Immunoaffinity Chromatography

Teacher's Guidebook

(Cat. # BE-507)

MATERIALS INCLUDED

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

- 12 Column: Immunoaffinity Columns with Rubber Stoppers
- 1 vial IMC Protein Extract 1
- 1 vial IMC Protein Extract 2
- 1 bottle Immunoaffinity Buffer
- 1 bottle Immunoelution Buffer
- 1 vial Immunoneutralization Buffer
- 80 Centrifuge Tubes (1.5ml)

SPECIAL HANDLING INSTRUCTIONS

- Store Immunoaffinity Columns and Protein Extracts at 4°C.
- All other reagents can be stored at room temperature.

ADDITIONAL EQUIPMENT REQUIRED

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

TIME REQUIRED

- 1-2 hours

OBJECTIVES

- Teaches immunoaffinity chromatography
- Hands-on lab activity to purify antigen & antibody

BACKGROUND

Immunoaffinity chromatography is a popular technique used in many scientific fields that uses the high specificity of antigen and antibody interaction to isolate and purify functional proteins. The immunoaffinity chromatography procedure uses antibodies immobilized on a solid support over which biological samples are passed and the antigen, specific for the immobilized antibody, is captured. Non-specific proteins and peptides are washed away and the antigen is then eluted. Immunoaffinity chromatography can also be used to purify or enrich antibodies. In this case the specific antigen is coupled to the column and the sera containing the antibody of interest is passed over the column.

The antibody can be immobilized to a solid support by numerous techniques, including chemical coupling of the antibody or antigen to an activated solid support through amines or sulfhydryl residues. There are currently various commercial activated agaroses available on the market that use a multitude of different coupling chemistries.

Another technique uses a solid support coated with an antibody binding protein, such as Protein A or G, which captures and immobilizes the antibodies. The antibody is then covalent linked to the resin with the aid of a chemical crosslinker.

The bacterial proteins, protein A and protein G are the most commonly used antibody 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

Immunoaffinity chromatography has many uses, including isolation of specific proteins to determine their physical properties and capture protein complexes. A popular use of immunoaffinity chromatography is in the identification of novel proteins that interact and complex with a known protein. During the chromatography 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 immunoaffinity chromatography experiment to isolate a protein of interest. Each group examines two different samples to determine which sample has the protein of interest. This kit is provided with a fluorescent tagged protein to make visualization simple.

TEACHER'S PRE EXPERIMENT SET UP

Always wear gloves and protective clothing throughout the whole experiment.

1. Before opening the IMC Protein Extract vials, centrifuge the vials for 5 minutes to bring down all pellets to the bottom of the tubes.

2. Add 300µl Immunoaffinity Buffer to each IMC Protein Extract vial. Soak the pellet for 5 minutes with periodical vortexing to dissolve the pellet completely.

3. Label six tubes with "Protein Extract 1" and six tubes with "Protein Extract 2". Aliquot 50µl Protein Extract into the appropriate tube and supply each group with one of each protein extract tube.

4. Supply each group with two Immunoaffinity Columns.

5. The columns are centrifuged in 1.5ml centrifuge tubes. The caps may interfere with centrifugation; therefore remove the caps from two centrifuge tubes for each group with a razor blade. Supply each group with 2 capless 1.5ml centrifuge tubes.

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

MATERIALS FOR EACH GROUP

Supply each group with the following components.

- 2 Immunoaffinity Columns
- 50µl Protein Extract 1
- 50µl Protein Extract 2
- 3ml Immunoaffinity Buffer
- 0.5ml Immunoelution Buffer
- 0.1ml Immunoneutralization Buffer
- 8 Centrifuge Tubes (1.5ml)
- 2 Capless Centrifuge tubes
- 2 Rubber stoppers

PROCEDURE

Always wear gloves and protective clothing throughout the whole experiment.

1. Label the two Immunoaffinity Columns with P1 or P2 and your group number/ name.

The immunoaffinity column resin is the antibody coupled to agarose beads.

2. Remove the cap from the top of the column and keep in a safe place until required. Snap off the bottom tab. Place the columns in the capless 1.5ml tubes. Centrifuge for 5 seconds at 1,000xg to remove the storage buffer.

3. Remove the storage buffer from the 1.5ml tube and discard.

4. Wash the Immunoaffinity Resin: Place the columns back into the capless 1.5ml tubes. Pipette 0.3ml Immunoaffinity Buffer on to the resin. Allow the buffer to enter the resin and then centrifuge for 5 seconds at 1,000xg.

5. Remove the Immunoaffinity Buffer from the 1.5ml tube and discard.

6. Seal the bottom of the columns with the supplied rubber stoppers. Add the following reagents to the appropriate column:

Tube#	**P1**	**P2**
Immunoaffinity Buffer	250µl	250µl
Protein Extract 1	50µl	----
Protein Extract 2	----	50µl

7. Close the column with the supplied cap. Put all columns on a rotator or shaker. Incubate the tubes at room temperature for 30 minutes with gentle rotation or shaking.

8. Label the 1.5ml centrifuge tubes as P1-Sup, P1a, P1b, P1c and P2-Sup, P2a, P2b, P2c.

9. Remove the caps and rubber stoppers from the columns and return the columns to the capless 1.5ml tubes. Centrifuge the columns for 5 seconds at 1,000xg.

10. Carefully transfer the supernatant from the 1.5ml tubes to tube P1-Sup and P2-Sup accordingly. This is the flow through.

11. *Wash the resin*: Return the columns to the capless 1.5ml tubes and add 0.3ml Immunoaffinity Buffer to the columns and allow it to enter the resin. Centrifuge 5 seconds at 1,000xg.

12. Remove the wash buffer from the 1.5ml tube and discard. Repeat steps 11 and 12 twice.

13. Centrifuge the column and tube for 5 seconds at 1,000xg to remove any residual wash buffer.

14. Add 10µl Immunoneutralization Buffer to tubes P1a, P1b, P1c, P2a, P2b and P2c.

15. *Elute the sample*: Return the columns to the capless 1.5ml tubes. Add 50µl Immunoelution Buffer to each column. Centrifuge the columns for 5 seconds at 1,000xg. Transfer the elution to the P1a and P2a tubes. Close the tubes and tap 3-4 times to ensure complete neutralization.

16. Repeat above elution step 14 twice for the P1b and P2b, then P1c and P2c elutions.

17. Observe all fractions, supernatant and resin under UV light by pipetting the samples directly on to the UV transilluminator. Record your results in the table below. (Note-best visibility is obtained with the use UV transilluminator)
NOTE: *Fluorescence may be enhanced by viewing samples directly on the UV Transilluminator. The plastic tubes also fluoresce and masks the sample fluorescence.*

RESULTS, ANALYSIS & ASSESSMENT

1. Insert your observations about the green intensity of the samples in the table below:

	Protein Extract 1	**Protein Extract 2**
Flow through	*Strong to weak fluorescence*	*Strong fluorescence*
Elution 1	*Strong fluorescence*	*Weak or no fluorescence*
Elution 2	*Fluorescence (weaker than elution 1)*	*Weak or no fluorescence*
Elution 3	*Fluorescence (weaker than elution 2)*	*Weak or no fluorescence*

2. Explain your results:

Protein extract 1 contains the fluorescent-tagged antigen that is specifically recognized by the bound antibody. This is shown by limited or no fluorescence in the supernatant of flow through and the increase in fluorescence during elution.

Protein extract 2 contains a fluorescent protein; however this protein does not have any antigenic sites recognized by the immobilized antibody. As a result the majority of the fluorescence remains in the flow through or supernatant.

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Human	Total IgG	++++	++++	Hamster	Total IgG	++	++
	IgG_1	++++	++++	Guinea Pig	Total IgG	++++	++
	IgG_2	++++	++++	Rabbit	Total IgG	++++	+++
	IgG_3	+	++++	Horse	Total IgG	++	++++
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Flow through		
Elution 1		
Elution 2		
Elution 3		

2. Explain your results:

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