Immunoprecipitation Technique

(BE-506)

MATERIALS INCLUDED WITH THE KIT

This kit has enough materials and reagents for 24 students (six groups of four students).

Checklist

- ❑ 1 vial Cellular Extract
- ❑ 1 vial Antibody: BE Antibody 1
- ❑ 1 vial Antibody: BE Antibody 3
- ❑ 1 vial Resin (Protein-A)
- ❑ 1 bottle PBS
- ❑ 60 Centrifuge Tubes (1.5ml)

SPECIAL HANDLING INSTRUCTIONS

- Store Cellular Extract, Antibodies 1 and 3 and Resin (Protein-A) at 4°C.
- All other reagents can be stored at room temperature.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.

ADDITIONAL EQUIPMENT REQUIRED

- Low Speed Centrifuge for 1.5-2ml tubes
- Rotator to hold 1.5ml tubes or a Shaking Incubator
- UV light box

TIME REQUIRED

- 2-3 hours

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OBJECTIVES

- Teaches immunoprecipitation technique
- Hands-on immunoprecipitation activity to isolate specific protein with an antibody.

BACKGROUND

Immunoprecipitation is a popular technique used in many scientific fields that uses the high specificity of antibodies to isolate functional proteins. The immunoprecipitation procedure removes soluble peptides and proteins from a solution that react specifically with an antibody and they can then be subsequently analyzed.

The name, immunoprecipitation, is a misnomer as the removal of the antigen (protein or peptide) is not dependent on the formation of an insoluble antibody:antigen complex. In fact the antibody:antigen complex is removed with the aid of an antibody binding protein coupled to an insoluble resin, such as agarose beads.

The bacterial proteins, protein A and protein G are the most commonly used antibody binding proteins. Protein A is a 42kDa protein that is a normal constituent of *S. aureus* bacteria cell walls. Protein A has 4 binding sites for antibodies, of which only can be used at any one time, which bind to the constant region of the antibody. Protein G is a 30-35kDa protein that is found in the cell wall of β-hemolytic streptococci of the C and G strains. Both protein A and G have been cloned and modified to eliminate other protein binding domains, such as albumin. Protein A and G bind the constant domain of antibodies, but there specificity differ between species and subclass. The table below highlights the differences.

Species	Antibody Class	Protein A	Protein G	Species	Antibody Class	Protein A	Protein G
Mouse	Total IgG	++++	++++	Rat	Total IgG	+	++
	IgG_1	+	+++		IgG_1	-	+
	IgG_{2a}	++++	++++		IgG_{2a}	-	++++
	IgG_{2b}	++++	++++		IgG_{2b}	-	++
	IgG_3	+++	+++		IgG_{2c}	++	+++
Human	Total IgG	++++	++++	Hamster	Total IgG	++	++
	IgG_1	++++	++++	Guinea Pig	Total IgG	++++	++
	IgG_2	++++	++++	Rabbit	Total IgG	++++	+++
	IgG_3	+	++++	Horse	Total IgG	++	++++
	IgG_4	++++	++++	Cow	Total IgG	++	++++
Goat	Total IgG	+	++	Pig	Total IgG	+++	++
Chicken	Total IgG	-	-	Sheep	Total IgG	+	++

Table 1: Relative affinity of Protein A and Protein G for Immunoglobulins

The immunoprecipitation basically involves the addition of the antibody and agarose beads with immobilized protein A/G to a cell lysate or sample. After an incubation period the sample is centrifuged and the agarose beads pellet with the antibody:antigen complex and the remaining proteins and cellular material is removed. Non-specific proteins are washed away in phosphate buffered saline (PBS) that is supplemented with a mild detergent.

Immunoprecipitation has many uses, including isolation of specific proteins to determine their physical properties, monitoring protein processing and modification, by using radiolabels and pulse chase experiments. A popular use of immunoprecipitation is in the identification of novel proteins that interact and complex with a known protein. During the immunoprecipitation procedure associated proteins are isolated with its interacting antigen and these can be identified by electrophoresis and other downstream applications, such as mass spectroscopy.

Students will carry out a simple immunoprecipitation experiment to isolate a protein of interest. This kit is provided with a fluorescent-tagged protein to make visualization simple.

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TEACHER'S PRE EXPERIMENT SET UP

Wear heat protective gloves when making the agarose solution.

1. Before opening the Cellular Extracts, BE Antibody 1 and BE Antibody 3 vials, centrifuge the vials for 5 minutes to bring down all pellets to the bottom of the tubes.

2. Add 200µl PBS to each vial of the Cellular Extract, BE Antibody 1 and BE Antibody 3. Soak the pellets for 5 minutes with periodical vortexing to completely dissolve the pellets.

3. Chill the PBS on ice or at 4°C.

4. Aliquot reagents for each student group according to the next section.

NOTE*: Instruct students to keep all reagents cold (on ice or at 4°C).*

MATERIALS FOR EACH GROUP

Supply each group with the following components. Components shared by the whole class and should be kept on a communal table.
30µl Cellular Extract
30µl BE Antibody 1
30µl BE Antibody 3
1 vial Resin (Protein-A) (Shared with whole class)
3ml PBS
6 Centrifuge Tubes (1.5ml)

PROCEDURE

Always wear gloves and protective clothing throughout the whole experiment.

1. Label 3 centrifuge tubes as IP-1, IP-2 and IP-3 with your group name.

2. Vortex the Resin (Protein-A) tube briefly. Using a wide bore tip (cut the tip with a blade or a pair of scissors), pipette 15µl Resin (Protein-A) suspension to each labeled tube.

3. *Wash the Resin (Protein-A):* Pipette 0.1ml PBS to each tube. Centrifuge for 5 seconds at 1,000xg. Carefully remove and discard the supernatant ***without*** disturbing the agarose beads.

4. Set up three tubes as in the following table:

Tube	IP-1	IP-2	IP-3
PBS	280µl	290µl	280µl
Cellular Extract	10µl	10µl	10µl
BE Antibody 1	10µl	----	----
BE-Antibody 3	----	----	10µl

5. Close all tubes tightly. Put all tubes on a rotator. Incubate the tubes at 4°C for 60 minutes with gentle rotation.
   If your laboratory does not have a rotator, place on a shaker or incubate the tubes at 4°C and invert the tubes 3-4 times every 5-10 minutes.

6. Label 3 tubes for collecting the supernatants as "IP-1 Sup", "IP-2 Sup", and "IP-3 Sup".

7. Centrifuge all tubes 5 seconds at 1,000xg. Carefully transfer the supernatants to the labeled tubes ***without*** disturbing the agarose beads.

8. Add 300µl PBS to each tube. Vortex briefly or tap the tubes with fingers to suspend the agarose beads.

9. Centrifuge all tubes 5 seconds at 1,000xg. Carefully remove and discard the supernatant ***without*** disturbing the agarose beads.

10. Repeat above step 8 and step 9.

11. Observe the pellets and supernatants under UV light. For optimal visibility a UV transilluminator is recommended.

12. OPTIONAL: Proportional amounts of the samples can be prepared for protein electrophoresis and viewed on an SDS polyacrylamide gel. G-Biosciences Protein Electrophoresis Kit (BE-406) is recommended.

RESULTS, ANALYSIS & ASSESSMENT

1. Observe the pellets and supernatants under UV light. Which tube contains green fluorescence protein and why?

   IP-1 pellet, IP-2 and IP-3 supernatants contain strong green fluorescence. IP-1 supernatant contains weak or no green fluorescence. IP-2 and IP-3 pellets contain no green fluorescence.

   The antigen extract contains the fluorescence dye labeled protein. IP-1 contains a primary antibody reactive with the fluorescent tagged. The protein-A agarose binds to the antibody and antigen complex through the protein-A and antibody interaction. So the fluorescence protein is precipitated in the pellet. There is no antibody in IP-2, and the fluorescent tagged protein will not bind to the Protein-A agarose and therefore remain in the supernatant. The antibody in IP-3 binds to the Protein-A agarose but will not bind to the antigen, so the fluorescent tagged protein remains in the supernatant.