Improved Affinity Resins for Immunoprecipitation.

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Abstract

Immunoprecipitation (IP) is a common biochemical technique used by researchers to isolate proteins of interest from crude cell extracts. This procedure generally involves capturing the protein of interest with either a specific antibody directly conjugated to an agarose resin or by capturing the antigen-antibody complex with protein A agarose resin. Low visibility of the pelleted agarose makes subsequent washes and removal of supernatants difficult. In addition, sample loss, due to low-visibility of the affinity matrix, makes accurate, quantitative results hard to achieve. We developed high visibility, high-contrast colored resins, which facilitate supernatant removal without sample loss. The resulting dye-resins show minimal non-specific binding of proteins from mammalian cell extracts. Application testing demonstrates results equivalent to standard IP matrices. These colored resins will allow researchers to produce higher quality IP data more conveniently.

Introduction

Goal – To develop affinity resins with enhanced visibility for use in immunoprecipitation (IP) and other molecular pull-down experiments.

Desired Attributes
- More visible than standard, non-colored affinity resins.
- Low background protein binding.
- Compatible with IP techniques.
- Compatible with co-IP techniques.

Approach
- Conjugate dyes to agarose beads.
- Test for binding of mammalian cell lysate proteins.
- Test performance in IP applications.

Background

Dye ligand affinity chromatography has been a common tool used by researchers for protein purification. Organic dye molecules, covalently attached to a solid matrix such as beaded agarose, can mimic physiological ligands for proteins. This enables proteins with affinity for the dye molecules to be easily separated from a heterogeneous biological preparation. Such affinity resins tend to bind large amounts of proteins with varying affinities.

In contrast, to develop a more visible resin for IP applications, our approach was to create an agarose resin with a covalently attached dye that bound only trace amounts of non-specific proteins.

Immunoprecipitation

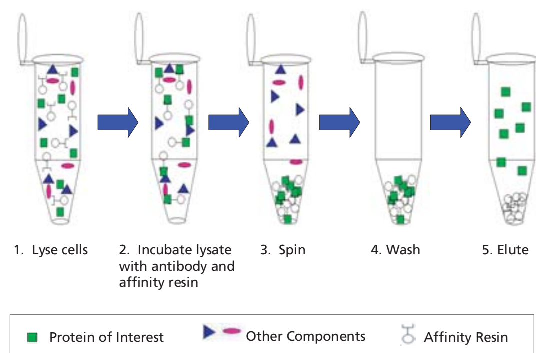

Fig. 1. Schematic representation of immunoprecipitation.

Agarose Resin Visibility

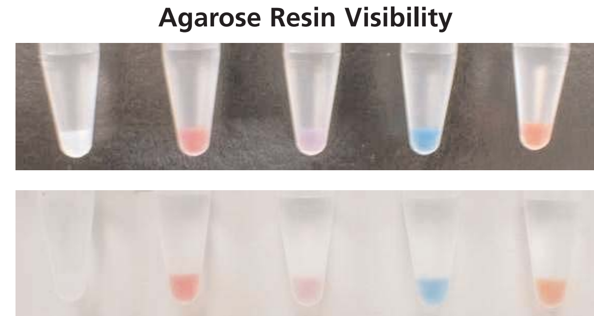

Fig. 2. Dyes covalently attached to agarose make agarose pellets more visible. The same samples were photographed with a black background (upper panel) and a white background (lower panel) to illustrate relative contrast.

Lysate Protein Binding

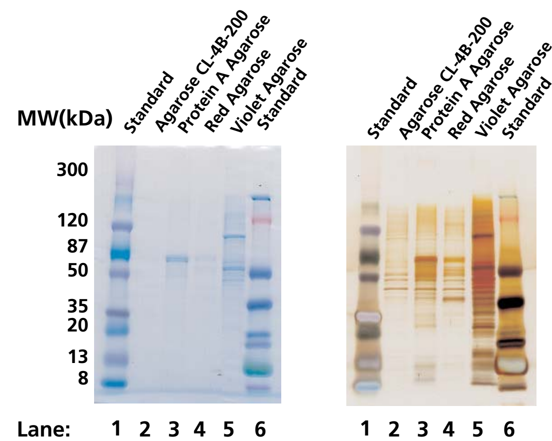

Fig. 3. Red agarose beads (Lane 4) bound low amounts of lysate protein. Agarose resin beads were incubated with COS7 lysates (10^7 cells in 1 ml RIPA buffer) for 1 hour at 4° C, washed, and proteins bound to the resins were eluted by boiling in Laemmli sample buffer and analyzed by SDS-PAGE. The gel was stained sequentially with colloidal blue stain (EZBlue™; left panel) and prolonged silver staining (right panel).

Application Testing

Red Protein A Agarose

Protein A is derived from *Staphylococcus aureus* and binds the Fc portion of IgG molecules. When covalently attached to a solid matrix, such as cross-linked agarose (also called Sepharose), it is used to capture and precipitate antigen-antibody complexes. We compared the performance of red protein A agarose to standard, non-colored protein A agarose for IgG binding (Fig. 4) and for immunoprecipitation of protein antigens (Fig. 5 and Fig. 6).

IgG Recovery

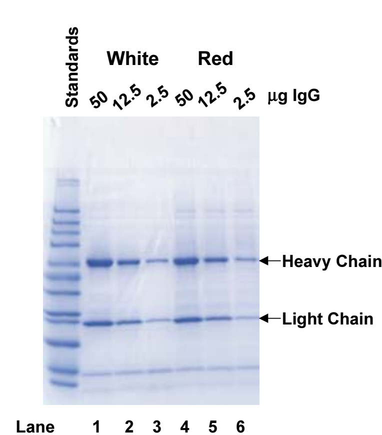

Fig. 4. Red protein A agarose and non-colored protein A agarose recovered a similar amount of IgG. Serial dilutions of ANTI-FLAG® M2 monoclonal antibody were spiked into COS7 lysates. The mouse IgG was recovered using non-colored protein A agarose **(White)** or red protein A agarose **(Red)** in a mock IP experiment. After washing, the bound proteins were eluted and subjected to SDS-PAGE. The gel was stained with colloidal blue stain (EZBlue™).

IP of Spiked Antigen

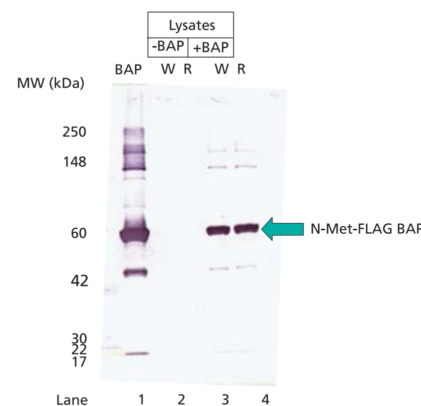

Fig. 5. Red protein A agarose and non-colored protein A agarose recovered a similar amount of spiked antigen from mammalian cell lysates. Purified N-Met-FLAG bacterial alkaline phosphatase (**BAP**, lane 1) was spiked into COS7 lysates (**+BAP**, lanes 4 and 5), incubated with anti-BAP mAb and immunoprecipitated with **white (W)** or **red (R)** protein A agarose. Lanes 2 and 3 did not have BAP spiked in the lysates **(-BAP)**. An immunoblot was probed with ANTI-FLAG® M2 Monoclonal Antibody Alkaline Phosphatase conjugate and visualized by addition of NBT/BCIP substrate.

IP of Expressed Protein

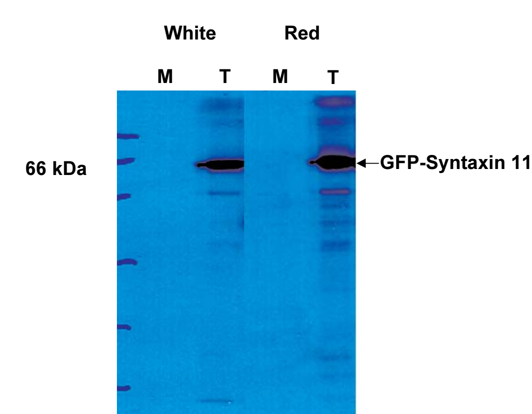

Fig. 6. Red protein A agarose and non-colored protein A agarose recovered a similar amount of expressed antigen from mammalian cell lysates. A GFP-syntaxin 11 fusion protein was immunoprecipitated with an anti-GFP antibody and either red protein A agarose **(Red)** or non-colored protein A agarose **(White)** from lysates of [^{35}S]methionine labeled HEK cells transfected with the GFP-syntaxin 11 gene **(T)**. A mock immunoprecipitation **(M)** served as a negative control. An autoradiograph of the recovered proteins after SDS-PAGE is shown. The film was exposed for 5 days.

Red Anti-FLAG M2 Agarose

ANTI-FLAG™ M2 agarose affinity gel has the M2 monoclonal antibody to the FLAG peptide conjugated to beaded agarose. Red anti-FLAG M2 agarose was shown to perform equivalently to standard, non-colored anti-FLAG M2 agarose for immunoprecipitation (Fig. 7a), and for co-immunoprecipitation to reveal protein-protein interactions (Fig. 7b).

Co-Immunoprecipitation

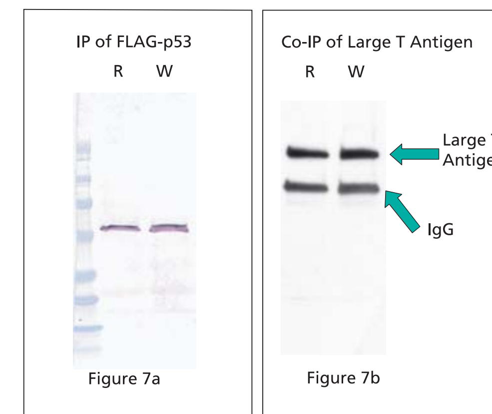

Fig. 7. Red anti-FLAG agarose and non-colored anti-FLAG agarose recovered a similar amount of Large T antigen by co-immunoprecipitation.

a. Immunoblot showing the IP of **FLAG-tagged p53** protein from lysates of COS7 cells transfected with FLAG-p53. IPs were carried out with red anti-FLAG M2 agarose **(R)** or non-colored anti-FLAG M2 agarose **(W)**. Detection/Visualization: anti-FLAG M2-AP conjugate; NBT/BCIP.

b. Immunoblot showing co-IP of **Large T Antigen**, an *in vivo* physiological binding partner of p53. Detection/Visualization: mouse anti-T Antigen (N-term.), 1° antibody; goat anti-mouse-HRP, 2° antibody; enhanced chemiluminesence.

Discussion

1. Resins with enhanced visibility were made by conjugating dyes to agarose.
2. Screening in mock IP experiments revealed that some enhanced visibility resins bound low amounts of non-specific background proteins.
3. A red protein A agarose was developed and demonstrated to be functionally equivalent to non-colored protein A agarose in IP applications.
4. A red anti-FLAG agarose was developed and demonstrated to be functionally equivalent to non-colored anti-FLAG agarose for IP and co-IP applications to reveal protein-protein interactions.

Conclusion

Affinity resins with enhanced visibility that bind low amounts of background protein have advantages over non-colored affinity resins:

1. More readily visible.
2. More rapid manipulation possible.
3. Improved visibility should lead to improved recovery of the resin and target molecules for more reproducible results.

Acknowledgements

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References

1. Lowe, C. R. and Pearson, J. C., Affinity chromatography on immobilized dyes. Methods Enzymol., 104, 97-113 (1984).
2. Scopes, R. K., Strategies for enzyme isolation using dye-ligand and related absorbents. J. Chromatog., 376, 131-140 (1986).
3. Harlow, E. and Lane, D., Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, p. 423-470 (1988).

Related Sigma Products

Product Name	Product Number
EZview™ Red Protein A Agarose Affinity Gel*	**P 6486**
EZview™ Red ANTI-FLAG M2 Agarose Affinity Gel*	**F 2426**
Protein A Agarose	P 2545
ANTI-FLAG® M2 Monoclonal Antibody Affinity Gel	A 2220
NBT/BCIP Alkaline Phosphatase Substrate	B 5655
EZBlue™ Gel Staining Reagent	G 1041
ANTI-FLAG® M2 mAb-AP Conjugate	A 9469

*Patent Pending