

## Western Blot Sample Preparation Protocol

### Lysis buffer recipe

**RIPA buffer:** 20mM Tris-HCL pH7.4, 150mM NaCl, 1mM EDTA, 1% Triton-X100, 1% sodium deoxycholate, 0.1% SDS. Just prior to use, add protease inhibitors: 1mM PMSF, 5ug/ml aprotinin and 5ug/ml leupeptin.

### Lysis of cultured cells

Cells should be harvested at ~80% confluency.

#### A. Adherent cells

1. Place Petri dish on ice and rinse twice with ice cold 1X PBS. Alternatively, trypsinize the cells, centrifuge in a cold test tube and wash cell pellet with ice cold 1X PBS twice to remove the trypsin.
2. Add 1ml of ice cold RIPA buffer per  $10^8$  cells to the cold petri dish and scrape cells free with a cold plastic cell scraper. Transfer suspension to a cold test tube. If the cells were trypsinized, add 1ml of ice cold RIPA buffer per  $10^8$  cells to the PBS-washed cell pellet to resuspend.
3. Lyse cells at 4oC for 20 min with constant agitation (best) or vortex every 3-5 min.
4. Centrifuge lysed cell suspension at 12,000 RPM for 20 min at 4oC. Discard lipid layer at top (if present) and cell pellet. Retain a 20ul aliquot for use in protein concentration estimation, and aliquot and freeze the cell lysate. Store at -80oC.

#### B. Non-adherent cells

1. Centrifuge cell culture at 1,000 RPM for 5 min to pellet cells. Discard supernatant, place test tube on ice and add ice cold 1X PBS to resuspend cell pellet. Centrifuge at 1,000 RPM for 5 min and discard supernatant. Repeat.
2. Add 1ml of ice cold RIPA buffer per  $10^8$  cells to resuspend the cell pellet.
3. Lyse cells at 4oC for 20 min with constant agitation (best) or vortex every 3-5 min.
4. Centrifuge lysed cell suspension at 12,000 RPM for 20 min at 4oC. Discard lipid layer at top (if present) and cell pellet. Prepare a small aliquot for use in protein concentration estimation and aliquot and freeze the cell lysate. Store at -80oC.

## Lysis of tissue

1. Dissect out tissue and to prevent degradation by proteases, quickly rinse tissue with ice cold 1X PBS to remove any blood, cut tissue into pieces and add liquid nitrogen to snap freeze the tissue pieces. Store at -80oC but do not seal the tube until the liquid nitrogen has boiled off.
2. When ready to lyse, add 300ul of ice cold lysis buffer per ~5g of tissue. Disrupt the tissue with a tissue homogenizer, rinse the blades with 2 x 200ul ice cold lysis buffer, and then incubate the homogenate at 4oC with constant agitation for 2 hours. A smaller or larger volume of lysis buffer can be used to increase or decrease the final lysate concentration, respectively. *Caution must be used so that the final concentration is not too low to be usable. The lysate will be further diluted 1:1 with 2X Laemmli sample buffer prior to running on a gel.*
3. Centrifuge lysed cell suspension at 12,000 RPM for 20 min at 4oC. Discard lipid layer at top and cell pellet. Prepare a small aliquot for use in protein concentration estimation and aliquot and freeze the cell lysate. Store at -80oC.

## Protein quantification

Perform a protein quantification assay such as a Bradford Protein Assay or use Bio-Rad's Protein Determination Kit to determine the concentration of the lysate.

## Sample preparation and gel run

1. When ready, dilute the lysate with 2X Laemmli sample buffer and record the new concentration.
2. Unless otherwise required by the experiment, boil each cell lysate in sample buffer at 100°C for 5 min to reduce and denature the sample.
3. Load equal amounts of protein (20-50ug) into the wells of an SDS-PAGE gel, along with a molecular weight marker.
4. Run gel and transfer to membrane per manufacturer's specifications.