

Application Note

EZview™ Red Protein A and ANTI-FLAG® M2 Affinity Gels: Immunoprecipitation with Enhanced Visibility Affinity Beads

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Introduction

The strength and specificity of interactions between biomolecules have been exploited for years in biochemical research. Affinity-based protein purification techniques have been used extensively to isolate and purify specific proteins or classes of proteins from complex biochemical mixtures, such as cell lysates.^{1,2} In recent years, small-scale, affinity-based molecular pull-down techniques, such as immunoprecipitation, have been used widely to study expression, modification, and interaction of proteins in a wide variety of biological systems.³

The underlying strategy behind the affinity-based molecular pull-down technique is to use the specific affinity of biomolecules as a method to select target molecules from a solution. An affinity ligand capture molecule, such as an antibody, is covalently attached to a particulate matrix, such as agarose, and the resulting conjugate is used to locate and bind its target, such as a particular antigen from a cell lysate or other biological or biochemical solution (Figure 1). The resulting agarose-bound protein complex is recovered and purified from other components of the solution by centrifugation and multiple washes to remove non-specific contaminants. In the case of immunoprecipitation, the affinity capture antibody can be directly attached to the particulate matrix, or soluble antibody may be captured by an antibody-binding protein, such as protein A, covalently attached to the matrix.

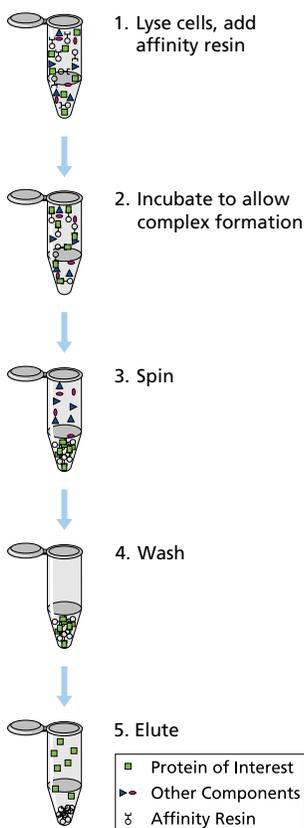


Figure 1. Affinity-based molecular pull-down technique.

A major disadvantage of affinity-based molecular pull-down and immunoprecipitation procedures, as commonly practiced, is that the affinity matrix is difficult to see in the microcentrifuge tubes used for the complex formation, purification, and wash steps. This difficulty in visualization leads to inefficient and tedious manipulations, and often results in loss of material and quantitative variability of results. For example, agarose or polyacrylamide beads generally are non-colored (i.e., translucent or white) and are difficult to see in standard opaque microcentrifuge tubes. Because of this poor visibility, the beads can be accidentally lost during the aspiration of the lysate supernatants and subsequent wash and aspiration steps necessary to remove residual, unbound proteins. Therefore, we developed the EZview™ Red Affinity Gels (patent pending), which are unique, colored agarose affinity beads for use in immunoprecipitation. These agarose affinity beads function the same as conventional agarose affinity beads, but have enhanced visibility to aid experimental manipulations and target protein recovery.

Materials and Methods

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO), unless otherwise stated.

Preparation of Lysates

COS-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Product Code: D 6171) containing 10% Fetal Bovine Serum (FBS; Product Code: F 2442) in 100-mm tissue culture plates (Product Code: C 6546). When the cells were initially confluent (10^7 cells/plate), the plates were set on ice, the medium was removed, and the adherent cells were washed twice with 10 ml of ice cold Dulbecco's Phosphate Buffered Saline (Product Code: D 8537) per plate. The cells were lysed directly on the plates by adding 1 ml of ice cold RIPA lysis buffer (150 mM NaCl, 1.0% NP-40 [Igepal], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris, pH 8.0) to each and scraping rapidly with a cell scraper. Mammalian Protease Inhibitor Cocktail (100 μ l; Product Code: P 8340) was added to each lysate and the lysates were used immediately or quick-frozen in liquid nitrogen and stored at -70 °C until use.

Immunoprecipitation

Lysates were clarified by centrifugation in a microcentrifuge for 10 minutes at $8,000 \times g$ at 4 °C. The clear supernatants were removed and incubated for 1 hour at 4 °C while mixing with soluble antibody. The mixture was then added to a 50 μ l packed gel volume of protein A agarose affinity beads (pre-washed and equilibrated in RIPA buffer) and incubated for an additional 1 hour with mixing at 4 °C. For immunoprecipitations using ANTI-FLAG® M2 agarose affinity beads, only one incubation for 1 hour with mixing at 4 °C was performed. The beads were collected by centrifugation for 30 seconds at $8,000 \times g$ and the supernatants were removed by aspiration. The pellets were washed three times with 1 ml of RIPA buffer per wash and collected by centrifugation as described. After aspirating the final wash supernatants, the affinity-bead pellets were each suspended in 25 μ l of RIPA buffer and 25 μ l of 2X Laemmli sample buffer (Product Code: S 3401) and analyzed by denaturing

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and/or immunoblotting as indicated.

Results and Discussion

It is desirable for many immunoprecipitation-based applications to keep non-specific protein binding low. Therefore, a number of dyes were covalently conjugated to agarose beads and tested for binding of proteins from mammalian cell lysates. A red dye was found that dis-

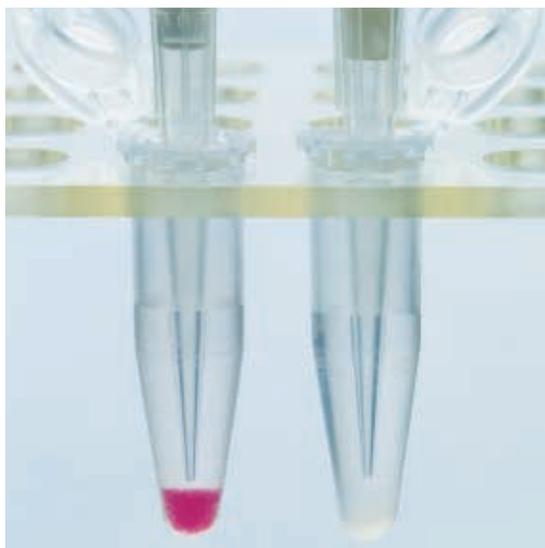


Figure 2. EZview™ Red Protein A Affinity Gel (left, Product Code: P 6486) has enhanced visibility compared to standard, non-colored Protein A Agarose (right, Product Code: P 2545).

played very low protein binding ability when attached to agarose and greatly enhanced the visibility of the agarose resin in normal, ambient light (Figure 2). This red dye was used to make EZview™ Red Protein A Agarose Affinity Gel (Product Code: P 6486), a highly visible red-beaded agarose protein A conjugated affinity resin. It was also used to make EZview™ Red ANTI-FLAG® M2 Affinity Gel (Product Code: F 2426), a highly visible red-beaded agarose ANTI-FLAG® M2 monoclonal antibody conjugated affinity gel. The performance of these unique, highly visible affinity beads was compared to standard, non-colored agarose affinity beads in immunoprecipitation (IP) applications.

We compared EZview™ Red Protein A Affinity Gel with standard protein A agarose affinity beads (Product Code: P 2545) for recovery of a mouse IgG in mock IPs from mammalian cell lysates. Different amounts of ANTI-FLAG® M2 monoclonal antibody (Product Code: F 3165) were spiked into COS-7 cell lysates and recovered with either non-colored or red protein A agarose affinity beads. The stained SDS-PAGE gel in Figure 3 shows that a similar amount of mouse IgG was recovered with both the EZview™ Red Protein A Affinity Gel and standard protein A agarose beads. In addition, little non-specific protein binding was observed with either affinity resin.

To examine recovery of a specific protein target antigen, we spiked purified FLAG-tagged bacterial alkaline phos-

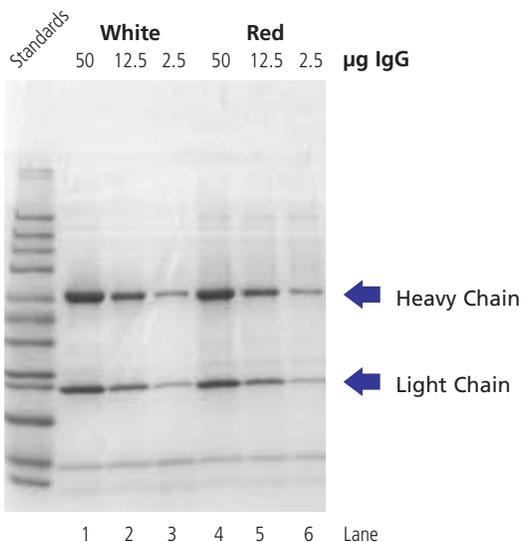


Figure 3. EZview™ Red Protein A Affinity Gel (Product Code: P 6486) and standard protein A agarose (Product Code: P 2545) recovered a similar amount of IgG. Different amounts (50 µg, 12.5 µg and 2.5 µg) of ANTI-FLAG® M2 monoclonal antibody (Product Code: F 3165) were spiked into COS-7 lysates (10⁷ cells in 1 ml RIPA buffer). The mouse IgG was recovered using standard protein A agarose (White, lanes 1-3) or EZview™ Red Protein A Affinity Gel (Red, lanes 4-6) 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™ Gel Staining Reagent; Product Code: G 1041).

phatase (N-Met-FLAG-BAP; Product Code: P 5975) into COS-7 cell lysates. We compared recovery of this fusion protein in IPs using anti-BAP monoclonal antibody (Product Code: B 6804) with either standard protein A agarose or EZview™ Red Protein A Affinity Gel. The IP complexes were

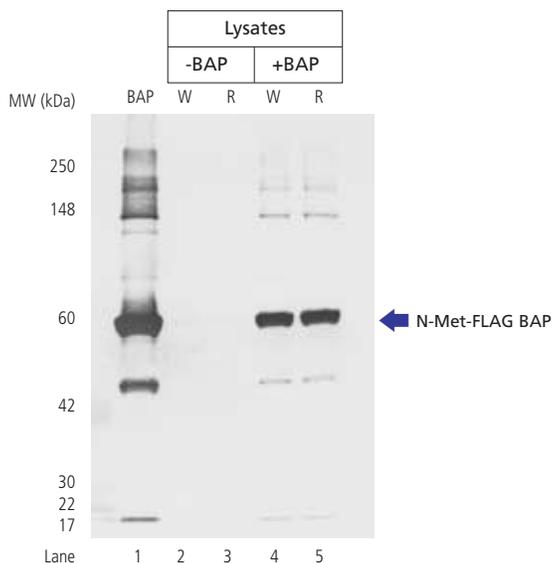


Figure 4. EZview™ Red Protein A Affinity Gel (Product Code: P 6486) and standard protein A agarose (Product Code: P 2545) recovered a similar amount of spiked antigen from mammalian cell lysates. Purified N-Met-FLAG bacterial alkaline phosphatase (BAP, lane 1; Product Code: P 5975) was spiked into COS-7 lysates at 5 µg/ml (+BAP, lanes 4 and 5), incubated with anti-BAP monoclonal antibody (Product Code: B 6804) and immunoprecipitated with standard protein A agarose [W] or EZview™ Red Protein A Affinity Gel [R]. Lanes 2 and 3 did not have BAP spiked in the lysates (-BAP). The immunoblot was probed with ANTI-FLAG® M2 Monoclonal Antibody Alkaline Phosphatase conjugate (Product Code: A 9469) and visualized by addition of BCIP/NBT substrate (Product Code: B 1911).

subjected to SDS-PAGE and analyzed by immunoblotting. Immunostaining of the Western blots revealed that a similar amount of N-Met-FLAG-BAP protein was recovered with both the standard protein A agarose and the EZview™ Red Protein A Affinity Gel (Figure 4). No differences in non-specific background protein binding were observed.

We next compared the performance of the EZview™ Red ANTI-FLAG® M2 Affinity Gel with standard ANTI-FLAG® M2 Monoclonal Antibody Affinity Gel (Product Code: A 2220) for co-immunoprecipitation of interacting proteins. Co-immunoprecipitation is a very useful technique for studying how proteins interact within a cell. Proteins that form a complex *in vivo* can be precipitated together, although only the target protein is bound directly by the affinity resin. A mammalian expression plasmid encoding a p53 fusion protein, amino terminally tagged with a FLAG peptide sequence, was transiently transfected into COS-7 cells. Cell lysates were immunoprecipitated with either standard ANTI-FLAG® M2 Monoclonal Antibody Affinity Gel or with EZview™ Red ANTI-FLAG® M2 Affinity Gel and analyzed by Western blotting and immunostaining for the FLAG-p53 fusion protein and for the large T antigen, an *in vivo* binding partner of p53. Figure 5 shows that the EZview™ Red ANTI-FLAG® M2 Affinity Gel recovered similar amounts of both the FLAG-p53 fusion protein (Figure 5A) and the large T antigen binding partner (Figure 5B) compared to the standard, non-colored ANTI-FLAG® M2 Monoclonal Antibody Affinity Gel. Also, no non-specific background protein binding was detected for either affinity gel preparation on either immunostained blot.

Conclusions

Two unique affinity resins for immunoprecipitation, EZview™ Red Protein A Agarose Affinity Gel and EZview™ Red ANTI-FLAG® M2 Affinity Gel were developed to have enhanced visibility with low non-specific protein binding capacity. These enhanced visibility immunoprecipitation resins have several advantages over standard, non-colored immunoprecipitation resins. The enhanced visibility of the EZview™ beads allows more rapid and less tedious manipulations in experiments that typically involve multiple centrifugation, aspiration, and wash steps. In addition, the ability to easily see the EZview™ affinity resins should lead to improved recovery of the resin and target molecules for more reproducible results and reduced need to repeat experiments due to accidental loss of the affinity gel.

About the Author

Tom Rutkoski and Max Huang are Sigma Undergraduate Co-op students from the Department of Biochemistry at The Pennsylvania State University, University Park, PA and the Biomedical Engineering Program at Washington University, St. Louis, MO., respectively. Ned Watson, Ph.D., is a senior scientist in Protein R&D at Sigma-Aldrich, St. Louis, MO.

ORDERING INFORMATION

Product Code	Product Name	Unit
F 2426	EZview™ Red ANTI-FLAG® M2 Affinity Gel*	1 ml 5x1ml
P 6486	EZveiw™ Red Protein A Affinity Gel*	1 ml 5x1 ml

*patent pending

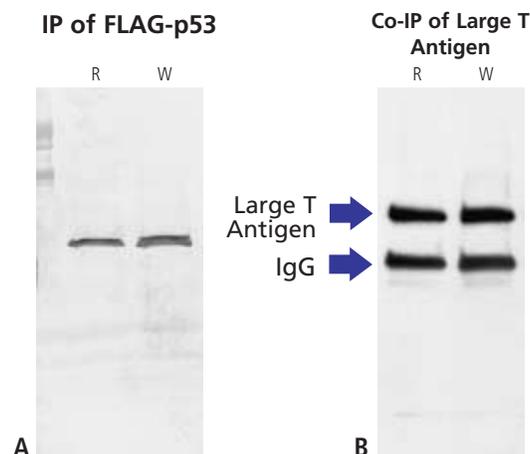


Figure 5. EZview™ Red ANTI-FLAG® M2 Affinity Gel (Product Code: F 2426) and standard ANTI-FLAG® M2 Affinity Gel (Product Code: A 2220) recovered a similar amount of large T antigen by co-immunoprecipitation. (A) Immunoblot showing the IP of FLAG-tagged p53 protein from lysates of COS-7 cells transfected with FLAG-p53. IPs were carried out with EZview™ Red ANTI-FLAG® M2 Affinity Gel [R] or standard ANTI-FLAG® M2 Affinity Gel [W]. The immunoblot was probed with ANTI-FLAG® M2 Monoclonal Antibody Alkaline Phosphatase conjugate (Product Code: A 9469) and visualized by BCIP/NBT (Product Code: B 1911). **(B)** Immunoblot showing co-IP of large T antigen, an *in vivo* physiological binding partner of p53. The immunoblot was probed with mouse anti-large T antigen (N-terminus) primary antibody (Research Diagnostics, Inc.) and goat anti-mouse IgG-horseradish peroxidase conjugate secondary antibody (Product Code: A 9044), and was visualized by enhanced chemiluminescence.

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References

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