Immunoperoxidase Staining

PROTOCOL

Santa Cruz Biotechnology, Inc.

A. TISSUE CULTURE CELLS

- Grow cultured cells in chambered culture slides overnight at 37° C.
- Wash briefly with PBS and fix cells by one of the following procedures:
  - 5 minutes in -10° C methanol, air dry; or
  - 2 minutes in cold acetone, air dry; or
  - 10–15 minutes in 1-4% formalin in PBS (keep wet).
- Wash in three changes of PBS.

Optional: Incubate for 5–7 minutes in 0.1–1% hydrogen peroxide in PBS to quench endogenous peroxidase activity. Wash in PBS twice for 5–7 minutes each.

B. FROZEN TISSUE SECTIONS

- Freeze tissue block, embedded in cryo-embedding medium, in liquid nitrogen according to standard procedures. Frozen tissue block may be stored at -70° C until ready for sectioning.
- Cut 4–10-micron thick tissue sections using a cryostat.
- Adhere sections to **electrostatically-charged micro slides** (e.g. sc-363562) kept at room temperature.
- Slides may be stored at -70° C. Thaw slides at room temperature prior to fixing and staining.
- Fix slides in cold acetone for 10 minutes and keep refrigerated (or choose other fixation procedure). Wash in three changes of PBS.

Optional: Incubate for 5–7 minutes in 0.1–1% hydrogen peroxide in PBS to quench endogenous peroxidase activity. Wash in PBS twice for 5–7 minutes each.

NOTE: For tissues containing high levels of endogenous biotin (which may result in higher background staining), we recommend following the Formalin-Fixed, Paraffin-Embedded Tissue Sections protocol, as endogenous biotin is normally destroyed in paraffin-embedded tissue.

C. FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUE SECTIONS

- Fix tissue in formalin and embed in paraffin blocks according to standard procedures.
- Cut 4–6 micron thick tissue sections using a microtome, and apply to **electrostatically-charged micro slides** (e.g. sc-363562).
- Deparaffinize in xylenes using three changes for 5 minutes each.
- Hydrate sections gradually through graded alcohols: wash in 100% ethanol twice for 15 minutes each, then 90% ethanol twice for 15 minutes each.
- Wash in deionized H_2O for 1 minute with stirring.
- Aspirate excess liquid from slides.

NOTE: Alternatively, other non-xylenes clearing agents can be used for tissue deparaffinization (e.g. Histo-Clear).

Optional: Antigen unmasking may be performed at this point. Certain antigenic determinants are masked by formalin fixation and paraffin embedding and may be exposed by one of several methods:

1. Heat treatment (recommended method): Place slides in a container and cover with 10 mM **sodium citrate buffer, pH 6.0** (sc-294091); or with 50 mM **glycine-HCl buffer** (sc-29096), pH 3.5, with 0.01% (w/v) **EDTA** (sc-29092). Heat at 95° C for 5 minutes. Top off with fresh buffer and heat at 95° C for 5 minutes (optimal incubation time may vary for each tissue type). Allow slides to cool in the buffer for approximately 20 minutes. Wash in deionized H_2O three times for 2 minutes each. Aspirate excess liquid from slides.
2. Pepsin: Incubate sections for 10–20 minutes in **0.1% Pepsin** (sc-476554) in 0.01 N HCl at room temperature. Wash slides several times in deionized H_2O. Aspirate excess liquid from slides.
3. Saponin: Incubate sections for 30 minutes in **0.05% Saponin** (sc-280079) in deionized H_2O at room temperature. Wash at least three times in PBS. Aspirate excess liquid from slides.

Optional: Incubate for 5–7 minutes in 0.1–1% hydrogen peroxide in deionized H_2O to quench endogenous peroxidase activity. Wash in PBS twice for 5–7 minutes each.

D. INDIRECT IMMUNOPEROXIDASE STAINING

- For indirect immunoperoxidase staining of tissue sections, we recommend the use of 3 staining protocols:

1. Avidin-Biotin Complex (ABC) staining protocol using conventional biotinylated secondary antibodies and the **ImmunoCruz® ABC Kit** (sc-516216).
2. Labeled Streptavidin Biotin (LSAB) staining protocol, using conventional biotinylated secondary antibodies and **Avidin D-HRP** (sc-516217).
3. Indirect immunoperoxidase staining using HRP-conjugated mouse IgG binding proteins, **m-IgGκ BP-HRP** (sc-516102), or **m-IgGλ BP-HRP** (sc-516132), in lieu of conventional anti-mouse secondary antibodies.

- All steps are carried out at room temperature in a humidified chamber. Allow all reagents to reach room temperature prior to use. Tissue sections should not be allowed to dry out at any time during the procedure. Use suction to remove reagents after each step, but avoid drying of specimens between steps. Use sufficient reagents to cover the specimens (approximately 100 µl per slide is usually adequate). Use the washing steps suggested to ensure no interference with the reagents at each step.

ABC Staining Protocol

- Incubate specimens for 1 hour in 5% normal blocking serum in PBS or in **UltraCruz® Blocking Reagent** (sc-516214). If a conventional secondary antibody is used, blocking serum should ideally be derived from the same species in which the secondary antibody was raised.
- Remove blocking serum or blocking reagent from slides.
- Incubate with primary antibody for 30 minutes at room temperature or overnight at 4° C. Optimal antibody concentration should be determined by titration; recommended range is 0.5–5.0 µg/ml diluted in PBS with 5% normal blocking serum, or diluted in **UltraCruz® Blocking Reagent** (sc-516214).

CONTINUED...

- Wash with three changes of PBS for 5–7 minutes each.
- Incubate for 30–45 minutes with biotin-conjugated secondary antibody at approximately 1 µg/ml diluted in either PBS with 1.5% of the appropriate blocking serum, or in **UltraCruz® Blocking Reagent** (sc-516214).
- Wash with three changes of PBS for 5–7 minutes each.
- Prepare the Avidin-Biotin Complex (ABC reagent) using the A and B reagents from the **ImmunoCruz® ABC Kit** (sc-516216), 30 minutes prior to using. Mix 50 µl of each A and B reagents in 2.5 ml of PBS and let the mix stand at room temperature for 30 minutes.
- Incubate for 30 minutes with the ABC reagent. Wash with three changes of PBS for 5–7 minutes each.
- Incubate in peroxidase substrate and chromogen mixture [(e.g. **hydrogen peroxide** (sc-203336) and **DAB** (sc-24982), mixed at the appropriate concentration in 0.1 M Tris-HCl pH 7.6, or use another chromogen of choice (browse our list of HRP and AP Substrates on our website, www.scbt.com)] until desired stain intensity develops. Optimal time for staining should be determined independently. Individual slides should be monitored to determine the proper development time.
- Wash sections in deionized H_2O.

Optional: Counterstain slides in **Gill's Hematoxylin Solution, No. 2** (sc-24973) for 5–10 seconds and immediately wash with several changes of deionized H_2O.

- When appropriate, dehydrate through alcohols and xylenes as follows: Soak in 90% ethanol twice for 3 minutes each, then 100% ethanol twice for 3 minutes each, then xylenes three times for 10 seconds each. Wipe off excess xylenes. Immediately add 1–2 drops of permanent mounting medium (e.g., **Organo/Limonene**: sc-45087), cover with a **glass coverslip** (sc-24975) and observe by light microscopy.

NOTE: For a listing of mounting media for immunohistochemistry, including **Organo/Limonene** (sc-45087) and **ImmunoHistoMount** (sc-45086) for immunoperoxidase staining, please browse the Mounting Media for Staining on our website, www.scbt.com.

LSAB Staining Protocol

- Incubate specimens for 1 hour in 5% normal blocking serum in PBS, or in **UltraCruz® Blocking Reagent** (sc-516214). If a conventional secondary antibody is used, blocking serum should ideally be derived from the same species in which the secondary antibody was raised.
- Remove blocking serum, or blocking reagent from slides.
- Incubate with primary antibody for 2 hours at room temperature or overnight at 4° C. Optimal antibody concentration should be determined by titration; recommended range is 0.5–5.0 µg/ml diluted in PBS with 5% normal blocking serum or **UltraCruz® Blocking Reagent** (sc-516214).
- Incubate for 30–45 minutes with biotin-conjugated secondary antibody at approximately 1 µg/ml diluted in either PBS with 1.5% appropriate blocking serum, or in **UltraCruz® Blocking Reagent** (sc-516214).
- Wash with three changes of PBS for 5–7 minutes each.
- Incubate for 30 minutes in ready-to-use **Avidin D-HRP** (sc-516217).
- Wash with three changes of PBS for 5-7 minutes each.
- Incubate in peroxidase substrate and chromogen mixture as described under the ABC Staining Protocol.
- Wash sections in deionized H_2O.
- Counterstain, dehydrate and mount slides as described under the ABC Staining Protocol.

Indirect Immunoperoxidase Staining Using Mouse IgG Binding Proteins

- For indirect immunoperoxidase staining of tissue sections using mouse IgG binding proteins instead of conventional anti-mouse secondary antibodies, we recommend the use of the Santa Cruz Biotechnology, Inc. HRP-conjugated mouse IgG kappa binding protein, **m-IgGκ BP-HRP** (sc-516102), or the HRP-conjugated mouse IgG lambda binding protein, **m-IgGλ BP-HRP** (sc-516132).
- Incubate specimens for 1 hour in **UltraCruz® Blocking Reagent** (sc-516214).
- Incubate with primary antibody overnight at 4° C. Optimal antibody concentration should be determined by titration; recommended range is 2.0–8.0 µg/ml diluted in **UltraCruz® Blocking Reagent** (sc-516214).
- Incubate for 90–120 minutes with either **m-IgGκ BP-HRP** (sc-516102), or **m-IgGλ BP-HRP** (sc-516132) diluted in **UltraCruz® Blocking Reagent** (sc-516214) (Starting dilution: 1:25, dilution range: 1;25-1:100. Optimal dilution to be determined by titration).
- Incubate in the peroxidase substrate and chromogen mixture, as described under the ABC Staining Protocol.
- Wash sections in deionized H_2O.
- Counterstain, dehydrate and mount slides as described under the ABC Staining Protocol.

E. DIRECT IMMUNOPEROXIDASE STAINING

- For direct immunoperoxidase staining of tissue sections, we recommend the use of the Santa Cruz Biotechnology, Inc. HRP-conjugated primary antibodies.
- All steps are carried out in a humidified chamber. Allow all reagents to reach room temperature prior to use.
- Tissue sections should not be allowed to dry out at any time during the procedure. Use suction to remove reagents after each step, but avoid drying of specimens between steps. Use sufficient reagents to cover the specimens (approximately 100 µl per slide is usually adequate).
- Incubate specimens for 1 hour at room temperature in 5% normal blocking serum in PBS, or **UltraCruz® Blocking Reagent** (sc-516214). Blocking serum ideally should be derived from the same species in which the primary antibody was raised.
- Remove blocking serum or blocking reagent from slides.
- Incubate with HRP-conjugated primary antibody overnight at 4° C. Optimal antibody concentration should be determined by titration; recommended range is 2.0–8.0 µg/ml diluted in PBS with 5% normal blocking serum or **UltraCruz® Blocking Reagent** (sc-516214).
- Wash with three changes of PBS for 5–7 minutes each.
- Incubate in peroxidase substrate and chromogen mixture [(e.g. **hydrogen peroxide** (sc-203336) and **DAB** (sc-24982), mixed at the appropriate concentration in 0.1 M Tris-HCl pH 7.6, or use another chromogen of choice (browse our list of HRP and AP Substrates on our website, www.scbt.com)] until desired stain intensity develops. Optimal time for staining should be determined independently. Individual slides should be monitored to determine the proper development time.
- Wash sections in deionized H_2O.
- Counterstain, dehydrate and mount slides as described under the ABC Staining Protocol.