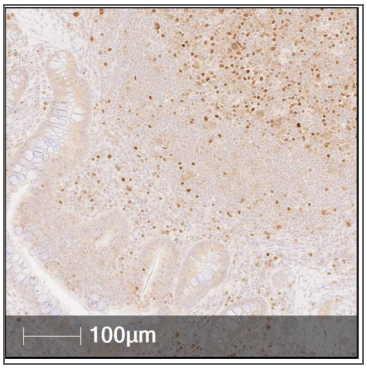


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**Protocol status:** Working
We use this protocol and it's working

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# In situ Blimp1 detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues

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## ABSTRACT

An immunohistochemistry (IHC) staining protocol for *in situ* identification of porcine Blimp1

## ATTACHMENTS

IHC_Pig_Blimp1_ManualAssay.pdf

## GUIDELINES

**Assay Controls:**

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

- IHC controls:

oNegative control (primary antibody only)

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

oNegative control (secondary antibody only)

§This slide receives co-detection antibody diluent in place of diluted primary antibody

oBatch 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.

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## 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)
- 5.5 Quart Digital Steamer (Hamilton Beach 37530Z)
- 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)

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

- Pro-Par Clearant (Anatech 510)
- Fixative

o10% 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)

oCo-Detection Target Retrieval Reagents (ACD 323165/323166)

oCo-Detection Antibody Diluent (ACD 33160)

- Dual Endogenous Enzyme Block (Dako S2003)
- Protein Block (Dako X0909)
- Anti-Blimp1 antibody; mouse IgG1; clone 3H2ER8; stock concentration 200 ug/mL (Santa Cruz sc-66015)
- EnVision+ System HRP Labelled Polymer Anti-Mouse (Dako K400111-2)
- Liquid DAB+ (Dako K346811-2)

oDAB+ Substrate Buffer

oDAB+ 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) 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.

## 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 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)
- Prepare steamer:
- ---------- Fill the bottom reservoir of steamer to the 'Fill' line
- ---------- Assemble the steamer, using both tiers of steam bowls. Do not place the divider between steam bowls, as an extra tall compartment is needed
- ---------- Pour 200 mL prepared Co-Detection Target Retrieval solution into staining dish and place into the steamer
- Preheat the prepared steamer, programmed for 1 hour
- ---------- Perform this step ~25 minutes before use so that retrieval solution can adequately heat to ~110°C

## Heat-Induced Epitope Retrieval

3 **Heat-Induced Epitope Retrieval**

- Leave slides in PBS-T at RT until steamer is preheated (retrieval solution reaches ~110°C)
- Once steamer has preheated, submerge slide rack in **preheated 1X Co-detection Target Retrieval solution 20 min**
- ---------- Slides should be maintained at ~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 slides from steamer
- 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

4 **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

5 **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:

- Turn off steamer
- Discard epitope retrieval 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**
- ---------- 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-Blimp1 antibody to Co-Detection Antibody Diluent at a dilution of 25 ug/mL (1:8 dilution if stock antibody concentration is 200 ug/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**
- ---------- 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-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

## 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 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

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