



The Power to Question

santa cruz biotechnology, inc.

Western Blotting Protocol



A. SAMPLE PREPARATION

Sample preparation procedures are provided for monolayer cells, suspension cells, and tissue samples. Follow the procedure suited to your needs.

MONOLAYER CELLS

- Grow cells to subconfluency in a 100 mm x 20 mm petri dish, remove culture medium and rinse cell monolayer with room temperature 1x PBS (10X liquid PBS: [sc-24946](#)). The following steps should be performed on ice or at 4° C using fresh, ice cold buffers.
- Add 0.6 ml of RIPA buffer ([sc-24948](#)) to the monolayer cells in the plate. Gently rock plate for 15 minutes at 4° C. Remove adherent cells with a cell scraper. Transfer the resulting lysate to a microcentrifuge tube.
- Wash the plate once with 0.3 ml of RIPA buffer and combine with first lysate. (Optional: Add 10 µl of 10 mg/ml PMSF (cat # [sc-3597](#)) stock and/or pass through a 21-gauge needle to shear the DNA.) Incubate 30–60 minutes on ice.
- Centrifuge cell lysate at 10,000xg for 10 minutes at 4° C. The supernatant fluid is the total cell lysate. Transfer the supernatant to a new microcentrifuge tube. This is your whole cell lysate. For increased protein recovery, resuspend the pellet in a small volume of RIPA, centrifuge and combine supernatants.

SUSPENSION CELLS

- Collect approximately 2.0×10^7 cells by low-speed centrifugation (e.g. 200xg) at room temperature for 5 minutes. Carefully remove culture medium.
- Wash the pellet with PBS at room temperature, and again collect by low-speed centrifugation. Carefully remove supernatant.
- Add 1.0 ml of ice cold RIPA buffer ([sc-24948](#)) with freshly added ([Protease Inhibitors](#)) and/or ([Phosphatase Inhibitors](#)). Gently resuspend cells in RIPA buffer with a pipet and incubate on ice for 30 minutes.
- Further disrupt and homogenize cells by hydrodynamic shearing (21-gauge needle), dounce homogenization or sonication, taking care not to raise the temperature of the lysate. (Optional: Add 10 µl of 10 mg/ml PMSF stock; [sc-3597](#)) Incubate 30 minutes on ice.
- Transfer to microcentrifuge tube(s) and centrifuge at 10,000xg for 10 minutes at 4° C. The supernatant fluid is the total cell lysate. Transfer the supernatant to a new microcentrifuge tube. This is your whole cell lysate. For increased protein recovery, resuspend the pellet in a small volume of RIPA, centrifuge and combine supernatants.

TISSUE SAMPLES

- Weigh tissue and dice into very small pieces using a clean razor blade. Frozen tissue should be sliced very thinly and thawed in RIPA buffer ([sc-24948](#)) containing ([Protease Inhibitors](#)) and/or ([Phosphatase Inhibitors](#)). Use 3 ml of ice cold RIPA buffer per gram of tissue.
- Further disrupt and homogenize tissue with a dounce homogenizer or a sonicator, maintaining temperature at 4° C throughout all procedures. (Optional: Add 30 µl of 10 mg/ml PMSF ([sc-3597](#)) stock per gram of tissue.) 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 clear lysate.

B. 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 ([sc-24945](#)) and boil for 2–3 minutes. Unused samples may be stored at -20° C.
- Load up to 10 µl of lysate per 1.0 mm of well width for gels of 0.75 mm thickness.
- We recommend the use of Cruz Marker™ molecular weight standards ([sc-2035](#)). Load 2 µl/well for 0.75 mm gels and 5 µl/well for 1.5 mm gels. When used with [Cruz Marker™ compatible secondary antibodies](#), internal standard bands will appear when the probed blot is exposed to detection reagent. Alternatively, use Prestained Molecular Weight Standards ([sc-2361](#)).
- Electrophorese according to standard protocols.

- Transfer proteins from the gel to a nitrocellulose or PVDF membrane using an electroblotting apparatus according to the manufacturer's protocols.

C. IMMUNOBLOTTING

- Block non-specific binding by incubating membrane in Blotto (either Blotto A or Blotto B; IgG-free BSA, [sc-2323](#), is recommended when using anti-bovine secondary antibodies) for 30–60 minutes at room temperature. Alternatively, the membrane may be blocked at 4° C overnight in a covered container, using Blotto without Tween-20.
- If using a phospho-specific antibody, add 0.01% (v/v) of each Phosphatase Inhibitor Cocktails A and B ([sc-45044](#) and [sc-45045](#)) to the blocking solution and the antibody diluent to inhibit phosphatases.
- Incubate the blocked membrane in primary antibody diluted in Blotto for 1 hour at room temperature. (For phospho-specific antibodies: Use Blotto B with 0.01% (v/v) of each Phosphatase Inhibitor Cocktails A and B ([sc-45044](#) and [sc-45045](#).) Optimal antibody concentration should be determined by titration. We recommend a starting dilution of 0.5–2.0 µg/ml. Wash membrane three times for 5 minutes each with TBST.
- Incubate the membrane for 45 minutes at room temperature with horseradish peroxidase (HRP) conjugated secondary antibody, or alkaline phosphatase (AP) conjugated secondary antibody ([Conventional Secondary Antibodies for Western Blotting](#)), diluted to 1:500–1:2000 in Blotto. If high backgrounds are observed, secondary antibody should be diluted further (up to 1:20,000). If Cruz Marker™ molecular weight standards ([sc-2035](#)) are used in the gel, the [Cruz Marker™ compatible secondary antibodies](#) must be used in order to visualize standards with ECL.
- Wash membrane three times for 5 minutes each with TBST and once for 5 minutes with TBS ([Buffers and General Solutions](#)).
- Incubate membrane in Chemiluminescence Luminol Reagent ([sc-2048](#)) according to Luminol data sheet, or visualize proteins using standard protocols. If luminol is used for visualization, an HRP-conjugated secondary antibody must be used.