HiPer® IgG Purification Teaching Kit

Product Code: HTI022

Number of experiments that can be performed: 5

Duration of Experiment

Storage Instructions

- The kit is stable for 6 months from the date of receipt
- Store 5X Sample Loading Buffer and Protein Ladder at -20°C
- Store Protein A Agarose Spin Columns, 10X Binding Buffer, Elution Buffer, Neutralizing Buffer, Serum,30% Acrylamide-Bisacrylamide Solution, 2.5X Tris SDS Buffer(pH 8.8), 5X Tris SDS Buffer (pH 6.8), 5X Tris-Glycine-SDS Gel Running Buffer, TEMED at 2-8°C
- Other kit contents can be stored at room temperature (15-25°C)

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Index

Aim:

To learn the technique of IgG purification based upon the affinity of IgG to immobilized Protein A agarose.

Introduction:

The HiPer®IgG Purification Teaching Kit is based on the affinity of IgG to immobilized Protein A agarose and is used for purification of polyclonal antibodies. When a suspension containing mixture of substances along with IgG are loaded on the column, the IgG binds to Protein A agarose matrix and is eluted by reducing the pH.The purification can be checked by electrophoresing the eluted fraction on SDS-PAGE.

Principle:

Affinity chromatography is a chromatographic process through which proteins are separated on the basis of their reversible interaction with a specific ligand coupled to a chromatographic matrix. The bound protein of interest is eluted by a solution containing free ligand. Affinity chromatography is a very effective molecular technique for purification of protein on the basis of its biological function. Through this chromatography the desired protein is isolated from a mixed solution depending upon the protein's specific binding affinity to ligands mounted in a gel matrix through which the protein mixture is allowed to run. In immunoaffinity chromatography procedure antibodies are immobilized on a matrix through which biological samples are passed and the antigen which is specific for the immobilized antibody is captured. For this reason this procedure is used to purify or enrich antibodies.

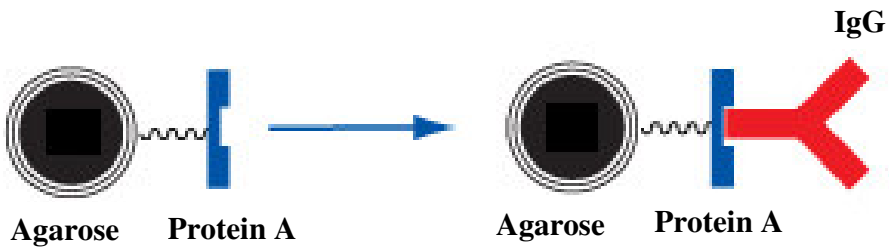


Fig 1: Schematic representation showing the binding of IgG to Protein A

Protein A is a cell wall component of *Staphylococcus aureus*. It consists of a single polypeptide chain of 42 kDa which specifically binds to the Fc region of IgGs.The binding site is located on the Fc region of immunoglobulin. Protein A is covalently coupled to agarose to prepare an affinity matrix for isolating IgGs from various species. As it binds specifically to the Fc region of IgG molecules it is used for the purification of IgG fractions from crude serum, ascites fluid, tissue culture supernatant etc.

In this kit the method of protein purification is demonstrated where IgG is purified from serum sample based upon its binding to Protein A agarose matrixfollowed by analysis on SDS-PAGE.

Kit Contents: This kit can be used to study the method of IgG purification from serum sample.

Table 1: Enlists the materials provided in this kit with their quantity and recommended storage

Sr. No.	Product Code	Materials Provided	Quantity	Storage
			5 expts	
1	ML037	Acrylamide/Bisacrylamide Solution 30% (29:1)	40 ml	2-8°C
2	ML039	2.5X Tris-SDS Buffer (pH 8.8)	35 ml	2-8°C
3	ML040	5X Tris-SDS Buffer (pH 6.8)	10 ml	2-8°C
4	MBT092	Prestained Protein Ladder	0.030 ml	-20°C
5	ML041	5X Tris-Glycine-SDS Gel Running Buffer	200 ml	2-8°C
6	TKC037	5X Sample Loading Buffer	0.1ml	-20°C
7	TKC393	Serum	4.0 ml	2-8°C
8	PW1139	Collection tubes, 2.0 ml	40 No	RT
9	ML116	Phosphate Buffered Saline	0.08 ml	R T
10	DS0064	Staining solution	125 ml	R T
11	DS0065	Destaining solution	125 ml	R T
12	MB003	Ammonium persulphate (APS)	0.15 g	R T
13	MB026	Tetramethylethylenediamine (TEMED)	0.15 ml	2-8°C
14	MB002	Agarose	0.3 g	R T
15	DBCA09	Protein A Agarose Spin Column	5 Nos	2-8°C
16	DS0095	10X Binding Buffer	20 ml	2-8°C
17	DS0096	Elution Buffer	10 ml	2-8°C
18	DS0097	Neutralizing Buffer	1 ml	2-8°C

Materials Required But Not Provided:

Glass wares: Measuring cylinder, Beaker
Reagents: Distilled water
Other requirements: Protein Electrophoresis apparatus, Micropipettes, Tips, 37°C Shaker, Centrifuge, Gel rocker, Crushed ice, Microwave/Burner/Hotplate

Storage:

HiPer®IgG Purification Teaching Kit is stable for 6 months from the date of receipt without showing any reduction in performance. On receipt, store Protein Ladder, 5X Sample Loading Buffer at -20°C. 30% Acrylamide-Bisacrylamide Solution, 2.5X Tris-SDS Buffer (pH 8.8), 5X Tris-SDS Buffer (pH 6.8), 5X Tris-Glycine-SDS Gel Running Buffer, TEMED, Protein A Agarose Spin Column, 10X Binding Buffer, Elution Buffer, Neutralizing Buffer and Serumshould be stored at 2-8°C. Other kit contents can be stored at room temperature (15-25°C).

Important Instructions:

1. Read the entire procedure carefully before starting the experiment.
2. **Preparation of 1X Binding Buffer:** To prepare 10 ml of 1X Binding Buffer, take 1 ml of 10X Binding Buffer and add 9 ml of Molecular Biology Grade water* before use.
3. **Preparation of 10% APS Solution:** Before starting the experiment, dissolve 0.15 g of Ammonium persulphate in distilled water to make a final volume of 1.5 ml. Store at 2-8ºC. Use within 3 months.
4. **Preparation of 1X Tris-Glycine-SDS Gel Running Buffer:** To prepare 500 ml of 1X Tris-Glycine-SDS Gel Running buffer, take 100 ml of 5X Tris-Glycine-SDS Gel Running Buffer and add 400 ml sterile distilled water*. Store at 2-8ºC. Mix well before use. The 1X Tris-Glycine-SDS Gel Running Buffer can be reused 4-5 times.
5. Clean the entire apparatus with detergent and then with distilled water*. Ensure that the plates are free of detergent.

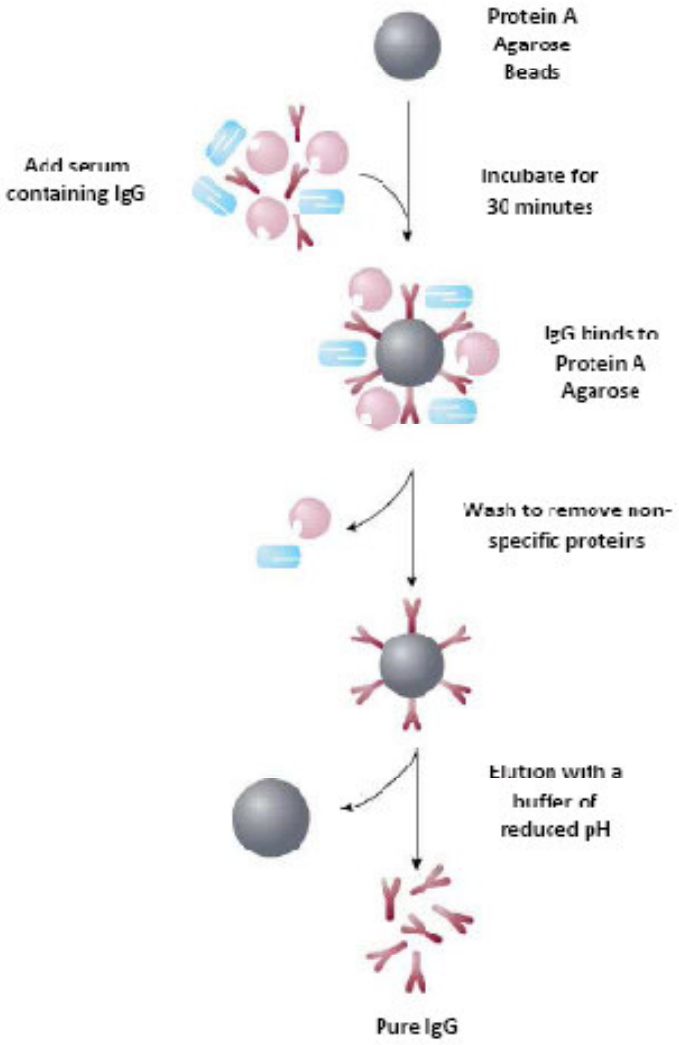
* Molecular biology grade water is recommended (Product code: ML024).

Procedure:

1. Equilibrate Protein A agarose spin column and buffers to room temperature. Remove the lower cap of the column and place it in the 2 ml collection tube. Centrifuge at 500 X g for 1 minute to allow elimination of the preservative.
2. Equilibrate the spin column with 0.4 ml of Binding Buffer and mix manually. Centrifuge at 500 X g for 1 minute and discard the flow through. Repeat this step once. Do not let the resin bed dry.
3. Close spin column outlet with cap.Add 0.5 ml of serum (containing the IgG to be purified) through the top of the spin column. Close the lid and keep sample and resin in contact for at least 30 minutes before removing the bottom cap. Mix manually inverting the spin column. Centrifuge at 500 X g for 1 minute and collect and label the flow through.
4. Transferthe spin column to a new collection tube. Add0.4 ml of Binding Buffer through the top to eliminate all the proteins that have not been retained in the column. Mix manually inverting the spin column. Centrifuge at 500 X g for 1 minute and discard the flow through. Repeat the washing step once for a total of two washes.
   Note: Collect all the washes and label them.
5. Transferthe spin column to a new collection tube and close the column outlet with cap.Add 0.4 ml of Elution Buffer and close the lid. Mix thoroughly for 10 minutes before removing the bottom cap. Centrifuge at 500 X g for 1 minute, collect the eluate and label it. You can repeat the elution step once for a total of two individual eluates.
6. Each 0.4 ml of eluted fraction can be neutralized by the addition of 40 µl of Neutralization Buffer. Assay protein concentration by measuring the absorbance at 280 nm and combine the fractions with highest absorbance.
7. Take 6 tubes and label them as “Crude extract”, “Flow-through”, “First Wash”, “Second Wash”, “Eluate 1” and “Eluate 2”. Take 20 µl of each sample from the respective tubes and add 5 µl of 5X Sample Loading Buffer to it. Boil the tubes containing Protein Samples at 100ºC in a boiling water bath.

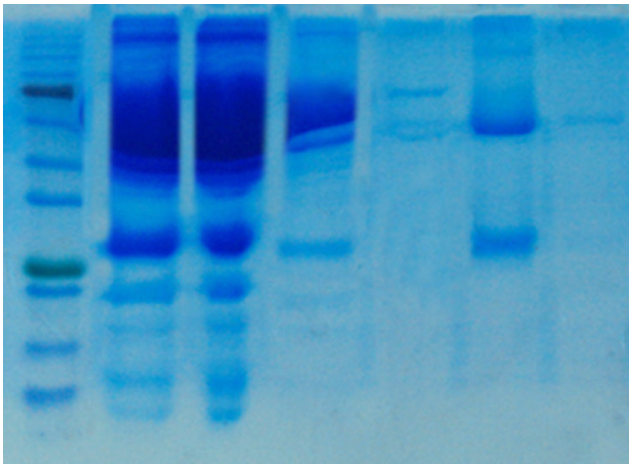
8. **SDS-PAGE:** Load 20 µl of the samples immediately after the heat treatment in the wells created by the comb in the Stacking Gel along with 5 µl of Prestained Protein Ladder. For the preparation ofgel, Staining and Destaining procedures, refer pages 8 -10.

Flowchart:



Observation and Result:

Lanes: 1 2 3 4 5 6 7



Lane 1: Protein Ladder
Lane 2: Crude extract
Lane 3: Flow-through
Lane 4: First wash
Lane 5:Second wash
Lane 6: Eluate 1
Lane 7: Eluate 2

Interpretation: The extent of IgG purification is determined by number of bands seen in "Eluate" on the gel. The "Crude extract" is impure and contains severalproteins and hence, many bands are seen. In "Eluate" two prominent bands are visible: one corresponds to IgG heavy chain (7olymer. 50 kDa) and the other to IgG light chain (approx. 25 kDa).

Troubleshooting Guide:

Sr. No.	Problem	Cause	Solution
1.	The purified IgG is degraded	It can be unstable in elution conditions.	Follow usage instructions for neutralizing the fractions of the eluted IgG.
2.	Column flows very slowly	There are air bubbles in sample or buffers that are blocking flow through pores.	Make sure that the sample and buffers don't contain foams.
3.	Bands are very faint	Loss of IgG during the experiment.	Make sure that there is no loss while collecting the "Eluate".
4.	High Background or many nonspecific bands on gel	Washing steps are not done properly.	Do the washing steps as mentioned in the brochure.

SDS-PAGE

Principle: To separate protein molecules of different shapes and sizes, they first have to be denatured so that the proteins no longer have any secondary, tertiary or quaternary structure. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone. SDS denatures all the proteins to their respective primary structure. SDS confers a negative charge to the polypeptide in proportion to its length.

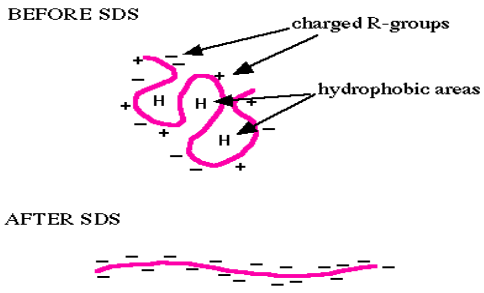


Fig2: Denaturation of protein by SDS

SDS treatment has two important features:

1. All proteins retain only their primary structure.
2. All proteins have a large amount of negative charge.

Polyacrylamide is the best gel recommended to provide such an environment. Polyacrylamide is a synthetic gel which is thermo-stable, transparent, strong and relatively chemically inert and can be prepared with a wide range of average pore sizes. It can withstand high voltage gradients and is feasible to various staining and destaining procedures and can be digested to extract separated fractions or dried for autoradiography and permanent recording. A polymer gel is formed of acrylamide monomers and the proteins are run through this gel by electrophoresis, hence this entire process is called Polyacrylamide Gel Electrophoresis (PAGE).

There are two layers of gel, namely Stacking or spacer gel, and Separating or resolving gel.
Stacking gel –The stacking gel contains large pores of polyacrylamide gel (generally 5%). This gel is prepared with Tris buffer of pH 6.8 which is of about 2 pH units lower than that of the electrophoresis buffer. This gel is formed over the separating gel.

Separating Gel –The separating gel contains small pores of polyacrylamide gel (5-30%). The Tris buffer used is of pH 8.8. In this gel, macro molecules separate according to their size.

The materials used in SDS-PAGE and their roles are as follows:

1. **Tris:** It is used as a buffer because it is an innocuous substance to most proteins. Its pKa is 8.3 at 20°C and reasonably a very satisfactory buffer in the pH range 7.0 – 9.0.
2. **Acrylamide:** This is a white crystalline powder and while dissolving in water, autopolymerisation takes place. It is a slow spontaneous process by which acrylamide molecules join together by head on tail fashion. But in presence of free radicals generating system, acrylamide monomers areactivated into a

free-radical state. These activated monomers polymerise quickly and form long chain of polymers. This kind of reaction is known as Vinyladdition polymerisation.

3. **Bisacrylamide (N,N'-Methylenebisacrylamide):** Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels. Chemically it has two acrylamide molecules coupled head to head fashion at their non-reactive ends.
4. **Sodium Dodecyl Sulphate (SDS):** SDS is the most common denaturing agent used to denature native proteins to individual polypeptides. When a protein mixture is heated to 100°C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus, polypeptides after treatment become a rod like structure possessing a uniform charge density that is same net negative charge per unit length.
5. **Ammonium Persulphate (APS):** APS is an initiator for gel formation.
6. **N, N, N', N'-tetramethylethylenediamine** (TEMED): Chemical 9olymerization of acrylamide gel is used for SDS-PAGE. It can be initiated by ammonium persulfate and the quaternary amine, N,N,N',N'-tetramethylethylenediamine (TEMED).

Procedure:

1. Assemble the electrophoresis unit such that the glass plates are clamped to the unit along with the spacers placed in-between them at two vertical edges.

2. Prepare 1% agarose (0.05g in 5ml of distilled water). Boil to dissolve the agarose and pour a thin horizontal layer at the lower edge of the plates to seal the assembly. Let it solidify by allowing it to cool down for 5-10 minutes

3. **Preparation of 12% Separating Gel-** To prepare separating gel, add the components as follows:

   30% Acrylamide-bisacrylamide Solution - 6 ml
   Distilled water* - 3 ml
   2.5X Tris-SDS Buffer (pH 8.8) - 6 ml
   10% APS Solution - 125 µl
   TEMED - 7.5 µl

   Pour the gel in-between the plates and allow it to solidify for an hour. Immediately after the gel is poured, add distilled water to level the gel.

4. After an hour pour off the water by inverting the casting assembly.

5. **Preparation of 5% Stacking Gel-** To prepare stacking gel, add the components as follows:
   30% Acrylamide-bisacrylamide Solution - 1.3 ml
   Distilled water* - 5.1 ml
   5X Tris-SDS Buffer (pH 6.8) - 1.6 ml
   10% APS Solution - 75 µl
   TEMED - 15 µl

After addition of TEMED gently mix all the components by swirling the beaker. Pour the stacking gel on top of the separating gel and immediately place the comb avoiding air bubbles. Allow it to solidify for 30 minutes.

Note: Acrylamide is a potential neurotoxin and should be treated with great care. Always wear an face mask and use gloves.

6. Pour 1X Tris-Glycine-SDS Gel Running Buffer in the unit such that the buffer connects the two electrodes, and hence completes the flow of current. Remove the comb from the Stacking Gel carefully.

7. Load 20 µl of the samples and 5 µl of Prestained Protein Ladder immediately after the heat treatment in the wells created by the comb in the Stacking Gel.

8. Connect the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode. Electrophorese at 100 volts and 10 mA until dye front reaches 0.5 cm above the sealing gel.

9. Carefully remove the gel from in-between the plates using spatula into the plastic tray containing distilled water. Wash the gel for 1 minute. Discard the water & proceed for staining destaining procedure.

* Molecular biology grade water is recommended (Product code: ML024).

Staining and Destaining of Gel:

1. After removing water, add 50 ml of Staining Solution in the tray containing gel, till the bands are visible. Sometimes the gel may have to be kept overnight in the staining solution for visualization of the bands.

2. Remove the gel from Staining Solution. The Staining Solution can be re-used 2-3 times.

3. Wash the gel by rinsing with distilled water till a considerable amount of stain leaches out from the gel. Keep changing the distilled water for 3-4 times.

4. Add 50 ml of Destaining Solution to the gel. Destaining should be carried out with constant moderate shaking.

5. Continue destaining till clear, distinct bands are observed.

6. Remove the gel from Destaining Solution. The Destaining Solution can be re-used 2-3 times.

Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com