TROUBLESHOOTING IMMUNOPRECIPITATION

Troubleshooting *This troubleshooting document gives the problem, possible cause and suggested solution for problems during the immunoprecipitation application:*

Problem: There is no binding/specific antigen detected

The antibody is not capable of immunoprecipitation	Try a different antibody, polyclonal antibodies generally perform better than monoclonal antibodies.
Not enough primary antibody is used	Determine the optimal concentration of primary antibody by titration.
There are too many competing proteins in the sample	Enrich the sample for the protein of interest, spin the lysate at 100,000g for 30 minutes before adding the antibody to remove insoluble proteins, membrane fragments, etc.
The antigen of interest is not present	Make sure you use the appropriate sample.
The antigen of interest is lost or denatured in the sample	Use the appropriate protease inhibitors. Prepare fresh lysates, avoid using frozen lysates.
The washes are too stringent	Reduce the number of washes. Reduce salt and detergent concentration or use a different detergent.
There are interfering substances present in the sample	Avoid lysates containing reducing agents (dithiothreitol (DTT), 2-mercaptoethanol or other), they may destroy antibody function. Extremes in pH and excessive detergent concentrations may also interfere with the antibody-antigen interaction.
The antibody binds weakly to either agarose beads	Use bridging antibody to enhance immunocomplex capture.
The incubation times are inadequate	The incubation times should be appropriate for the system. Optimize the incubation times, generally, the primary antibody and antigen of interest are incubated 1 hour to overnight at 2 ºC-8 ºC.
The sample has degraded by proteases	Include additional protease inhibitors in the lysis and wash buffers. Keep the sample cold at all times.
The antibody concentration is too low	Increase the concentration of the precipitating antibody.
The concentration of the protein of interest is too low	Increase the precipitating antibody concentration. Increase the cell lysate concentration. Metabolically label cellular proteins.
The antibody has low affinity for tagged protein	Use lower stringency wash buffers (for instance 150 mM NaCl with no detergent).
The tag sequence is not accessible to the precipitating antibody, this is due to conformation of the tagged gene protein	Use alternative insertion sites within the target protein for the tag sequence. To increase the avidity of antibody reaction, insert multiple tag sequences.
The antibody or protein incubation time is too short	Incubate with precipitating antibody for several hours at 4 ºC. Incubate with protein (G/A)-agarose overnight.
The target protein is not expressed in sample used Low level of target protein expression in sample	Check the expression profile of the target protein to ensure it will be expressed in the cells of your samples. Increase the amount of lysate used when there is low level of target protein expression. However, this may result in increased non-specific binding, so it is advisable to pre-clear the lysate before commencing with the IP procedure.
The target protein has not eluted from the beads	Ensure the correct elution buffer is used and that it is at the correct strength and pH for elution of the protein.
The antibody has not bound to the immune adsorbent beads	Ensure you are using the correct beads for the antibody isotype used.
An incorrect lysis buffer is used	Check the datasheet to see if the antibody detects the denatured or native protein and ensure the correct lysis buffer is used.

Problem: High background or unwanted proteins precipitate

Substances in sample bind non-specifically to either agarose beads, proteins or antibodies in general	Reduce the amount of sample loaded onto the beads. Pre-clear the lysate with the protein agarose conjugate without the antibody. This should clear the lysate of any proteins that are binding non-specifically to the beads. Some researchers also use an irrelevant antibody of the same species of origin and same Ig subclass to pre-clear the lysate. This step can be repeated to further reduce background.

	The beads are not pre-blocked enough with BSA. Make sure the BSA is fresh and incubate new beads for 1 hour with 1% BSA in PBS. Wash 3-4 times in PBS before using them. Prepare samples in serum-free media.
The antibodies are not specific enough	Use an affinity purified antibody, preferably pre-absorbed.
Too much antibody is used which leads to non-specific binding	Check the amount of antibody recommended. Decrease antibody concentration.
There are too many cells/too much protein/too much antibodies in the lysate leading to a lot of non-specific proteins in the eluate	Reduce the number of cells/lysate used. Determine the optimal concentration by titration.
The washing is inadequate	Make sure to wash the sample well at the relevant stages by placing a lid on the tube and inverting several times before centrifuging. Use more stringent washes, like 0.5 M LiCl, 1 M KSCN, 1.0 M NaCl, 0.2% SDS or 1% Tween 20. For one wash distilled water can be used. Increase the number of washes. Leave solid phase in the wash buffer for 10 minutes at each wash. The pellet should be transferred to a fresh tube prior to the last wash.
Equipment/buffers are contaminated	Use clean equipment and fresh buffers.
There is a carryover of proteins to the membrane that are not detergent soluble	Remove the supernatant immediately after centrifugations. This should leave insoluble proteins in the pellet. If resuspension occurs, centrifuge again.
Antigen degrates during immunoprecipitation	Ensure fresh protease inhibitors are added when sample is lysed.
Lysates are frozen before use	Make sure the lysate is not frozen, make fresh lysate.
There are aggregated proteins in the lysate	Centrifuge the lysate at 100,000g for 30 minutes before the addition of the antibody to remove the aggregated proteins.
The antigen consists of more than one polypeptide chain	Remember that the antigen may consist of more than one polypeptide chain, if specific proteins remain.
Monoclonal or affinity purified polyclonal antibody recognize homologous epitopes	Try monoclonal antibodies with different epitope.

Helpful links / references www.protocol-online.org