

Immunoprecipitation

A. Background: Protein precipitation refers to the formation of protein aggregates that can be isolated by centrifugation. Globular proteins can be precipitated by salt (ammonium sulfate), organic solvent (acetone), organic polymer (PEG), or trichloro-acetic acid. In the case of a mixture of proteins, these agents will not work since the proteins will precipitate simultaneously. Immunoprecipitation allows for a given protein to be precipitated selectively via an antibody-antigen reaction. The steps involved in this procedure include:

1. Lysing the cells with a detergent (for membrane bound proteins)
2. Binding of a given protein antigen to an antibody.
3. Precipitating the antibody-protein complex.
4. Washing the precipitate.
5. Dissociating the protein from the immune complex.

B. Reagents and Preliminary Procedures:

Lysis Buffer: per 100 mls:

50 mM Tris pH 8.0..... 5 mls of 1M Tris pH 8.0
5mM EDTA..... 1 ml of 0.5 M EDTA
150mM NaCl..... 3 ml of 5M NaCl
0.5% NP-40..... 5 mls of 10% NP-40
ddH₂O.....86 mls

Protease inhibitors (available from BMB):

Always use:

- PMSF: (stock = 100mM in ETOH) use 10-40µg/ml of LB
- EDTA: (stock = 50 mg/ml) use 0.2-0.5 mg/ml of LB
- Leupeptin: (stock = 1 mg/ml) use 0.7 µg/ml of LB

Recommended:

- Pepstatin: (stock = 0.5 mg/ml) use 0.7µg/ml of LB
- Aprotinin: (stock = 5 mg/ml) use 2-10 µg/ml of LB

PMSF: 100mM PMSF (TOXIC!!!!) in 95% EtOH; store at -20°C

TENN: per 100 mls:

50mM Tris pH 7.4..... 5 mls of 1M Tris pH 7.4
5mM EDTA..... 1 ml of 0.5M EDTA
0.5% NP-40..... 5 ml of 10% NP-40
150mM NaCl..... 3 mls of %M NaCl
ddH₂O..... 86 mls

SNNTE: per 100 mls:

5% sucrose..... 20 mls of 25% sucrose
50mM Tris pH 7.4..... 5 mls of 1M Tris pH 7.4
5mM EDTA..... 1 ml of 0.5M EDTA
0.1% NP-40..... 1 ml of 10% NP-40
0.5mM NaCl..... 10 mls of 5M NaCl
ddH₂O..... 63mls

NTE: per 100 mls:

50mM NaCl..... 1ml of 5M NaCl
10mM Tris pH 7.4..... 1 ML of Tris pH 7.4
1mM EDTA..... 0.5 mls of 0.5M EDTA
ddH₂O..... 97.5 mls

Sample Buffer (2x):

10% glycerol
2% SDS
0.02% Bromophenol blue
10% β-mercaptoethanol
0.125M Tris pH 6.8
Filter sterilize.

Staph. aureus cells: obtain from BMB.....10% cell suspension in ddH₂O or PBS/Azide (better); aliquot and store at -20°C. Stable 4-6 months. (Kessler, *J. Immunology* 117 (1976) : 1482)

Activation of Staph: (use freshly activated Staph for each IP time)

1. Thaw cells at room temperature
2. Spin down in microfuge for two minutes (mark tube where meniscus is)
3. Discard supernatant
4. Wash cell pellet with TENN. Resuspend cells in equal original volume of TENN.
5. Vortex.

C. Extraction of Cell Pellet:

1. Add PMSF and Leupeptin to lysis buffer (per 20ml of lysis buffer: add 0.2 ml of leupeptin, 0.2 ml of PMSF).
2. Resuspend pellet in lysis buffer (use 1 ml of lysis buffer if only one cell plate was used in the tube preparation, 2mls if two plates used. The number of mls of lysis

buffer may vary if the confluence of the cell plate varies or if the cells were previously determined to be low-protein producers.)

3. Keep resuspended cells on ice for several minutes. Vortex to resuspend totally.
4. Transfer to an eppendorf tube and spin in a **cold** microfuge for 5 minutes at 10,000 rpm. Supernatant has protein. **Store on ice.**
5. Test optical density and protein counts of extract supernatant:

optical density: (590nm): use the Bradford reagent:

- Dilute 1:4 and filter with whatman paper
- Use 0.8 dH₂O and 0.2 Bradford reagent.
- Add BSA standards at 1µg/ml; 2µg/ml; 4µg/ml; and 6µg/ml.
- Determine the amount of protein using a "ball-park" graphical analysis by plotting O.D.590 vs concentration of standards.

Protein counts: TCA precipitation for labelled protein counts:

- Aliquot 1 ml of trichloro-acetic acid into **three** tubes.
- Spin the extract and add 5µl of extract supernatant to the TCA.
- Let it sit on ice.
- Using the vacuum aspirator at the radioactive sink, place a filter at the bottom of the top piece of the precipitation apparatus and carefully pour the TCA sample onto the filter using excess TCA to rinse the vial and the inside of the filtering apparatus.
- Remove the filter with tweezers (RADIOACTIVE) and place it into a scintillation vile with 1ml of monofluor fluid.
- Rinse the filter apparatus and repeat the procedure with the other samples.
- Read the samples in the scintillation counter for 35 S-MET.

D. Pre-adsorption with Staph. aureus Protein A: Staph. aureus is a fixed bacterial culture (in this protocol) which had an attached protein (A) which sticks to the Fab portion of immunoglobins. If a mixture contains an Agn-Aby complex, the protein A will adhere to the Aby, and the Staph. aureus and the S.aureus-Agn-Aby complex may be precipitated out. The pre-absorption step consists of adding activated S.aureus to the cell extract before Aby addition. The cells will stick to any extraneous protein and will clear the supernatant of waste products when spun down. Pre-absorption is done prior to the Aby addition to clear up the Agn in the supernatant.

1. Add 0.04 ml of activated S.aureus cells/ml of extract (The extract is the supernatant from part C. Each cell line has a different extract). If 2 mls of lysis buffer are used, add 0.08 ml of acitvated S.aureus. (NOTE: Concerning the proper amount of S.aureus to use for your pre-absorbtion step; you can figure it out this way: Using your protein determination information, figure out exactly how many µgs of protein you have:

$$\frac{8\mu\text{g}/\mu\text{l}}{\text{"ball park amount"}} \times \frac{2000\mu\text{l}}{\text{Amount of extract}} = 16,000\mu\text{g}$$

Divide by 200 to obtain the amount of S.aureus to use for the pre-absorbption step.)

2. Incubate on ice for 10 minutes (put the extract into microfuge vials).
3. Spin 2 minutes in a microfuge at 25°C.
4. Remove supernatant and discard pellet (or save it "just in case" if desired).

E. Immune Reaction: The theory behind this step is similar to the pre-absorbption step except that this reaction occurs between the activated cells and the immune complex after antibody addition.

1. Aliquot the supernatant into Eppendorf tubes: The amount to add is specific. You want a total of 600 µg of extract protein per reaction vial. Use your "ball-park" protein determination result:
 $8\mu\text{g}/\mu\text{l} = 600\mu\text{g}/X\mu\text{l} \implies X = 75 \mu\text{l}$ of extract supernatant per reaction vial.
Bring up to 300µl with Lysis Buffer.

2. Add antibody to be in excess such that 2-5X polyclonal is added or 100-200X monoclonal antibody is added per samples (100µl of monoclonal Aby or 5µl of polyclonal Aby).
3. After antibody addition, incubate tubes on ice for 1 hour.
4. Add activated 10% S.aureus suspension (up to 20µl of cell suspension per tube). The protein A on the bacterial surface will attach to the antibody of the Agn-Aby complex.
5. Incubate on ice for 10-15 minutes.

F. Washing the Immunocomplex:

1. Spin the reaction tubes in the microfuge at room temperature for one minute at 10,000 rpm.
2. Discard supernatant using aspirator apparatus at the radioactive sink. Save pellet.
3. Resuspend the pellet in 0.5 ml of SNNTE buffer, It will be necessary to use the ultra vortex to suspend the cell pellet.
4. Spin for 1 minute at room temperature; discard supernatant.
5. Repeat steps (3) and (4) 2-3 times depending on the stability of the protein involved.
6. Resuspend the pellet in 0.5 ml of NTE buffer.
7. Spin down at room temperature for 1 minute.
8. Resuspend in 30µl of sample buffer for gel electrophoresis. Store at -20°C.

F. SDS - PAGE of ³⁵S - Labeled Protein Samples:

Preparation of the protein samples:

1. Just before loading, remove samples from the -20°C freezer.
2. Boil samples for 3 minutes along with a radioactive samples marker preparation.
3. Spin samples at 7,000 rpm for 1 minute.
4. Load supernatant onto a gel, using about 15 µl/well.

Gel Preparation:

·For 1 mini gel (10% acrylamide): 10 ml of running gel (X4 for large protein gel apparatus):

	<u>Running Gel</u>	<u>Stacking Gel</u>
·dH ₂ O	3.37 ml	2.76 ml
·1.5M Tris pH 8.8	2.5 ml	1.25 ml
·30 % acrylamide	3.3 ml	0.65 ml
·2% Bis	0.66 ml	0.26 ml
·10% SDS	100 µl	50 µl
·10% Am. Sulfate	70 µl	50 µl
·TEMED	7µl	5 µl

1. Follow a gel/mini gel protocol for mini gels, an overlay of dH₂O can be used on top of the running gel as it polymerizes. Polymerization for a mini gel takes about 30 minutes for the running gel portion and 15-20 minutes for the stacking gel portion.

NOTES:

· Make sure to wedge the plates with the appropriate size comb while the running gel is polymerizing.

· Remove all overlay dH₂O with a piece of whatman filter paper.

· Keep an eye on your stacking gel as it polymerizes.

· Make sure the protein in running at the correct concentrations:

5x stock Tris-Glycerine Protein Buffer:

120 g of tris base [0.12]

576 g glycerine [0.96]

40 g SDS [0.5%]

8 L ddH₂O

· Make sure to clean out wells before adding samples.

· The mini gels should run at a constant current of 30 mAmps, and the volts should start at about 80 and increase to 140-150t the end of the run.

G. Gel Staining, Enhancement, and Exposure to Film:

1. Disassemble apparatus. **CAUTION: Buffer is Potentially radioactive.**
2. Pull away side spacers.
3. Carefully pry the plates apart -- gel should stick to one of the plates.
4. Slip the gel into destaining solution for 15-30 minutes at room temperature with shaking. This precipitates the proteins, and it attaches to the gel.
Destaining solution:
7.5% Acetic acid
5% Methanol
5. Aspirate off the destaining solution into the radioactive sink.
6. Under the hood add ENHANCE to cover the gel. ENHANCE can be reused several times. Store in a foil covered bottle.
7. Put saran wrap over ENHANCE/gel container and cover it with foil.
8. Shake for 30-60 minutes at room temperature.
9. Save the ENHANCE and wash the gel with a slow drip dH₂O stream in the radioactive sink. The gel will turn very white and flaky.
10. Slip whatman paper under the gel (record orientation), cover it with saran wrap, and dry it in the gel dryer under vacuum at 80°C (mini gel takes about 30 minutes).
11. When dry, expose to film at - 80°C.