

IP PROTOCOL

Wash P150 (P100) plate of cells 2x with 8 ml (5 ml) PBS

Wash P150 (P100) plate of cells 1x with 8 ml (5 ml) IP wash

Lyse cells on plate in 1% NP-40 lysis buffer.
1 ml/P150 plate (0.5 ml/P100) plate on a rocker @ 4° C for 20 min.

Transfer supe to eppendorf tube & spin full speed (13,000g) 10 min @ 4° C

Transfer supe to new tube & aliquot if desired.
Min final volume 500 ul/tube so it will rock.

Add Ab & protein A beads (or protein G depending on species and subclass of Ab) and rock 90 min @ 4° C.

For rabbit serum use minimum of 8 ul solid beads for each ul serum.

For monoclonals use ~5 ul solid beads for up to 3 ug Ab.

Always use a minimum of 10 ul beads / IP.

Spin 30 sec-1min on setting #3 in swinging bucket microfuge @ 4° C. Toss supe.

Wash 2x with 1 ml IP lysis buffer.

Wash = add 1 ml buffer, invert 2-3x by hand, DO NOT VORTEX,
spin 30 sec-1min on setting #3 in swinging bucket microfuge @ 4° C. Toss supe.

Wash 3x with 1 ml PBS.

Add GSD (glycerol, SDS, DTT), boil, & load on gel.

Add > volume GSD than of solid beads.

If no other buffer is added, use 1:3 GSD made up with dH₂O.

IP WASH

(1% glycerol, 20 mM Tris pH 8.0, 135 mM NaCl)

500 ml = 6.25 g glycerol, 10 ml 1M Tris, 3.94 g NaCl

IP LYSSE

1% NP-40 in IP Wash buffer

Coating Buffer to block beads

30 mM Tris, pH 8.0

.02 % Na azide

10% BSA

dH₂O

For 100 ml

5 ml 1M Tris, pH 8.0

2 ml 1% Na azide (100 x)

10 g BSA

dH₂O to 100 ml

Weigh out 0.15 g in eppendorf tube

Fill to 1.3 ml line w/coating buffer

Add to solid dry beads + suspend

Leave @ room temp for 1/2 hr. to swell

wash 3x w/ddH₂O

resuspend 1:1 in ddH₂O

for storage add Na azide to .02%

Lysing a Plate of Cells

Materials	Location
IP Wash = IP lyse w/o NP40	Egon's bench
IP Lyse (new)	side door of frig
Aprotinin (A)	egg carton of frig; in Epp
PMSF (phenol/methyl	room temp on bench
100 mM Vanadate (V) Na ₃ VO ₄	egg carton; in Epp
scraper	
GSD	freezer; in Epp
Falcon tube (15 ml)	
ice bucket	next to hood

Procedure

1. Aspirate or pipet off the media. This can be done outside of hood.
2. Wash with 5 ml PBS (for P100; 10ml for P150); swirl; aspirate.
3. Wash with 5 ml IP Wash; swirl; remove. Drain thoroughly by resting plate at a 45° angle to the vertical; remove.

In some cases, scrape after IP Wash, spin, remove sup and add lysis buffer. eg. when you want to lyse a P150 in 0.5ml of lysis buffer.

4. In a 15ml Falcon add the following to make lysis buffer:
 - 1ml new IP lyse (depends on the size of plate)
 - 5 λ /ml Aprotinin
 - 10 λ /ml PMSF (add this while vortexing contents in Falcon b/c PMSF does not mix well with IP lyse)
 - 1mM Vanadate (vortex tube while adding)

Ingredients which are kept in Eppendorfs should be spun down to collect the condensate and debris. Frozen chemicals can be thawed in the bath for a short time.

Add 0.5ml of lysis buffer per P100.

5. Rock at 4°C for 20 min (rotate plate after 10 min so that liquid will cover entire plate). If hot, put Saran wrap under plates to protect rocker.

6. Scrape (use 1 scraper per cell type). Transfer to eppendorf. Spin 10 min, 4°C, 13,000xg.

Scraping is required if you want to obtain as much lysate as possible, such as when lysing in small volumes. Otherwise, you can just use a pipetman to draw lysate.

7. Keep samples on ice to maintain activity of proteins! Also to decrease activity of proteases. Transfer supernatant (sup) to new Epp. *Cell debris is in pellet. Soluble proteins are in sup.*

8. For 1D gel, transfer 100λ of sample to separate Epp. Boil in 50λ GSD for 3 min. Spin for 1 sec. *Boiling also helps denature proteins.*

If these samples are run on a gel, the markers must be mixed with the IP lyse mixture from step 4 as a "buffer" to insure that the samples have the same conditions of salts, etc.

Appendix

I. Protease Inhibitors

Aprotinin-serine protease inhibitor. non-covalent binding to active site.
PMSF- serine protease inhibitor. half-life of 30min in H₂O. covalently modifies active site at serine.

Vanadate- tyrosine phosphatase inhibitor. non-covalently modifies.

Phenylarseneoxide- tyrosine phosphatase inhibitor (covalent)/cysteine inhibitor.

NaF- ser/thr phosphatase inhibitor

II. Centrifuges

Name	Tubes	Temp	Use	speed
Beckman	15/50ml Falcon	adjust		rpm
Bucket or swinging	epp	4 C	pellet beads (1") pellet cells intact (30sec-1min)	setting 3 0-10,000g
Angle	epp	4 C or RT	lysates	13000xg

III. Storage

Use O-ring tubes for long-term storage. Don't use these for general samples that will be loaded immediately.

IV. GSD

GSD can be used in its concentration form all the way to 1:6 dilution. In very dilute GSD, the decrease in glycerol leads to difficulty in loading sample. Optimum dilution is 1:3, 1:4. Glycerol increases density of sample to separate sample from the buffer.