Western (Immuno-) Blotting

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** (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 x 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 microfuge 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

SUPPORT PRODUCTS
Western (Immuno-) Blotting

Mouse IgG Binding Proteins

Secondary Antibodies

Cruz Marker™ Molecular Weight Standards

Western Blotting Chemiluminescence Luminol Reagent

Prestained Molecular Weight Standards

Blocking Reagents for Western Blotting Applications

Gel Incubation Trays

Adult Tissue Extracts for Western Blotting

Control IgGs and IgG Conjugates

Buffers and General Solutions

Nuclear Extracts for Gel Shift and Western Blotting

Whole Cell Lysates for Western Blotting

PhosphoCruz™ Protein Purification System

Acetylation Specific Antibodies

Support Products

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, 5-10 µg transfected lysate, 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 mouse IgG binding proteins or CruzMarker™ MW Tag 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 UltraCruz® Nitrocellulose Pure Transfer Membranes, UltraCruz® PVDF Transfer Membrane (sc-3723), or Immobilon®-FL PVDF membrane (sc-516541) using an electroblotting apparatus according to the manufacturer's protocols.

C. IMMUNOBLOTTING FOR INDIRECT DETECTION

- Block non-specific binding by incubating membrane in Blotto (either **TBS Blotto A**: sc-2333 or **TBS Blotto B**: sc-2335) for 30–60 minutes at room temperature (UltraCruz® Gel Incubation Trays in multiple colors and 200ml or 120ml sizes available). Alternatively, the membrane may be blocked at 4° C overnight in a covered container, using Blotto without Tween-20. For near-infrared detection, block the Immobilon®-FL PVDF membrane with **UltraCruz® Blocking Reagent** (sc-516214) for 1 hour at room temperature.
- 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, or diluted in **UltraCruz® Blocking Reagent** (sc-516214) for near-infrared (NIR) detection, for 1 hour at room temperature, with shaking. (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 primary antibody concentration should be determined by titration. We recommend a primary antibody starting dilution of 0.5-2.0 µg/ml. Wash membrane three times for 5 minutes each with TBST.
- If **Cruz Marker™ molecular weight standards** (sc-2035) are used in the gel and the primary antibody is a mouse IgG kappa light chain, mix the primary antibody with the unconjugated **Cruz Marker™ MW Tag** (sc-516729) in either Blotto, or UltraCruz® Blocking Reagent as appropriate. Incubate the membrane in this mixture for 1 hour at room temperature, with shaking. Optimal Cruz Marker™ MW Tag dilution range is 1:1000 to 1:2000. **NOTE**: *This step is required for Indirect NIR detection. It is optional for Indirect ECL detection, depending on which secondary reagent is used to detect the standards (see notes under Indirect ECL Detection, below).*
- Wash membrane three times for 5 minutes each with TBST.

Indirect Enhanced Chemiluminescence (ECL) Detection

- Incubate the membrane for 45 minutes at room temperature with horse-radish peroxidase (HRP) conjugated secondary antibody, or mouse IgG binding protein (**m-IgGκ-BP-HRP**: sc-516102, or **m-IgGλ-BP-HRP**: sc-516132), diluted to 1:500-1:2000 in Blotto. If high

backgrounds are observed, secondary antibody or detection reagent should be diluted further (up to 1:20,000).

NOTE: If **Cruz Marker™ molecular weight standards** (sc-2035) are used in the gel, and the membrane *has not* been incubated with the **Cruz Marker™ MW Tag** (sc-516729) or **Cruz Marker™ MW Tag-HRP** (sc-516732), then the **Cruz Marker™ compatible mouse IgG binding protein** (sc-516102-CM or sc-516132-CM) must be used in order to visualize standards with ECL.

NOTE: If **Cruz Marker™ molecular weight standards** (sc-2035) are used in the gel, and the membrane *has* been incubated with the **Cruz Marker™ MW Tag** (sc-516729), then the mouse IgG binding protein **m-IgGκ BP-HRP** (sc-516102) 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.
- Incubate membrane in **Chemiluminescence Luminol Reagent** (sc-2048) according to Luminol datasheet, or visualize proteins using standard protocols.

Indirect RGB Fluorescent or Near-Infrared (NIR) Detection

For RGB Fluorescent or Indirect Near-Infrared (NIR) Western blotting detection, we highly recommend using our kappa or lambda chain mouse IgG binding proteins and a compatible imaging/detection system.

		m-IgGκ BP	m-IgGλ BP
RGB Fluorescent	CFL 488	sc-516716	sc-516190
	CFL 555	sc-516177	sc-516191
	CFL 594	sc-516178	sc-516192
	CFL 647	sc-516179	sc-516193
Near-Infrared	CFL 680	sc-516180	sc-516194
	CFL 790	sc-516181	sc-516195

NOTE: For optimal results, use low fluorescence PVDF membranes, such as **Immobilon®-FL PVDF**: sc-516541, and **UltraCruz® Blocking Reagent**: sc-516214 for all blocking, primary antibody and mouse IgG binding protein incubations. All RGB Fluorescent or NIR detection steps should be performed in the dark.

NOTE: For two-color Western blots using primary antibodies with kappa or lambda light chains, primary antibodies and their corresponding secondary detection reagents may be incubated simultaneously.

- Following the primary antibody incubation step, incubate the membrane for 1 hour at room temperature with the appropriate mouse IgG binding protein in **UltraCruz® Blocking Reagent** (sc-516214), 0.01% SDS for Immobilon®-FL PVDF membrane only. Recommended mouse IgG binding protein dilution is 1:10,000. Do not use SDS if using nitrocellulose membranes.

NOTE: If **Cruz Marker™ molecular weight standards** (sc-2035) are used in the gel, and the membrane *has* been incubated with the **Cruz Marker™ MW Tag** (sc-516729), then a CFL-conjugated mouse IgGκ binding protein must be used in order to visualize standards.

- Use clean forceps to gently handle the blot from the corner without creasing the membrane. Do not write on the blot with pen or marker, as the ink can fluoresce and cause background.
- Wash membrane three times for 5 minutes each with TBST and once for 5 minutes with TBS.
- Place membrane on top of blotter paper and let dry for 5-10 minutes.
- Proceed with imaging the Western blot using preferred method. The membrane can be stored in 1X TBS or 1X PBS for up to 48

hours in the dark at 4° C. Adjust the settings to optimize signal detection. Refer to the manufacturer guidelines for your detection instrument.

D. IMMUNOBLOTTING FOR DIRECT DETECTION

Direct Enhanced Chemiluminescence (ECL) Detection

For direct ECL detection, we recommend the use of SCBT's horseradish peroxidase (HRP)-conjugated primary antibodies.

- Block non-specific binding by incubating membrane in Blotto (either **TBS Blotto A**: sc-2333 or **TBS Blotto B**: sc-2335) for 30–60 minutes at room temperature (UltraCruz® Gel Incubation Trays in multiple colors and 200ml or 120ml sizes available). Alternatively, the membrane may be blocked at 4° C overnight in a covered container, using Blotto without Tween-20.
- Incubate the blocked membrane with HRP-conjugated primary antibody diluted in Blotto for 2 hours at room temperature with shaking. Optimal antibody concentration should be determined by titration; recommended concentration range is 0.5-2 µg/ml diluted in Blotto. If **Cruz Marker™ molecular weight standards** (sc-2035) are used in the gel, mix the HRP-conjugated primary antibody with the **Cruz Marker™ MW Tag-HRP** (sc-516732) in Blotto. Incubate membrane in this mixture for 2 hours at room temperature, with shaking. Optimal Cruz Marker™ MW Tag dilution range is 1:1000 to 1:2000.
- Wash membrane three times for 5 minutes each with TBST and once for 5 minutes with TBS.
- Incubate membrane in **Chemiluminescence Luminol Reagent** (sc-2048) according to Luminol datasheet, or visualize proteins using standard protocols.

Direct RGB Fluorescent or Near-Infrared (NIR) Detection

For Direct RGB Fluorescent or NIR detection, we highly recommend using SCBT's primary antibodies conjugated to Alexa Fluor® dyes, and a compatible imaging/detection system.

RGB Fluorescent	Alexa Fluor® 488
	Alexa Fluor® 546
	Alexa Fluor® 594
	Alexa Fluor® 647
Near-Infrared	Alexa Fluor® 680
	Alexa Fluor® 790

NOTE: For optimal results, use low fluorescence PVDF membranes (such as **Immobilon®-FL PVDF**: sc-516541), and **UltraCruz® Blocking Reagent** (sc-516214) for both, blocking and dilution of Alexa Fluor® conjugated primary antibodies. All RGB Fluorescent or NIR detection steps should be performed in the dark.

- Block non-specfic binding by incubating membrane with **UltraCruz® Blocking Reagent** (sc-516214) for 1 hour at room temperature, with shaking.
- Incubate the blocked membrane with the appropriate Alexa Fluor® conjugated primary antibody diluted in **UltraCruz® Blocking Reagent** (sc-516214) for 2 hours at room temperature, in the dark, with shaking. Optimal antibody concentration should be determined by titration; recommended concentration range is 0.2-2 µg/ml. If **Cruz Marker™ molecular weight standards** (sc-2035) are used in the gel, mix the Alexa Fluor® 680, or the Alexa Fluor® 790-conjugated primary antibody with the **Cruz Marker™ MW Tag-Alexa Fluor® 680** (sc-516730) or with the **Cruz Marker™ MW Tag-Alexa Fluor® 790** (sc-516731) in UltraCruz® Blocking Reagent. Incubate membrane in this mixture for 2 hours at room

temperature, in the dark, with shaking. Optimal Cruz Marker™ MW Tag dilution range is 1:1000 to 1:2000.

- Use clean forceps to gently handle the blot from the corner without creasing the membrane. Do not write on the blot with pen or marker, as the ink can fluoresce and cause background.
- Wash membrane three times for 5 minutes each with TBST and once for 5 minutes with TBS.
- Place membrane on top of blotter paper and let dry for 5-10 minutes.
- Proceed with imaging the Western blot using preferred method. The membrane can be stored in 1X TBS or 1X PBS for up to 48 hours in the dark at 4° C. Adjust the settings to optimize signal detection. Refer to the manufacturer guidelines for your imaging instrument.