CD Creative Diagnostics®

IMMUNOPRECIPITATION TROUBLESHOOTING TIPS

IP troubleshooting tips

Solve your IP problems with these troubleshooting tips, covering common causes of high background, no or low proteins detected following IP, antibody fragments blocking the signal of interest.

Problems Index

➢ High background

Possible causes and corresponding solutions

- Sample problems

1. Using frozen cells

Try to avoid frozen cells, using fresh material whenever possible. If frozen material has to be used, use froze n lysates instead of cells. But to co-IPs, frozen cells could be less of a problem.

2. Unpurified sample

Samples from tissues typically suffer from more contamination by lipids, carbohydrates or nucleic acids, and may generate significantly more background than samples from cultured cells. The more complex the sampl e, the more background binding can occur.
Firstly, you can reduce sample complexity by extracting proteins from selected cellular compartments. Secondly, pre-clearing step is essential. Pre-clearing can reduce the background by adhesion of sample co mponents to the resin.

- Problems in binding step

1. Non-specific binding of antibody to protein A/G

If biological fluids are used as samples (biological fluids may contain antibodies which will bind to Protein A/ G), use certain IP kit that doesn't use Protein A or G, but relies on direct immobilization of the antibody to the resin.

2. Non-specific binding of proteins to the beads

Beads are not pre-blocked enough with BSA. Verify the BSA (fraction V) is fresh and incubate fresh beads 1 hour with 1% BSA in PBS. Wash 3-4 times in PBS before using them.

3. Overusing antibody or sample leading to non-specific binding

Check the recommended amount of antibody suggested. Try using less antibody. Reduce cells/lysate u sed. Generally 10-500 µg cell lysate recommended.

4. Protein aggregates remain

Spin at 100.000 g for 30 minutes to remove protein aggregates.

5. Long incubation time

When using primary antibody, shorten the incubation step to 45 minutes. When using Protein A resin, shorten the incubation step to 30 minutes.

- Problems in washing step

1. Contamination

Problems related to actin contamination can be avoided by adding 10 mM ATP to lysis and wash buffers. Transfer the matrix to a fresh tube after the last washing step in order to avoid carry-over of contamination.

2. Improper washing stringency and steps

Adjust washing stringency and steps. Move toward a higher stringency buffer and increase the numberof wa shing steps. Use up to 1% Tween-20 (a nonionic detergent), up to 0.2% SDS (an anionic and therefore, char ged detergent), or up to 1 M NaCl. Alternating these high stringency washes with distilled water may help to reduce background or nonspecific binding.

- **No or low proteins detected following IP**

Possible causes and corresponding solutions

- Low binding of antigen

1. Inappropriate binding buffer

The solution in which the binding reaction is performed is very crucial. Detergents, salts and other additives may reduce non-specific binding but may also decrease yields. Use an appropriate buffer, making pH, salt c oncentration and any co-factors suitable for the interactions.

2. Washes too harsh

Reduce the number of washes. Reduce salt and detergent concentration or use a different detergent.

3. Unsuitable incubation times

Incubation times should be suitable for the system. Generally, the primary antibody and antigen of interest a re incubated 1 hour to overnight at 2-8°C.

4. Antibody used unsuitable for IP

If antibody used easily inactivated or binds weakly to antigen, it would be difficult find sufficiently mild conditi ons to establish and stabilize the essential binding interaction for the duration of incubation and wash steps r equired to perform an IP. Polyclonal antibodies often perform better in IPs than monoclonal antibodies. Some antibodies simply do not work in IP (native antigen), although they may be effective for assays such as Western blotting (denatured antigen). Whenever possible, purchase commercial antibodies that have been v alidated for IP.

5. Polyclonal antibody not affinity purified

If a polyclonal antibody has not been affinity purified, the antigen-specific antibody may represent only 1-2% of the total IgG present. While this type of antibody sample may work fine for Western blotting, it is problematic for IP because all IgG molecules (irrespective of antigen-specificity) in the sample will compete for the same Protein A/G binding sites on the beads. Most of the antibody bound to the solid support will be non-specific, and antigen yields will be low.

6. Insufficient amount of primary antibody used

Optimize the concentration of primary antibody by titration.

7. Too many competing proteins in sample

Before adding the antibody, spin the lysate at 100,000g for 30 minutes to remove insoluble proteins, membrane fragments, etc.

8. Interfering substances present in sample

Lysates containing dithiothreitol, 2-mercaptoethanol or other reducing agents will destroy antibody function and must be avoided. Extremes in pH and excessive detergent concentrations can also interfere with the antibody-antigen interaction.

9. Antigen of interest not exist or lost

Prepare fresh lysates. Avoid using frozen lysates. Use appropriate protease inhibitors in sample. Ensure sample is proper.

10. Protein of interest is obstructed by antibody heavy or light-chains

Secondary antibody recognizes denatured/reduced primary antibody released during the IP procedure or endogenous IgGs.

Using secondary antibodies, recognizing the heavy and light-chain of the primary antibody for WB detection of IP samples will always result in two bands (the heavy-chain at 50kDa and the light-chain at 25kDa).

To avoid interference by the antibody chains we recommend using secondary antibodies, that specifically recognize native (non-reduced) primary antibodies.

- Low elution of antigen

11. The interaction between antigen and antibody/protein is too strong to break by conventional elution buffers

If there is a low antigen yield and you doubt that the antigen is still bound on the beaded support, try this way to confirm it: boil a small aliquot of the beads in an extremely harsh buffer such as SDS-PAGE sample buffer. Then the beads can be centrifuged, using the supernatant analyzed by SDS-PAGE/Western blotting to confirm the presence or absence of the antigen. If the antigen is present, it may be worth investigating different elution buffers of increasing strength to determine conditions whereby the antigen can be eluted without being inactivated.

- **Antibody Fragments Blocking the Signal of Interest**

Possible causes and corresponding solutions

1. Antibody not immobilized on resin

   Protein A or G binding of antibody followed by covalent attachment with a crosslinker; orients the antigen bin ding sites outward, but can result in significant loss of antibody function. This choice is compatible with unpu rified antibodies.

   Direct immobilization of antibody onto an activated resin - non-oriented, but greater retention of antibody fun ction. This choice requires a purified antibody because the resin reacts with amines found on all proteins.

2. Detection reagent non-specific to native antibody

   Use a detection reagent that does not recognize the denatured antibody fragment.

Contact Us

1-631-624-4882

1-631-938-8221

info@creative-diagnostics.com

45-1 Ramsey Road, Shirley, NY 11967, USA