Protocol

Preparing Immunoprecipitations for Immunoblotting

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Immunoprecipitated proteins can be readily analyzed by immunoblotting. Proteins can be efficiently eluted from the Protein A or similar beads by addition of the SDS-PAGE sample loading buffer and heating at 95°C. This elution procedure will also remove the capturing antibody from the beads unless the antibody was cross-linked to the beads. Alternatively, the immunoprecipitated proteins as well as non-cross-linked capture antibodies can be eluted from the beads using low (2.1–2.8) or high (10–11) pH conditions. Incubation of the immunoprecipitates with the excess of the competing peptide allows the elution of the captured proteins without contamination of the sample with the antibodies present in the immunoprecipitates. However, this option is not always available, and the cost of competing peptide can be prohibitive for the routine immunoprecipitation/immunoblotting experiments. In this protocol, elution of the immunoprecipitated proteins from the beads is performed by mixing Protein A or similar beads containing the immunoprecipitated protein antigens of interest with SDS-PAGE sample buffer and boiling to prepare samples for protein gel electrophoresis.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Protein A or similar beads containing the immunoprecipitated protein antigens of interest
SDS-PAGE sample buffer for immunoblotting (6×) <R>

Prepare a 2× solution.

Equipment

Heating block set at 95°C or boiling water bath
Ice
Microcentrifuge tubes
Pipetman tip (200 µL) or a syringe needle attached to the aspiration tubing
Refrigerated microcentrifuge

From the Antibodies collection, edited by Edward A. Greenfield.

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METHOD

1. Perform immunoprecipitation and washing of the beads as described in Protocol: **Immunoprecipitation** (DeCaprio and Kohl 2018). Keep the samples on ice.
2. Aspirate the last wash buffer close to the beads using the 200-µL Pipetman tip or a syringe needle attached to the aspiration tubing.

   To prevent the aspiration of the beads, it is helpful to slowly lower the tip down against the side of the tube instead of inserting it into the liquid.
3. Perform a final centrifugation for 15 sec at 15,000*g*. Estimate the volume of the remaining liquid and beads in the tubes, and adjust to one-half of the desired sample volume by adding an appropriate amount of wash buffer.

   Use identical microcentrifuge tubes with a known volume of water to estimate the volume of your sample (e.g., tubes with 10, 15, 20, 25 µL, etc.). It is usually convenient to load the immunoprecipitated sample on the gel if the final volume of the sample is between 30 and 100 µL, depending on the type of gel comb used. Therefore, the volume of the beads and buffer should be between 15 and 50 µL.
4. Add an equal volume of the 2× SDS-PAGE sample buffer to the samples and mix by gentle tapping. Avoid mixing of the sample by pipetting or vortexing because some of the beads can stick to the pipette tip or the walls of the tubes.
5. Heat the samples in the heat block or water bath for 5 min.
6. Centrifuge the samples for 15 sec at 15,000*g* to pellet the beads and to collect any condensate.

   *The samples are now ready for electrophoresis (Protocol: **Resolving Proteins for Immunoblotting by Gel Electrophoresis** [Litovchick 2018]).*

TROUBLESHOOTING

Problem: When developing the immunoblot of immunoprecipitated protein samples, high-molecular-weight bands and overall higher background staining of the lanes are detected.

Solution: This is caused by incomplete reduction of the disulfide bonds between the heavy and light chains of the capturing immunoglobulin molecules. It is important to use fresh β-mercaptoethanol in the sample buffer when preparing the immunoprecipitated samples for immunoblotting and to heat the samples for a full 5 min at 95°C before loading on the gel.

Note that most of the "same species" immunoprecipitation/immunoblotting kits available from commercial providers rely on the complete denaturation and reduction of the capturing immunoglobulins in the sample. Some of the secondary detection reagents in these kits can recognize only the native, nondenatured form of the immunoglobulins that binds to the antigen during the immunoblotting procedure. Other secondary detection reagents only detect the antibody light chain, and the incomplete denaturation of the capturing antibody will result in detection of the additional bands on the gel.

Problem: There is increased background in the lanes and appearance of the aberrant bands on the immunoblot prepared using immunoprecipitated samples.

Solution: Loading the beads onto the gel may cause this. When loading the immunoprecipitated samples on the gel for immunoblotting, it is important not to disturb the beads. The beads may clog the gel-loading tip, resulting in a loss of the sample.

DISCUSSION

If acidic elution buffer was used to remove the immunoprecipitated proteins from the beads, the pH of the samples will have to be adjusted before loading them onto the gel. The easiest way to do so is to

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take advantage of the pH dependency of the Bromophenol Blue dye present in the sample buffer, which can be used as a pH indicator. When acidic samples are mixed with an equal volume of the 2× SDS-PAGE sample buffer, the blue color of the buffer will turn yellow. Adding one-tenth of the volume of 2 M Tris–HCl buffer (pH 6.8) to the samples should result in appearance of the blue color. If the dye color remains yellow, another one-tenth sample volume of 2 M Tris–HCl can be added to the sample.

The immunoprecipitates prepared using peptide elution can be directly mixed with an equal volume of the 2× SDS-PAGE sample buffer, heated, and loaded onto the gels. The presence of the peptide in the eluates usually does not interfere with subsequent immunoblotting unless the protein of interest is of a very low molecular weight and migrates close to the peptide that may be retained in the gel.

The presence of capturing antibody in the protein sample for immunoblotting can interfere with detection of the antigen. This is discussed in Introduction: **Immunoblotting** (Litovchick 2020).

RECIPES

SDS-PAGE Sample Buffer for Immunoblotting (6×)

Reagent	Quantity (for 10 mL)	Final concentration
Tris (2 M, pH 6.8)	1.8 mL	360 mM
SDS	1.2 g	12%
Glycerol	6 mL	60%
Bromophenol blue	1.5 mg	0.015%
β-mercaptoethanol (14.7 M)	1.8 mL	18%

Prepared buffer without β-mercaptoethanol can be stored at room temperature. If an SDS precipitate forms, it must be dissolved by heating. After adding β-mercaptoethanol, solutions can be stored for 1–2 wk at 4°C or for 1–2 mo at −20°C.

REFERENCES

DeCaprio J, Kohl TO. 2018. Immunoprecipitation. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot098640.

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