Research Applications

Delta Biolabs, LLC recommends the following procedures and secondary reagents for use with our primary antibodies: (1) Western (immuno-) blotting, (2) immunoprecipitation/Western blots, (3) immunoperoxidase cell staining, (4) ELISA assays, (5) methods for the use of peptides to neutralize antibody activity and, (6) preparation of solutions.

1. WESTERN (IMMUNO) BLOTTING

Sample Preparation

Monolayer cells

- Remove medium and rinse 100 mm cell culture plate with PBS at room temperature. All the following steps should be done on ice or at 4° C using ice cold buffers.
- Add 0.6 ml of RIPA buffer, with freshly added inhibitors, to a 100 mm cell culture plate. Scrape plate with a cell scraper. Transfer the lysate to a microcentrifuge tube with a syringe, fitted with a 21 gauge needle.
- Wash the plate once with 0.3 ml of RIPA buffer, combine with first lysate, and pass through the 21 gauge needle to shear the DNA. Add 10 µl of 10 mg/ml PMSF stock. Incubate 30-60 minutes on ice.
- Microcentrifuge cell lysate at 10,000xg for 10 minutes at 4° C. The supernatant fluid is the total cell lysate.

Suspension cells

- Collect approximately 2.0 x 10^7 cells by low speed centrifugation at room temperature for five minutes. Carefully remove medium.
- Wash the pellet with PBS at room temperature, and again collect by low speed centrifugation. Carefully remove PBS.
- Add 1.0 ml of ice cold RIPA buffer with freshly added inhibitors. Mix gently with a pipette and incubate on ice for 30 minutes.
- Disrupt and homogenize cells by passage through a 21 gauge needle, dounce homogenization or sonication, taking care not to raise the temperature of the lysate. Add 10 µl of 10 mg/ml PMSF stock. Incubate 30 minutes on ice.
- Transfer to microcentrifuge tubes and centrifuge at 10,000xg for 10 minutes at 4° C. The supernatant fluid is the total cell lysate.

Tissue samples

- Weigh tissue and dice into very small pieces using a clean razor blade. Frozen tissue can be sliced very thinly and thawed in lysis buffer containing inhibitors. Use 3 ml of ice cold RIPA buffer per gram of tissue.
- Disrupt and homogenize tissue with a dounce homogenizer or a polytron device. Maintain temperature at 4° C throughout all procedures. Add 30 µl of 10 mg/ml PMSF stock per gram of tissue and incubate on ice for 30 minutes.
- Transfer to microcentrifuge tubes, centrifuge at 10,000xg for 10 minutes at 4° C. Remove supernatant and centrifuge again. The supernatant fluid is the total cell lysate. A longer centrifugation may be necessary to obtain a clarified lysate.

Electrophoresis

- Mix sample (40-60 µg whole cell lysate, 10-20 µg nuclear extract, or 10-20 ng purified protein per lane) with an equal volume of 2x electrophoresis sample buffer and boil for 5 minutes. Unused samples may be stored at -20° C for up to several months.
- Load up to 10 µl of lysate per 1.0 mm of well width for gels of 0.75 mm thickness.
- If desired, use molecular weight standards of choice according to manufacturer's instructions. Carry out electrophoresis according to standard protocols.
- Transfer proteins from the gel to nitrocellulose membrane or PVDF, using an electroblotting apparatus according to the manufacturer's protocols.

Immunoblotting

- Block non-specific binding by incubating membrane in Blotto (either Blotto A or Blotto B) for 60 minutes at room temperature. Alternatively, the membrane may be blocked at 4° C overnight in a covered container, using Blotto without Tween-20.
- Incubate in primary antibody diluted in Blotto for 1 hour at room temperature. Optimal antibody concentration should be determined by titration. We recommend a starting dilution of 0.5-2.0 µg/ml Blotto; final optimal concentration may be as low as 0.1 µg/ml. Wash membrane four times for 5 minutes each with TBS, 0.05% Tween-20.
- Incubate for 45 minutes at room temperature with horseradish peroxidase (HRP) conjugated secondary antibody, or alkaline phosphatase (AP) conjugated secondary antibody diluted in Blotto. If high backgrounds are observed, the secondary antibody should be diluted further, as low as 1:20,000. Wash membrane four times for 5 minutes each with TBS, 0.05% Tween-20, and once for 5 minutes with TBS.
- Incubate membrane in Amersham ECL® reagents for one minute.
- Drain membrane, remove excess ECL® reagent, and seal in plastic wrap.
- Apply phosphorescent tape to the outside of the plastic and label with permanent black marker.

- Expose to ECL® hyperfilm in darkroom both for one minute and 5 minutes.
- Develop as usual for XRAY.
- (ECL® is a registered trademark of Amersham Biosciences)

2. IMMUNOPRECIPITATION/WESTERN BLOT

- Prepare a total cell lysate as described under Western blot procedure (section 1).
- Preclear whole cell lysate (optional step) as follows. To approximately 1 ml of whole cell lysate, add 0.25 µg of the appropriate control IgG (normal mouse, rat, rabbit or goat IgG, corresponding to the host species of the primary antibody), together with 20 µl of resuspended volume of the appropriate agarose conjugate (Protein A-Agarose, Protein G-Agarose, Protein A/G-Agarose, or Protein L-Agarose). Incubate at 4° C for 30 minutes.
- Pellet beads by centrifugation at approximately 1,000xg for 5 minutes at 4° C. Transfer supernatant (cell lysate) to a fresh 1.5 ml microcentrifuge tube at 4° C.
- To 1 ml of the above cell lysate, or approximately 100-500 µg of total cellular protein, add 10 µg of primary antibody agarose conjugate (i.e. 5 µl volume of packed beads) and incubate at 4° C for 1 hour to overnight with mixing such as end over end rotation.
- Alternatively, if primary antibody agarose conjugate is not available, incubate 1 ml cell lysate with 1-10 µl (i.e., 0.2-2 µg) primary antibody (optimal antibody concentration should be determined by titration) for 1 hour at 4° C. Add 20 µl of resuspended volume of the appropriate agarose conjugate (Protein A-Agarose, Protein G-Agarose, Protein A/G-Agarose, or Protein L-Agarose). Cap tubes and incubate at 4° C on a rocker platform or rotating device for 1 hour to overnight.
- Collect pellet by centrifugation at approximately 1,000xg for 5 minutes at 4° C. Carefully aspirate and discard supernatant.
- Wash pellet 4 times with either RIPA buffer (more stringent) or PBS (less stringent), each time repeating centrifugation step above.
- After final wash, aspirate and discard supernatant and resuspend pellet in 40 µl of 1x electrophoresis sample buffer.
- Boil samples for 5 minutes. Load up to 5-10 µl of sample per 1.0 mm well width for gels of 0.75 mm thickness.
- Continue with electrophoresis and immunoblotting as described under Western blotting procedure (section 1).

3. IMMUNOPEROXIDASE CELL STAINING

Tissue Culture Cells

- Grow cultured cells on sterile glass cover slips or 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 (recommended method); or
  - 2 minutes in cold acetone, air dry; or
  - 10 minutes in 1% formalin in PBS (keep wet).
- Wash in four changes of PBS.
- Optional: Incubate for 5-10 minutes in 0.1-1% hydrogen peroxide in PBS to quench endogenous peroxidase activity. Wash in PBS twice for 5 minutes each.

Frozen Tissue Sections

- Freeze tissue in OCT block in liquid nitrogen according to standard procedures. The frozen block may be stored at -70° C for up to 2 weeks before sectioning.
- Clean glass slides with 95% ethanol, treat with subbing solution, and air dry. Can use pre-treated slides.
- Cut 4 to 10 micron thick cryostat sections of tissue block. Adhere sections to room temperature slides. 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 5-10 minutes in 0.1-1% hydrogen peroxide in PBS to quench endogenous peroxidase activity. Wash in PBS twice for 5 minutes each.

NOTE: Frozen sections may have relatively high levels of endogenous biotin which can result in high background staining. The endogenous biotin is normally destroyed in paraffin-embedded tissue.

Paraffin-Embedded Tissue Sections

- Deparaffinize and hydrate tissue sections by one of the following three methods:
- Heat treatment (recommended method): Place slides in a container and cover with 10 mM sodium citrate buffer, pH 6.0; or with 50 mM glycine-HCl buffer, pH 3.5, with 0.01% (w/v) EDTA. 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.
- Pepsin: Incubate sections for 10-20 minutes in 0.1% pepsin in 0.01 N HCl buffer, pH 2.5, at room temperature. Wash slides several times in deionized H_2O. Aspirate excess liquid from slides.
- Saponin: Incubate sections for 30 minutes in 0.05% saponin in deionized H_2O at room temperature. Wash at least three times in PBS. Aspirate excess liquid from slides.

- Optional: Incubate for 5-10 minutes in 0.1-1% hydrogen peroxide in deionized H_2O to quench endogenous peroxidase activity. Wash in PBS twice for 5 minutes each.

NOTE: Delta Biolabs antibodies are only suggested for use on formalin-fixed, paraffin-embedded tissue sections if the catalog description states "IHC (including paraffin-embedded sections)".

Immunoperoxidase Staining

- For immunoperoxidase staining of tissue sections, we recommend the use of the ABC Staining Systems which utilize preformed avidin-biotinylated horseradish peroxidase complex as a detection reagent.
- All steps are carried out at room temperature in a humidified chamber. Allow all staining system reagents to reach room temperature prior to use. Tissue sections should not be allowed to dry out at any time during the procedure. Aspirate or blot 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).

ABC Staining Systems

- Incubate specimens for 30 minutes in 1.5% normal blocking serum in PBS. Blocking serum ideally should be derived from the same species in which the secondary antibody is raised. Remove blocking serum from slides.
- Incubate with primary antibody for 30 minutes at room temperature. Optimal antibody concentration should be determined by titration; recommended range is 0.5-5.0 µg/ml diluted in PBS with 1.5% normal blocking serum. Wash with two changes of PBS for 5 minutes each.
- Incubate for 30 minutes with biotin-conjugated secondary antibody, or at approximately 1 µg/ml diluted in PBS with 1.5% normal blocking serum. Wash with two changes of PBS for 5 minutes each.
- Incubate for 30 minutes with avidin biotin enzyme reagent. Wash with three changes of PBS for 5 minutes each.
- Incubate in peroxidase substrate for 2-10 minutes, or until desired stain intensity develops. Wash sections in deionized H_2O for 5 minutes.
- Dehydrate through alcohols and xylenes as follows: Soak in 95% ethanol twice for 10 seconds each, then 100% ethanol twice for 10 seconds each, then xylenes three times for 10 seconds each. Wipe off excess xylene. Immediately add 1-2 drops of permanent mounting medium (e.g., Permount). Cover with a glass coverslip. Observe by light microscopy.

4. ELISA ASSAYS

- Coat microtiter plates with target protein diluted in 1xPBS. Optimal concentrations should be determined by titration, but for purified antigens, 100 µl per well at 1 µg/ml is usually sufficient. Incubate 1 hour at room temperature or overnight at 4° C covered with parafilm.
- Aspirate to remove antigen solution, wash three times with PBS containing 0.05% Tween-20, and slap plate dry. Add 200 µl/well of blocking buffer (2% BSA with 0.05% TWEEN-20) to block non-specific protein binding. Incubate for 1 hour at room temperature, or overnight at 4° C covered with parafilm.
- Aspirate to remove blocking solution, wash three times with PBS containing 0.05% Tween-20, and slap plate dry. Strip wells or plates are usually stable in resealable plastic storage bags for 4 weeks at 4° C.
- Add test antibody samples and controls at 100 µl/well diluted in blocking buffer. Antibodies may be serially diluted for determining titer. Incubate 1 hour at room temperature.
- Aspirate to remove samples, wash three times with PBS containing 0.05% Tween-20, and slap plate dry.
- Add 100 µl/well of HRP conjugated secondary antibody diluted to 1:2000-1:10,000 in blocking buffer. Optimal antibody concentration is determined by titration. Incubate 1 hour at room temperature.
- Aspirate to remove reagent, wash three times with PBS containing 0.05% Tween-20, and slap plate dry, making sure the bottoms of the plates are dry.
- Add 100ul/well of TMB substrate and develop for 30 minutes at room temperature.
- Add 100ul/well of 1M HCL to stop the reaction and read plates on microtiter plate reader at 450nm.

5. PEPTIDE NEUTRALIZATION

- Blocking peptides are available as controls for all Delta Biolabs affinity-purified rabbit polyclonals raised against peptide antigens. Antibody binding to antigen may be neutralized by pre-absorption with the blocking peptide.
- Determine the most dilute antibody concentration at which a consistently positive result is achieved for the desired test.
- For neutralization, combine antibody (at a concentration determined by the above method) with a five-fold (by weight) excess of blocking peptide in a small volume of PBS. Incubate for 2 hours at room temperature or overnight at 4° C.
- Following neutralization, dilute antibody/peptide mixture into appropriate working solution and proceed with the specific protocol for the desired test.

6. GENERAL SOLUTIONS

- **Tris buffered saline (1x TBS):** 10 mM Tris-HCl, pH 8.0; 150 mM NaCl.
- **Phosphate buffered saline (1x PBS):** 9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, and 150 mM NaCl. Adjust pH to 7.4 with NaOH.
- **Electrophoresis sample buffer (2x):** Mix 1.0 ml glycerol, 0.5 ml ß-mercaptoethanol, 3.0 ml 10% SDS, 1.25 ml 1.0 M Tris-HCl, pH 6.7, and 1-2 mg bromophenol blue. Store frozen in small aliquots. Alternatively, make buffer without ß-mercaptoethanol and store at room temperature. Add ß-mercaptoethanol just before using.
- **Diaminobenzidine tetrahydrochloride (DAB):** Dissolve 5 mg DAB (Sigma Chemicals) in 100 ml 100 mM Tris-HCl, pH 7.6, and add 0.1 ml 0.3% hydrogen peroxide. Prepare fresh DAB solution daily.
- **RIPA buffer:** 1x PBS, 1% Nonidet P-40 (Amaresco) or Igepal CA-630 (Sigma Chemicals), 0.5% sodium deoxycholate, 0.1% SDS. This may be made in large volumes. Add inhibitors at time of use from the following stock solutions: 1) 10mg/ml PMSF in isopropanol (add at 10 µl/ml RIPA) 2) Aprotinin (Sigma cat # A6279, available as liquid, add at 30 µl/ml RIPA) 3) 100 mM sodium orthovanadate in frozen aliquots (add at 10 µl/ml RIPA).
- **Subbing solution:** 0.3% (w/v) gelatin, 0.05% chromium potassium sulfate in distilled H_2O.
- **Blotto A (general use):** 5% milk, TBS, 0.05% Tween-20
- **Blotto B (for use with anti-phosphotyrosine antibodies):** 1% milk, 1%BSA, TBS, 0.05% Tween-20.