In situ Ki-67 detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues

An immunohistochemistry (IHC) staining protocol for *in situ* identification of porcine Ki-67

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) 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.

Equipment:

- Pipettes/pipette tips – volumes ranging between 2-1000 uL
- Drying oven (able to reach & hold 60℃)
- 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)
- Decloaking Chamber NxGen (Biocare Medical DC2012/DC2012-220V)
- 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)
    - 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)
- RNA-Protein Co-Detection Ancillary Kit (ACD 323180)
    - Co-Detection Target Retrieval Reagents (ACD 323165/323166)
    - Co-Detection Antibody Diluent (ACD 33160)
- Dual Endogenous Enzyme Block (Dako S2003)
- Protein Block (Dako X0909)
- Anti-Ki-67 antibody; mouse IgG1; clone B56; stock concentration 250 ug/mL (BD 550609)
- EnVision+ System HRP Labelled Polymer Anti-Mouse (Dako K400111-2)
- Liquid DAB+ (Dako K346811-2)
    - DAB+ Substrate Buffer
    - DAB+ Chromogen
- Gill's Hematoxylin I (American Master Tech Scientific HXGHE1LT)

- Refrax Mounting Medium (Anatech 711)
- #1 thickness cover glass (Fisherbrand 12-545-F)

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
    - Batch control
        - If performing staining across multiple batches, include serial sections of one tissue in each batch

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.

Protocol

Before starting the assay:

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

Baking

- **Bake slides 20 min 60℃**

While slides bake:

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

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 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℃ up to 1 month)
- Prepare the steamer
- 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
- Preheat the prepared decloaking chamber, programmed for 15 min at 95℃
    - Chamber will take exactly 15 min to preheat, and there will be a 2 min window to add slides before chamber pressurizes & locks

Heat-Induced Epitope Retrieval

- Leave slides in PBS-T 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℃**
    - 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), click “Skip” on screen since slides are loaded, & 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 **PBS-T**
- Leave slides in PBS-T

While slides incubate in 1X co-detection target retrieval solution:

- Discard deparaffinizing & rehydrating reagents
- Add ~200 mL PBS-T to one staining dish
- Prepare humidified slide staining tray by adding water to bottom & placing lid on top

Hydrophobic Barrier

- **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 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

Tissue Quenching

- Decant slides and again place flat in slide staining tray
- Incubate with **Dual Endogenous Enzyme Block 10 min RT**
    - 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 epitope retrieval reagents
- Add ~200 mL PBS-T to each of two staining dishes

Protein Blocking

- Decant slides and again place flat in slide staining tray
- Incubate with **Protein Block 20 min RT**
    - 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 anti-Ki-67 antibody to Co-Detection Antibody Diluent at a dilution of 2.5 ug/mL (1:100 dilution if stock antibody concentration is 250 ug/mL). Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting.

Primary Antibody

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

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-mouse HRP polymer 30 min RT**
    - 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

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 7 min RT**
    - 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
    - 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

- 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

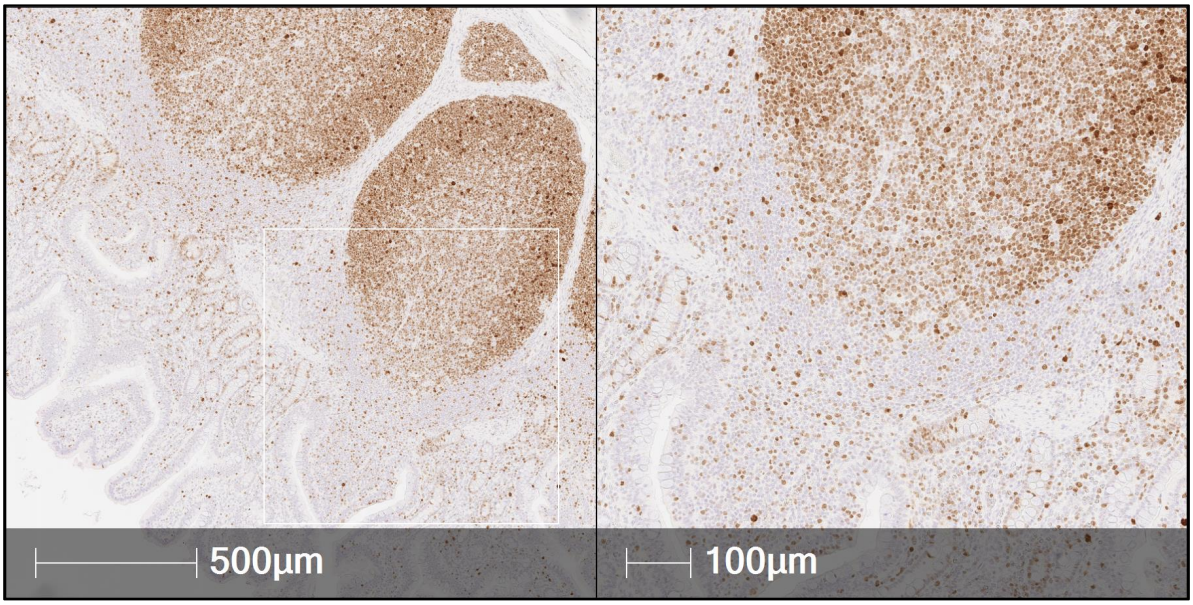
- Submerge slides in fresh **95% ethanol 1 min RT**
- Submerge slides in fresh **100% ethanol 1 min RT**
- Submerge slides in fresh **100% ethanol 1 min RT**
- Submerge slides in fresh **100% ethanol 1 min RT**
- Submerge slides in fresh **Pro-Par 5 min RT**
- Submerge slides in fresh **Pro-Par 5 min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

Results

Ki-67 protein staining in ileum of an ~8-week-old, weaned pig. Tissue was fixed in 4% PFA ~24 hours. Staining localizes primarily to follicles and crypts.



Contributions

Assay development: Jayne Wiarda

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