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Immunoprecipitation Starter Pack

Instructions for Use

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1 Introduction

Immunoprecipitation is a highly specific and effective technique for analytical separations of target antigens from crude cell lysates. By combining immunoprecipitation with other techniques, such as SDS-PAGE and immunoblotting, it can be used to detect and quantify antigens, determine relative molecular weights, monitor protein turnover and post-translational modifications, and check for enzyme activity.

The pack consists of nProtein A Sepharose™ 4 Fast Flow and Protein G Sepharose 4 Fast Flow, which are affinity resins for the efficient and rapid isolation of antibodies from various crude cell extracts. By using their high specificity for IgGs from a wide range of mammalian species, nProtein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow offer effective and rapid removal of immune complexes formed between an antigen and its specific antibody in the immunoprecipitation reaction.

2 Resins properties

Native protein A is produced by fermenting a selected strain of *Staphylococcus aureus*. The recombinant protein G is produced in *Escherichia coli*. The proteins are immobilized to Sepharose 4 Fast Flow by the CNBr method.

Sepharose 4 Fast Flow is a stable, highly crosslinked agarose matrix providing minimal non-specific adsorption.

The resulting resins are characterized by high binding capacities and low ligand leakage under a wide range of pH and buffer conditions.

3 Specificity

Protein A and protein G have different binding selectivities depending on the origin of the IgG. Use the following table to select between nProtein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow according to the source and sub-type of your specific antibody.

Table 3.1: Relative binding strength of polyclonal IgG from various species to protein A and protein G measured by competitive ELISA.

Species	Subclass	Protein A[1]	Protein G
Human	IgA	variable	-
	IgD	-	-
	IgE	-	-
	IgG_1	++++	++++
	IgG_2	++++	++++
	IgG_3	-	++++
	IgG_4	++++	++++
	IgM	variable	-
Avian egg yolk	IgY	-	-
Cow		++	++++
Dog		++	+
Goat		-	++
Guinea pig	IgG_1	++++	++
Hamster		+	++
Horse		++	++++
Koala		-	+
Llama		-	+
Monkey (rhesus)		++++	++++
Mouse[2]	IgG_1	+	++++
	IgG_{2a}	++++	++++
	IgG_{2b}	+++	+++
	IgG_3	++	+++

Species	Subclass	Protein A[1]	Protein G
	IgM	variable	-
Pig		+++	+++
Rabbit	No distinction	++++	+++
Rat[3]	IgG_1	-	+
	IgG_{2a}	-	++++
	IgG_{2b}	-	++
	IgG_3	+	++
Sheep		+/-	++

[1] ++++ = strong binding; ++ = medium binding; - = weak or no binding.
[2] IgG_1 from mouse binds more strongly to protein G than to protein A.
[3] Note that IgG from rat binds to protein G coupled to Sepharose 4 Fast Flow.

4 Getting started

To obtain satisfactory results using immunoprecipitation, all procedures involved must be empirically optimized. For example, selecting cell lysis conditions is very critical and has to be optimized with regard to cell type and how the antigen is to be used. Whereas cells without cell walls (e.g., animal cells) are easily disrupted by treatment with mild detergent, other cells may need some type of mechanical shearing such as sonication or homogenization.

The parameters listed below (lysis buffers, incubation times, volumes, and concentrations) should therefore be regarded as guidelines for initial experiments.

Preparing the resins

nProtein A Sepharose 4 Fast Flow and Protein G Sepharose 4 Fast Flow are supplied preswollen in 20% ethanol. Wash the resin three times with lysis buffer and/or elution buffer. Centrifuge at 12 000 × g for 20 seconds between the washes and discard the supernatant. Prepare a 50% slurry by mixing equal volumes of resin and lysis buffer. Store at 4°C and mix well before use.

Cell lysis

Step	Action
1	

Adherent cells	Cells in suspension
• Remove all culture medium and wash twice with ice-cold PBS. • Discard the supernatants and drain well.	• Collect cells by centrifugation at 1000 × g for 5 minutes and discard the culture medium supernatant. • Resuspend the pellet in ice-cold PBS, centrifuge and discard the supernatant. • Repeat the wash once.

Step	Action
2	

Adherent cells	Cells in suspension
• Place the tissue culture dish on ice. • Add ice-cold lysis buffer[1] to a concentration of 10^6 to 10^7 cells/mL (1 mL to a cell culture plate, Ø 10 cm). • Incubate on ice for 10 to 15 minutes with occasional rocking.	• Suspend the washed pellet in ice-cold lysis buffer[1] at a concentration of 10^6 to 10^7 cells/mL (approximately 10 cell volumes lysis buffer). • Incubate on ice for 10 to 15 minutes with gentle mixing.

[1] See section *Chapter 5 Buffers and solutions, on page 11* for help when selecting lysis buffer.

3	Transfer the cells to a suitable homogenization tube.
4	Further disrupt the cells by sonication, homogenization or passage through a 21 Gauge needle. Keep the cells on an ice bath to prevent the temperature from rising.
5	Centrifuge at 12 000 × g for 10 minutes at 4°C to remove particulate matter.
6	Transfer the lysate (the supernatant) to a fresh tube. Keep on ice.

Pre-clearing (optional)

Antibodies present in the cell lysate may also bind to the resin and thus interfere with subsequent analysis. In such a case pre-clearing may be desired.

Step	Action
1	Add 50 to 100 µL nProtein A Sepharose 4 Fast Flow or Protein G Sepharose 4 Fast Flow suspension (50% slurry) to 1 mL cell lysate in an Eppendorf™ tube. Higher volume (500 to 1000 µL) of resin might be necessary when working with serum samples due to the large amount of IgG present.
2	Gently mix for 1 hour at 4°C.
3	Centrifuge at 12 000 × g for 20 seconds. Save the supernatant.

Couple antigen to antibody

Step	Action
1	Aliquot samples (500 µL) in new Eppendorf tubes.
2	Add polyclonal serum (0.5 to 5 µL), hybridoma tissue culture supernatant (5 to 100 µL), ascites fluid (0.1 to 1 µL) or purified monoclonal or polyclonal antibodies (add the volume corresponding to 1 to 5 µg). For controls, use non-immune antibodies that are as close to the specific antibody as possible (for example, polyclonal serum should be compared to normal serum from the same species).
3	Gently mix for 1 hour at 4°C.

Precipitation of the immune complexes

Step	Action
1	Add 50 µL nProtein A Sepharose 4 Fast Flow or Protein G Sepharose 4 Fast Flow suspension (50% slurry). ***Note:*** *It is possible to work with volumes down to 10 µL.*
2	Gently mix for 1 hour at 4°C.
3	Centrifuge at 12 000 × g for 20 seconds and save the pellet.
4	Wash the pellet three times with 1 mL lysis buffer and once with wash buffer. Centrifuge at 12 000 × g for 20 seconds between each wash and discard the supernatants. Be very careful when removing the supernatants to avoid loss of the beads!

Dissociation and analysis

Step	Action
1	Suspend the final pellet in 30 µL sample buffer.
2	Heat to 95°C for 3 minutes.
3	Centrifuge at 12 000 × g for 20 seconds to remove the beads. Carefully remove the supernatant.
4	Add 1 µL 0.1% bromphenol blue.

Step	Action
5	Analyze the supernatant by SDS-PAGE, followed by protein staining and/or immunoblotting for detection. Radiolabeled antigens are detected by autoradiography.

5 Buffers and solutions

Lysis buffers

Cell lysis must be harsh enough to release the target antigen, but mild enough to maintain its immunoreactivity. Selecting lysing conditions is therefore very critical and has to be individually optimized.

Some commonly used lysis buffers are listed below. NP-40 (IGEPAL™ CA-630) and RIPA buffer release most soluble cytoplasmic or nuclear proteins without releasing chromosomal DNA and are a good choice for initial experiments. Some parameters that affect the extraction of an antigen include salt concentration (0 to 1 M), non-ionic detergents (0.1% to 2%), ionic detergents (0.01% to 0.5%) and pH (6 to 9). For more detailed information about optimization of lysis procedures, see *Harlow and Lane 1999*.

Name	Description	Stringency
Low salt	1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 1 mM PMSF	+
Mammalian Protein Extraction Buffer	Tris-based buffer, 10 mM NaCl, detergent mixture (NP-40, Tween™), pH 7.5	+
Yeast Protein Extraction Buffer	Tris-based buffer, 50 mM NaCl, detergent mixture (NP-40, Tween) pH 7.5	+
NP-40 (IGEPAL CA-630)	150 mM NaCl, 1% IGEPAL CA-630 50 mM Tris, pH 8. 0, 1 mM PMSF	++
RIPA	150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM PMSF	+++
High salt	500 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 1 mM PMSF	++++

Other buffers/solutions	
PBS	1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4
Wash buffer	50 mM Tris, pH 8
Sample buffer (reducing)	1% SDS, 100 mM DTT, 50 mM Tris, pH 7.5

6 Common problems and general tips

Choice of antibody	• Polyclonal serum contains antibodies against multiple epitopes of an antigen, which helps stabilize the antigen-antibody-nProtein A/ Protein G Sepharose 4 Fast Flow complexes, but which also constitutes a problem with regard to high background during analysis. • Monoclonal antibodies are more specific, which reduces background but sometimes means that less stable immune complexes are formed due to lower affinity. This can be overcome by using pools of different monoclonal antibodies.
Target antigen cannot be detected due to incomplete release during lysis.	• Try harsher lysis conditions (see *Harlow and Lane 1999* for detailed procedures).
High level of background proteins on SDS-PAGE Specific: polyclonal serum may contain antibodies that recognize other antigens.	• Purify the antibody by affinity chromatography. • Try a different antibody, or a different antibody lot. Lot variation does occur specially for polyclonal antibodies.

Non-specific: binding of proteins to nProtein A/ Protein G Sepharose 4 Fast Flow or/and the plastic tubes or presence of protein aggregates that coprecipitate with the immune complex.	• Precoat plastic tube with lysis buffer prior to addition of cell lysate. • Add saturating amount of competitive protein (i.e., BSA, gelatin, acetone powders). • Spin the lysate at 100 000 × g for 30 minutes to remove aggregated proteins prior to the addition of the antibody. • Spin the antibody at 100 000 × g for 30 minutes to remove particulate matter. • Spin the antigen-antibody complex at 10 000 × g for 10 minutes prior to addition of nProtein A/ Protein G Sepharose 4 Fast Flow to remove protein aggregates. • Try a different antibody. • Use more stringent washing conditions such as: 1 M sodium chloride, 1 M potassium thiocyanate, 0.5 M lithium chloride, 0.2% SDS or 1% Tween 20. Alternate between high and low salt wash buffer, or wash the beads with distilled water. Prolong washing times and/or increase the number of washes. • Titrate the optimal amounts of cell lysate, antibody and nProtein A Protein G Sepharose 4 Fast Flow.
Protein A or Protein G ligand leakage.	• Boiling in SDS buffer at 95°C for an extended time is a very harsh treatment and might in some instances cause leakage of protein ligand. Consider using alternative methods for dissociation of immune complexes from the resin, such as using buffer of 0.1 M glycine-HCl, pH 2.5 to 3.1. Prior to use, wash with elution buffer (such as 0.1 M glycine- HCl, pH 2.5 to 3.1), followed by lysis buffer to remove minute amounts of Protein A or Protein G ligands that leak from the resin.

General tips.

- Consistent sample preparation is vital to reduce variation and obtaining consistent results.
- Protein A Mag Sepharose or Protein G Mag Sepharose are other products that can be used for immunoprecipitation. They are Sepharose based magnetic beads with protein A or protein G ligand, and ideally suited for immunoprecipitation.

7 References

Harlow, E. and Lane, D. Using antibodies: laboratory manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York (1999)

Juan S. Bonifacino1, Esteban C. Dell'Angelica, Timothy A. Springer in Current Protocols in Molecular Biology, (Ausubel, F. M. et al. Eds.), John Wiley and Sons, New York, Unit 10.16 (2001)

Cytiva , Affinity Chromatography Handbook, Principles and Methods, Code No. 18102229.

Cytiva , Antibody Purification, Handbook, Code No. 18103746.

8 Further information

Please visit *cytiva.com/protein-purification* for more information. Useful information is also available in the Handbooks 18103746 and 18102229, see the chapter below.

9 Ordering information

	Pack Size	Code No.
Immunoprecipitation Starter Pack	2 × 2 mL	17600235
– nProtein A Sepharose 4 Fast Flow, 2 mL		
– Protein G Sepharose 4 Fast Flow, 2 mL		

Related products		
nProtein A Sepharose 4 Fast Flow	5 mL	17528001
	25 mL	17528004
Protein G Sepharose 4 Fast Flow	5 mL	17061801
	25 mL	17061802
nProtein A Sepharose CL-4B	1.5 g	17078001
Protein A Mag Sepharose	500 µL	28944006
	4 × 500 µL	28951378
Protein G Mag Sepharose	500 µL	28944008
	4 × 500 µL	28951379
Mammalian Protein Extraction Buffer	500 mL	28941279
Yeast Protein Extraction Buffer Kit	1 kit	28944045
Affinity Chromatography Handbook Principles and Methods	1	18102229
Antibody Purification, Handbook	1	18103746

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71501754 AG V:7 10/2020