**SINGLE-PROTEIN (PROTEIN COMPLEX) PHOSPHORYLATION SITE MAPPING**

***Materials***

Cells/tissue source (e.g., cell line, tumor, extracted bodily tissue)

Lysis/IP buffer (e.g.

0.5% (v/v) NP-40

1% (v/v) Triton X-100

150 mM NaCl

50 mM Tris·Cl, pH 7.4

1 mM EDTA

1 mM EGTA)

Protease and phosphatase inhibitors:

1 mM of Na3VO4

1 mM aprotinin

1 mM leupeptin

1 mM pepstatin

10 mM NaF

1 mM PMSF

2.5 mM sodium pyrophosphate

1 mM β-glycerophosphate

Bradford protein assay kit (BioRad)

Antibody targeting protein of interest

Protein A (GE Healthcare, cat. no. 17-0963-03) or G (GE Healthcare, cat. no. 17-0618-02) agarose beads

1× SDS sample buffer

31.25 mM Tris·Cl, pH 6.8

5% glycerol

1% (w/v) SDS

0.36 M 2-mercaptoethanol

0.0025% (v/v) bromphenol blue

SDS-PAGE Tris-glycine polyacrylamide minigels 10-well, 1-mm (10% fixed or 4% to 20% gradient; Lonza, cat. no. 58511)

Tris-glycine SDS running buffer (1× formulation: 25 mM Tris·Cl, 192 mM glycine, 0.1% SDS, pH 8.3)

Coomassie blue stain (15% methanol, 10% acetic acid, 2 g Coomassie Brilliant Blue)

Coomassie destain (15% methanol, 10% glacial acetic acid)

50% (v/v) acetonitrile/H2O (LC-MS grade)

Stainless steel mortar with ceramic pestle

Platform rocker with circular motion

15-ml conical polypropylene centrifuge tubes

Refrigerated centrifuge and microcentrifuge

End-over-end rotator

95◦C heat block or water bath

Minigel SDS-PAGE apparatus (BioRad or other vendor) with power supply

37◦C shaking incubator

***Lyse cells***

1. Prepare cells to make sure they are in log phase. Lyse a sufficient amount of cells (∼107) in ∼5 ml of lysis /IP buffer with protease/phosphatase inhibitors to produce at least 10 mg of protein

*For lysis of frozen tissue (use* ∼*100 mg to produce 10 mg of protein), grind it using liquid*

*nitrogen in a stainless steel mortar with a ceramic pestle until it is a powder, let liquify at*

*4*◦*C, and then immediately add lysis buffer containing protease/phosphatase inhibitors.*

2. Incubate lysate for 45 min at 4◦C with gentle rocking on a platform rocker in a 15-ml

conical tube.

3. Centrifuge 15-ml tube with lysate for 20 min at 14,000 × *g*, 4◦C, to remove cell

debris. Keep supernatant.

4. Take a small aliquot of the supernatant and determine the protein concentration using

the Bradford assay

5. Transfer to a new tube, dilute the protein lysate with lysis/IP buffer (containing

protease and phosphatase inhibitors) to ∼2 mg/ml (5 ml total), and keep at 4◦C.

***Perform immunoprecipitation***

6. Add ∼8 to 10 μg of antibody, or enough to clear the lysate of the protein of interest

(this amount will vary across different antibodies) and incubate the solution on an

end-over-end rotator 2 hr to overnight at 4◦C.

7. Wash 50 μl of protein A (or G) agarose beads per immunoprecipitation to be performed with 1 ml of lysis/IP buffer by agitating five times, centrifuging 3 min at 2500 × *g*, 4◦C, and discarding buffer. Repeat wash three times, then make a 1:1 slurry in lysis/IP buffer, at 4◦C

8. Add 80 μl of the bead slurry (40 μl pure beads) to the lysate-antibody solution and incubate for 2 hr on an end-over-end rotator at 4◦C

9. Centrifuge 2 min at 2500 × *g*, 4◦C, then remove supernatant completely.

10. Wash the protein–antibody beads complex with 1 ml of lysis/IP buffer by rocking for 3 min at 4◦C.

11. Centrifuge 2 min at 2500 × *g*, 4◦C, remove supernatant, then repeat the wash described in steps 10 to 11 three times.

12. Elute the proteins from beads by adding ∼80 μl of 1× SDS sample buffer (twice the volume of pure beads) and incubating for 5 min at 95◦C.

*Proteins can also be eluted from bait protein/antibody complex using competition with*

*a peptide (e.g., FLAG or HA for tagged bait proteins), small molecule, or pH change in*

*some cases instead of SDS sample buffer.*

***Perform SDS-PAGE (preferred for obtaining protein of highest purity)***

13. Load sample (in 1× SDS sample buffer) on an SDS-PAGE gradient or fixed percentage

polyacrylamide minigel appropriate for purifying the specific molecular weight of the protein of interest.

14. Run mini SDS-PAGE at ∼120 V until the 55-kDa pre-stained marker band is separated from the band above and below (short gel run, ∼1/6 distance of mini gel lane).

15. Stain the gel with Coomassie blue stain for 1 hr at room temperature and destain the gel overnight at 4◦C with at least five solvent changes in the first 2 hr.

16. Excise the protein gel sections above and below the IgG heavy chain band to avoid antibody contamination and peptide signal suppression, put each band in a 1.5-ml plain microcentrifuge

tube, and wash with 150 to 200 μl of 50% acetonitrile/50% water for 15 min and discard supernatant. Repeat wash once.

**ACETONE PRECIPITATION OF PROTEIN SAMPLES (FOR IP ELUTION**

**WITH PEPTIDE OR SMALL MOLECULE)**

Acetone precipitation cleans protein samples of both salts and detergents (ThermoScientific,

2009). It should be used only when proteins are eluted by competition without IgG

contamination, since excess IgG will suppress phosphopeptides of interest.

***Additional Materials***

Acetone

Protein sample

5% (w/v) trifluoroacetic acid (TFA)

Acetone-compatible tubes (polypropylene only)

pH paper

***Precipitate protein***

1. Cool the required volume of acetone to −20◦C in freezer.

2. Place protein sample in acetone-compatible tube.

3. Add four times the sample volume of cold (−20◦C) acetone to the tube.

4. Vortex tube and incubate for 60 min at −20◦C.

5. Centrifuge 10 min at 14,000 g, 4◦C.

6. Decant and properly dispose of the supernatant, being careful to not dislodge the protein pellet.

7. Repeat steps 3 to 6 one more time.

8. Allow the acetone to evaporate from the uncapped tube at room temperature for 30 min. Do not over-dry pellet, or it may not dissolve properly.