HiPer® Immunoprecipitation Teaching Kit

Product Code: HTI016

Number of experiments that can be performed: 5

Duration of Experiment

Storage Instructions

- The kit is stable for 12 months from the date of manufacture
- Store 5X Sample Loading Buffer and Protein Marker at -20°C
- Store 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, Antigen, Antibody and Protein A Sepharose Beads at 2-8°C
- Other kit contents can be stored at room temperature (15-25°C)

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Aim:

To learn the technique of immunoprecipitation which involves the precipitation of the antigen-antibody complex by Protein A beads

Introduction:

Immunoprecipitation (IP) is a widely used procedure in immunology where a protein or antigen is precipitated out of a solution using an antibody that specifically binds to that antigen. This precipitation is done by an immunoglobulin binding protein, such as protein A or protein G, immobilized to a solid support or bead eg. agarose or sepharose. During this procedure the protein A or G coupled agarose beads bind to the antibody-antigen complex and the complex is precipitated and removed from the solution by centrifugation of the beads. Finally, the protein complex is eluted out from the beads and verified by SDS-PAGE (SDS-Polyacrylamide gel electrophoresis) and immunoblotting. Protein A is highly stable surface receptor with molecular weight of 45 KDa. It can be produced by S. aureus or by recombinant DNA technology.

Principle:

Immunoprecipitation is a routinely used procedure by which peptides or proteins which specifically react with an antibody are removed from a solution. The name of the technique is a misnomer as the interaction of the peptide or protein with the antibody does not cause precipitation and also the removal of the antigen from solution does not depend upon the formation of an insoluble antibody-antigen complex. The precipitation is caused by an immunoglobulin binding protein, protein A or protein G, immobilized to a solid support or bead like agarose or sepharose. The protein A or G binds to the antibody-antigen complex and the complex is precipitated and removed from the solution by spinning down the beads. Beaded agarose or agarose resin is a highly porous material and has a very high antibody binding capacity of all support materials available for immunoprecipitation. Furthermore, this resin is durable as it can withstand centrifugation up to 5000 x g and temperatures up to 120°C without significant loss of structure. The resin has varying shapes and sizes and can be modified for coupling of an appropriate ligand, Protein A or Protein G which are immunoglobulin (Ig)-binding proteins. Both protein A and G show high specificity for the heavy chains on the Fc region of antibodies and effectively orients the immobilized antibodies with antigen-binding sites facing outward. Protein A or G are attached to a beaded support and used as affinity ligand for binding of antibodies during immunoprecipitation procedure. The basic principle of immunoprecipitation reaction is shown in Fig 1.

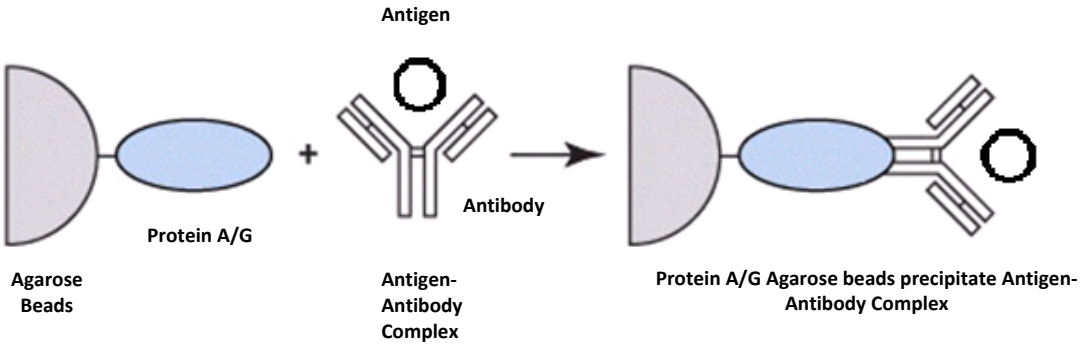


Fig 1: Schematic representation of the principle of immunoprecipitation

The antigen-antibody complex is eluted out from the Protein A/G coupled agarose beads by heating and can be analyzed through SDS-PAGE and immunoblotting.

Immunoprecipitation is widely used to study protein-protein interactions, post-translational modifications, expression level of a protein of interest, biochemical characteristics etc. Moreover, this method facilitates the

detection of rare proteins which otherwise would be very difficult to detect as they can be concentrated up to 10,000-fold by immunoprecipitation.

In this kit the method of immunoprecipitation is demonstrated where an antigen is incubated with its specific antibody and the antigen-antibody complex is precipitated by Protein A sepharose beads followed by analysis on SDS-PAGE.

Kit Contents:

This kit can be used to study the immunoprecipitation method.

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

Sr. No.	Product Code	Materials Provided	Quantity 5 expts	Storage
1	ML037	Acrylamide-Bisacrylamide Solution 30% (29:1)	44 ml	2-8°C
2	ML039	2.5X Tris-SDS Buffer (pH 8.8)	36 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	0.085 ml	-20°C
7	DS0064	Staining solution	125 ml	RT
8	DS0065	Destaining solution	125 ml	RT
9	MB003	Ammonium persulphate (APS)	0.15 g	RT
10	MB026	Tetramethylethylenediamine (TEMED)	0.2 ml	2-8°C
11	MB002	Agarose	0.3 g	RT
12	TKC371	Protein A Sepharose Beads	0.6 ml	2-8°C
13	TKC372	Antigen	0.6 ml	2-8°C
14	TKC373	Antibody	0.06 ml	2-8°C
15	TKC081	10X Assay Buffer	1 ml	2-8°C
16	TKC374	10X Wash Buffer	3 ml	2-8°C
17	PW146	Micro Centrifuge Tube-B (1.5 ml)	10 Nos.	RT

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® Immunoprecipitation Teaching Kit is stable for 12 months from the date of manufacture without showing any reduction in performance. On receipt, store Prestained 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 Sepharose Beads, Antigen, Antibody, 10X Assay and Wash Buffers should 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 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 2-8°C. Use within 3 months.
3. **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 2-8°C. Mix well before use. The 1X Tris-Glycine-SDS Gel Running Buffer can be reused 4-5 times.
4. Clean the entire apparatus with detergent and then with distilled water*. Ensure that the plates are free of detergent.
5. **Preparation of 1X Assay Buffer: (1 ml):** Dilute 0.1 ml of 10X Assay Buffer with 0.9 ml of Autoclaved MilliQ.
6. **Preparation of 1X Wash Buffer: (1 ml):** Dilute 0.1 ml of 10X Wash Buffer with 0.9 ml of Autoclaved MilliQ.

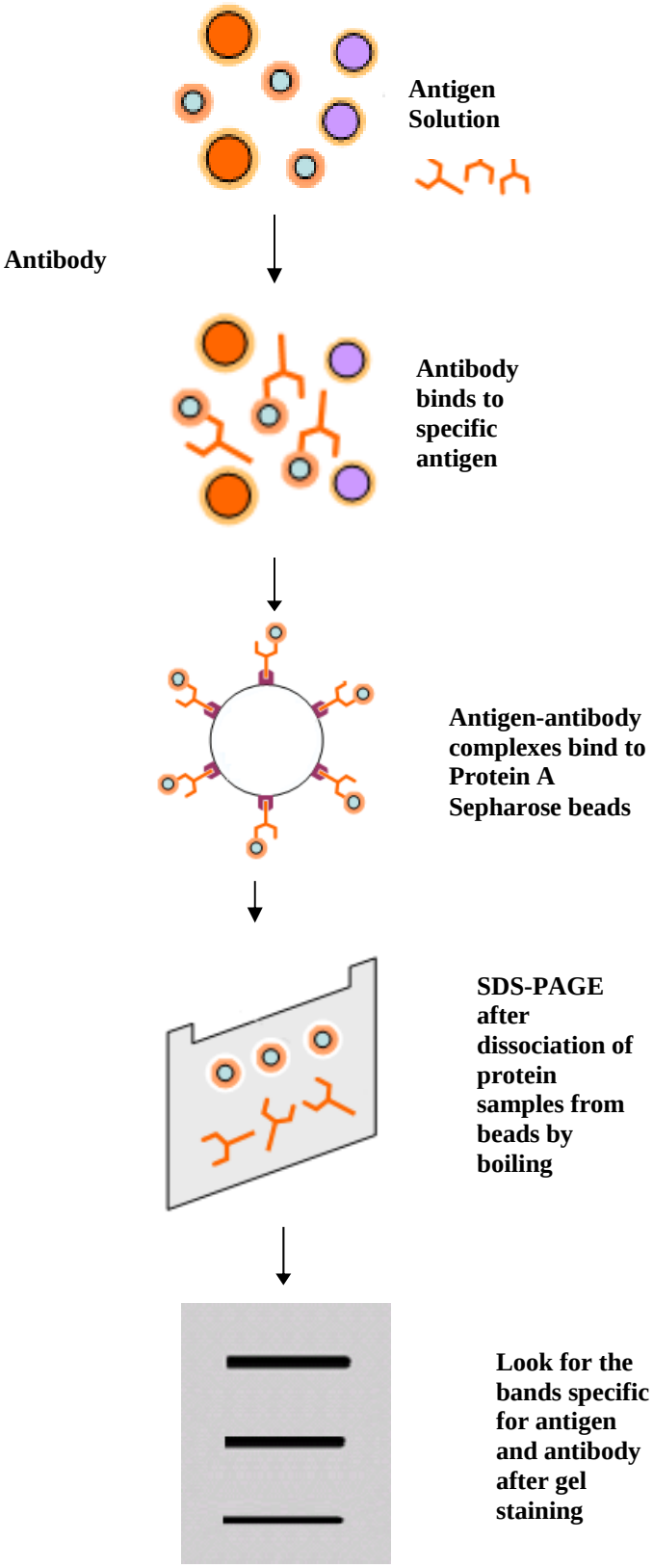
* Molecular biology grade water is recommended (Product code: ML024).

Procedure:

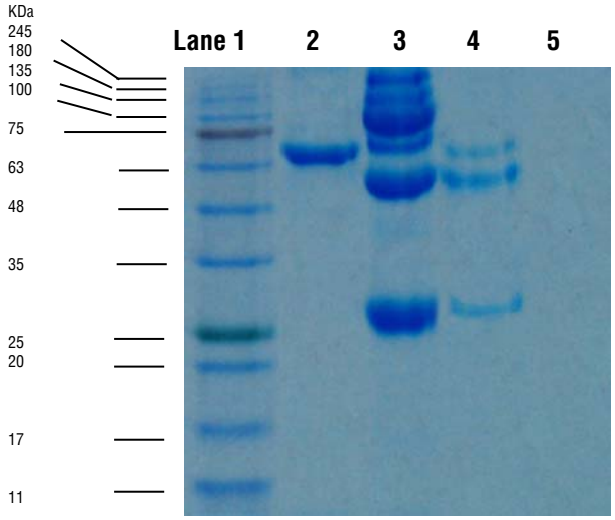
1. Take two Micro centrifuge tubes (1.5 ml), label them as Sample 1 and Sample 2 and add 450 µl of 1X Assay buffer to each tube.
2. Add 50 µl of Antigen to both the tubes. Mix gently by inverting.
3. Add 10 µl of Antibody to the tube labeled as Sample 1 and mix well by inverting the tubes.
4. Keep both the tubes at room temperature for an hour on a gel rocker.
5. After the incubation add 50 µl of protein A Sepharose beads to both the tubes.

   NOTE: Make sure to mix Protein A Sepharose beads by gently inverting the tube. While adding beads always use cut tips.
6. Keep both the tubes at room temperature for an hour on a gel rocker.
7. After the incubation centrifuge both the tubes for 5 minutes at 3000 rpm (1000 g) at room temperature. Discard the supernatant carefully with a pipette.
8. Wash the pelleted beads with 0.5 ml of 1X Wash Buffer by first gentle mixing of the beads with the buffer and then by centrifuging the tubes at 3000 rpm for 5 minutes.
9. Repeat the above step twice.
10. Finally, add 40 µl of 1X Assay Buffer and 10 µl of 5X Sample loading buffer to the pelleted beads.
11. Boil both the tubes for 5 minutes and quick chill the samples by placing them immediately on ice.
12. Centrifuge the tubes at high speed (~ 10000 rpm) for 3 minutes at room temperature and take out the supernatants in fresh tubes.
13. For antigen and Antibody sample preparation, add 1µl of each in 19 µl of 1X Assay buffer and then add 5 µl of 5X Sample Loading Buffer. Load 5 µl of Prestained Protein Ladder, 20 µl each of the supernatants, antigen and antibody onto SDS-PAGE. For the preparation of gel, Staining and Destaining procedures, refer pages 10- 12.

Flowchart:


Antigen Solution
Antibody
Antibody binds to specific antigen
Antigen-antibody complexes bind to Protein A Sepharose beads
SDS-PAGE after dissociation of protein samples from beads by boiling
Look for the bands specific for antigen and antibody after gel staining

Observation and Results



Lane 1: Protein Marker
Lane 2: Antigen
Lane 3: Antibody
Lane 4: Sample 1 (Treated with antibody)
Lane 5: Sample 2 (Not treated with antibody)

Interpretation:

Lane 2 has one band at ~66 kD which is that of the antigen. Lane 3 has two bands, which indicate the heavy chain and light chain of the antibody. Lane 4 has three bands corresponding to the antigen-antibody complex that is immunoprecipitated by Protein A sepharose beads. These bands are absent in lane 5 as sample 2 is not treated with antibody and as a result Protein A beads don't have IgG molecules to capture.

Troubleshooting Guide:

Sr. No.	Problem	Possible Cause	Solution
1	After centrifugation, the quantity of pellet is very less	Exact amounts of beads are not added	Mix the protein A Sepharose slurry properly with cut tips
2	Bands are very faint	Loss of reaction mixture	While washing, make sure that the beads are not taken out with Sepharose beads
3	Bands cannot be seen properly on the gel after destaining	Insufficient amount of antibody used or the incubation time is not adequate.	Follow the procedure properly to avoid these problems.
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.

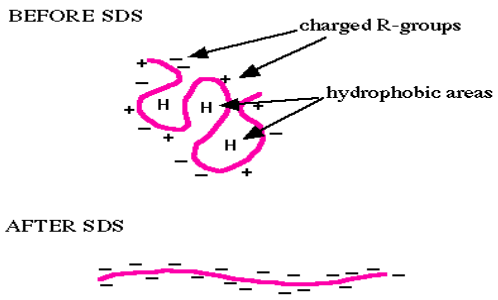


Fig2: Denaturation of protein by SDS

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 are activated into a free-radical state. These activated monomers polymerise quickly and form long chain of polymers. This kind of reaction is known as Vinyl addition 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 polymerisation 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	- 18 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	- 10 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 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

Storage temperature

Do not use if package is damaged

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