place flat in slide staining tray
- Incubate with **Protein Block 20 min RT**

o ----------Invert bottle immediately before use; apply drops 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 **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

While slides incubate with protein block:

- Discard tissue quenching reagents
- Prepare primary antibody by adding IgA antibody to 1% BSA in PBS at a dilution of ~0.033 ug/mL (1:40,000 dilution if stock concentration is 1 mg/mL). Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting.

Primary Antibody

7 **Primary Antibody**

- Decant slides and again place flat in slide staining tray
- Incubate with **diluted primary antibody overnight at 4°C**

o ----------Apply drops 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**

While slides are incubating with primary antibody:

- Discard protein blocking reagents

Secondary Antibody

8 The next day:

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

Secondary Antibody

- Decant slides and again place flat in slide staining tray
- Incubate with **anti-goat HRP polymer 30 min RT**

o ----------Invert bottle immediately before use; apply drops 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 **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

While slides are incubating with secondary antibody:

- Discard remaining primary antibody reagents
- Add ~200 mL PBS-T to each of two staining dishes

Chromogen Detection

9 Immediately before chromogen detection:

- Prepare diluted DAB chromogen by adding 1 drop DAB substrate per 1 mL substrate buffer. Total volume to use is dependent on tissue sizes. Make sure to mix reagents thoroughly. Store in the dark due to light sensitivity

Chromogen Detection

- Decant slides and again place flat in slide staining tray
- Incubate with **diluted DAB chromogen 2 min RT**

o ----------Pipette well to mix immediately before use; pipette appropriate volumes 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 **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

While slides are incubating with DAB chromogen:

- Discard secondary antibody reagents
- Add ~200 mL PBS-T to each of two staining dishes
- Add ~200 mL 25% hematoxylin to one staining dish

o ----------Prepare by combining 150 mL distilled water with 50 mL Gill's Hematoxylin

- Add ~200 mL distilled water to each of three staining dishes
- Add ~200 mL 95% ethanol to a staining dish in a fume hood
- Add ~200 mL 100% ethanol to each of three staining dishes in a fume hood
- Add ~200 mL Pro-Par to each of three clearing agent dishes in a fume hood

Counterstaining

10 **Counterstaining**

- Submerge slide rack in **diluted hematoxylin 15 sec 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

11 **Mounting**

- Submerge slides in fresh **95% ethanol 1 min RT**
- Submerge slides in fresh **100% ethanol 1 min RT**
- Submerge slides in fresh **100% ethanol 1 min RT**
- Submerge slides in fresh **100% ethanol 1 min RT**
- Submerge slides in fresh **Pro-Par 5 min RT**
- Submerge slides in fresh **Pro-Par 5 min RT**
- Submerge slides 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 bright-field microscope

While slides are air drying:

- Discard chromogen detection and counterstaining reagents