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Capture of Tagged Proteins and Complexes with Enhanced Visibility Affinity Resins

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Program #632.9

Abstract

It is often desirable to isolate native or recombinant proteins from biochemical preparations by small-scale affinity capture techniques, such as immunoprecipitation (IP). We developed a line of affinity capture resins with enhanced visibility (EZview Affinity Gels), which facilitate manipulations, such as washing and removal of supernatants from small affinity resin pellets, while reducing the possibility of sample loss.

In this work we demonstrate the utility of these enhanced visibility resins to capture epitope-tagged recombinant proteins and biochemically-tagged proteins for proteomic analysis. We show that these enhanced visibility resins are equivalent to standard, non-colored resins in terms of target protein capture and non-specific background, but with improved handling characteristics. In addition, we demonstrate the utility of an enhanced visibility streptavidin affinity capture resin (EZview Streptavidin Affinity Gel) to bind biotinylated antibodies for analysis of protein expression, modification and protein-protein interactions.

Introduction

Goal – To test novel affinity resins with enhanced visibility for use in immunoprecipitation (IP; Fig. 1) to study protein-protein interactions.

Approach

- Test direct capture of an epitope-tagged target protein with an antibody affinity resin.
- Test direct capture of a biotinylated protein with a streptavidin affinity resin.
- Test direct capture of a target protein with a biotinylated antibody bound to a streptavidin affinity resin.
- Test utility for protein-protein interaction studies.

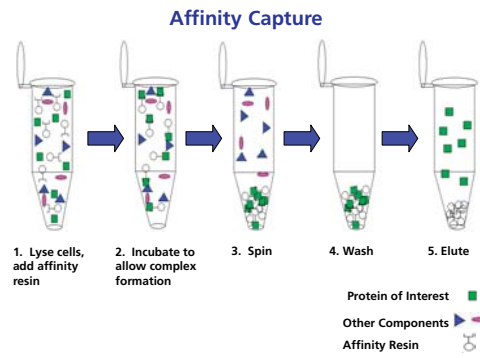


Fig. 1. Schematic representation of affinity-based molecular pull-down methods, such as immunoprecipitation.

Background

We developed highly visible colored resins, EZview™ Red Affinity Gels (Fig. 2), to improve manipulations during experiments using small-scale affinity capture techniques, such as IP. These novel agarose affinity resins contain covalently attached affinity capture molecules, such as specific antibodies, and a covalently attached dye.

Enhanced Visibility Affinity Gel



Fig. 2. Enhanced visibility of EZview™ Red Affinity Gel (patent pending).

Direct Affinity Capture Resins

Specific antibodies can be covalently attached to a solid matrix, such as agarose, by various chemical methods to form affinity resins that directly capture the antigen target proteins. The coupling method can require harsh conditions and lead to antibody molecules bound in undesired orientations or inactive antibodies.

Antibodies and other proteins can be easily modified by the attachment of biotin at specific sites. Subsequently, biotinylated antibodies can be rapidly and tightly bound to streptavidin agarose to form specific affinity resins. This approach can be applied to many different types of antibodies and proteins to generate a variety of different specific affinity resins.

EZview Affinity Gel Performance

We made enhanced visibility antibody affinity gels and streptavidin affinity gels and compared them to analogous standard affinity gels for the capture of target proteins.

Antibody Affinity Gel

We compared standard and EZview Red ANTI-FLAG antibody agarose affinity gels for immunoprecipitation of a FLAG epitope-tagged protein from a cell lysate (Fig. 3).

The enhanced visibility (EZview) affinity gel performed similarly to the standard affinity gel.

Affinity Capture of an Epitope-Tagged Protein: Standard vs. EZview ANTI-FLAG Affinity Gel

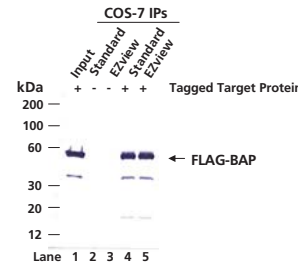


Fig. 3. EZview Red Affinity Gel performs similar to standard affinity gel for direct capture of epitope-tagged proteins. Tagged target protein (FLAG-tagged bacterial alkaline phosphatase, FLAG-BAP) was either spiked at 5 µg/ml (+), or not spiked (-), into COS-7 cell lysates (10⁷ cell in 1 ml RIPA buffer) and captured using either standard or EZview Red ANTI-FLAG Affinity Gel. A Western blot of an SDS-PAGE gel is shown. The blot was probed with ANTI-FLAG M2 monoclonal antibody conjugated to alkaline phosphatase and developed with BCIP/NBT substrate.

Streptavidin Affinity Gel

We compared standard and EZview Red Streptavidin agarose affinity gels for affinity capture of a biotin-tagged protein from a cell lysate (Fig. 4).

The enhanced visibility (EZview) affinity gel performed similarly to the standard affinity gel.

Affinity Capture of a Biotinylated Protein: Standard vs. EZview Streptavidin Affinity Gel

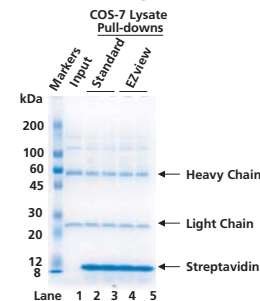


Fig. 4. EZview Red Streptavidin Affinity Gel and standard streptavidin agarose capture a similar amount of a biotinylated antibody. Biotinylated ANTI-FLAG M2 monoclonal antibody (Input, lane 1) was spiked into COS-7 lysates (10⁷ cells in 1 ml RIPA buffer) at 24 µg/ml. The antibody was captured using standard streptavidin agarose (Standard, lanes 2 and 3) or EZview Red Streptavidin Affinity Gel (EZview, lanes 4 and 5). After washing the affinity beads, the bound proteins were eluted and subjected to SDS-PAGE. The gel was stained with colloidal blue stain (EZBlue™ Gel Staining Reagent).

Biotinylated Antibody with Streptavidin Affinity Gel

Biotinylated ANTI-FLAG M2 monoclonal antibody was incubated with EZview Red Streptavidin Affinity Gel under saturating conditions and washed to remove unbound antibody. This affinity gel (EZview SA) was compared to EZview Red ANTI-FLAG Affinity Gel (EZview M2) for capture of a FLAG-tagged target protein (FLAG-p53(Δ1-71)) expressed in COS-7 cells.

Target/Bait Protein Capture

The streptavidin affinity gel with the ANTI-FLAG M2 antibody attached through biotin performed at least as well as the affinity gel with the M2 antibody covalently attached for capture of the FLAG-tagged target/bait protein, as assessed by Western blot (FLAG-p53(Δ1-71); Fig. 5, left panel).

Interacting Protein Capture

Immunostaining of a duplicate blot of the same samples for a known p53-interacting protein (Large T Antigen), expressed endogenously in COS-7 cell, revealed that the interacting protein was co-immunoprecipitated with the target/bait protein from the transfected cell lysate (Fig. 5, right panel).

Capture of Interacting Proteins from Cells

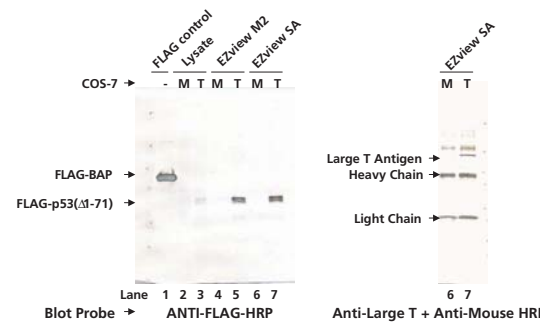


Fig. 5. Biotinylated antibody attached to EZview Red Streptavidin Affinity Gel efficiently captures target protein (FLAG-p53(Δ1-71)) and interacting protein (Large T Antigen) expressed in COS-7 cells. COS-7 cells were mock-transfected (M) or transfected (T) with a FLAG-tagged p53 construct using Escort II transfection reagent. After 3 days expression, the cells were lysed and immunoprecipitated with EZview Red ANTI-FLAG M2 Affinity Gel (EZview M2) or biotinylated ANTI-FLAG M2 monoclonal antibody (mAb) bound to EZview Red Streptavidin Affinity Gel (EZview SA). The captured material was analyzed by Western immunoblotting. The blot on the left was probed with ANTI-FLAG M2 mAb conjugated to horseradish peroxidase (HRP). The blot on the right was probed with Anti-SV40 Large T Antigen mAb followed by rabbit Anti-Mouse-HRP conjugate. Both blots were developed with TMB substrate.

Identification of Interacting Proteins by MALDI-MS

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) has become a powerful tool in proteomics for identifying proteins by signature masses of derived tryptic digest peptides.

Isolation of Interacting Proteins

We immunoprecipitated an expressed FLAG-tagged target/bait protein from transfected COS-7 cell lysates, using biotinylated ANTI-FLAG M2 monoclonal antibody and EZview Red Streptavidin Affinity Gel as above. The target/bait protein and the co-immunoprecipitated protein bands were excised after SDS-PAGE analysis (Fig. 6) and subjected to in-gel reduction and alkylation, followed by in-gel trypsin digestion.

Interacting Protein Isolation

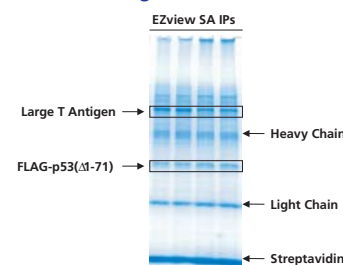


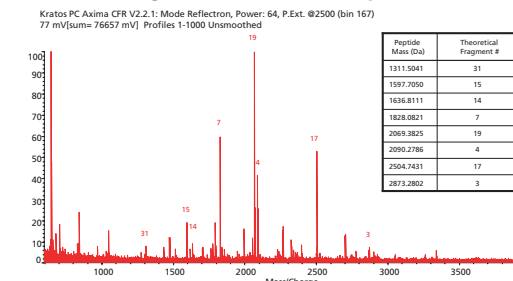
Fig. 6. Isolation of target and interacting proteins expressed in COS-7 cells. The FLAG-p53(Δ1-71) target/bait construct was transfected and expressed in COS-7 cells as in Fig. 5. Lysates were immunoprecipitated with biotinylated ANTI-FLAG M2 mAb bound to

EZview Red Streptavidin Affinity Gel. The final wash and elution of bound material was done in spin columns, to allow more concentrated recovery of proteins. The eluted proteins were separated by SDS-PAGE and visualized by EZBlue Gel Stain. The bands indicated by the rectangular boxes were cut from the gel, destained, and subjected to in-gel reduction with tributylphosphine and alkylation with iodoacetamide, followed by in-gel digestion with Proteomics Grade Trypsin (dimethylated). The resulting tryptic fragments were analyzed by MALDI-MS (see Fig. 7).

MALDI-MS Analysis

The MALDI-MS spectra of the experimental tryptic peptides and the identified theoretical peptide fragments are shown below for the target/bait protein (FLAG-p53(Δ1-71); Fig. 7A) and the co-immunoprecipitated protein (Large T Antigen; Fig. 7B).

MALDI-MS Analysis A. Target/Bait Protein: FLAG-p53(Δ1-71)



MALDI-MS Analysis B. Interacting Protein: Large T Antigen

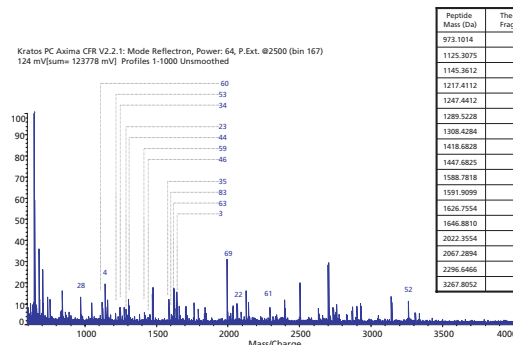


Fig. 7. Identification of interacting proteins by MALDI-MS analysis of tryptic peptides. After in-gel trypsin digestion, the peptide fragments were processed using ZipTips™ (Millipore), and were analyzed by MALDI-MS using a Kratos Axima CFR mass spectrometer in the reflectron mode. The mass spectra from the 500-4,000 Dalton mass range are shown with the theoretical tryptic fragment numbers and corresponding experimental peptide masses indicated in the tables on the right of each mass spectrum. The identified peptide fragment numbers are shown above the corresponding peaks in each spectrum. From the FLAG-p53(Δ1-71) protein digest, 8 of 28 theoretical trypsin cleavage fragments were identified for a sequence coverage of 28%. From the SV40 Large T Antigen protein digest, 17 of 56 theoretical trypsin cleavage fragments were identified for a sequence coverage of 30%.

Sequence Coverage (500-4,000 Dalton Mass Range)

Protein	Peptides Identified	Theoretical Fragments	Sequence Coverage
FLAG-p53(Δ1-71)	8	28	28%
Large T Antigen	17	56	30%

The peptides identified from the tryptic digests allowed identification of the proteins from known sequences in protein databases.

Discussion

- 1) Resins were made with enhanced visibility by conjugating low-protein binding dyes to agarose.
- 2) The EZview Red Affinity Gels were functionally equivalent to standard agarose affinity gels in IP applications.
- 3) An epitope-specific affinity gel, made by attaching an epitope-specific biotinylated antibody to a streptavidin affinity gel, functions as well as a covalently attached antibody affinity gel for IP of an epitope-tagged target/bait protein.
- 4) Interacting proteins can be identified by immunoblotting and/or MALDI-MS analysis of tryptic peptides after capture by a biotinylated antibody attached to EZview Red Streptavidin Affinity Gel.

Conclusion

Affinity resins with enhanced visibility are easy to see and use and can improve quantitative recovery of the resin and target molecules for more reproducible results.

One such resin, EZview Red Streptavidin Affinity Gel, allows simple creation of specific affinity gels by simple and tight binding through biotin on specific antibodies or other proteins that have been chemically biotinylated.

We demonstrate that these affinity resins have utility in study of protein-protein interactions by immunoblotting and by modern proteomic techniques, such as identification of interacting proteins by MALDI-MS analysis for identification of signature tryptic peptides.

Acknowledgements

We would like to thank Stephanie Uder, Ken Heuermann, Rick Mehig and members of the Sigma-Aldrich Biotechnology Protein R&D group for discussions and suggestions during this work. Also, we thank the Sigma-Aldrich Undergraduate Co-op program for support of Patty Lindbloom, Max Huang and Tom Rutkoski, who also participated in the development of the EZview Red Affinity Gels.

References

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Materials

Product Name	Product Number
ANTI-FLAG® M2 Affinity Gel	A 2220
EZview™ Red ANTI-FLAG® M2 Affinity Gel*	F 2426
Streptavidin Agarose	S 1638
EZview™ Red Streptavidin Affinity Gel*	E 5529
3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate for Membranes	F 9291
ANTI-FLAG® M2 mAb-AP Conjugate	A 9469
ANTI-FLAG® M2 mAb-HRP Conjugate	A 8592
Rabbit Anti-Mouse IgG-HRP Conjugate	A 9044
NBT/BCIP Alkaline Phosphatase Substrate	B 5655
EZBlue™ Gel Staining Reagent	G 1041
ProteoPrep™ Reduction and Alkylation Kit	PROT-0R
ProteoProfile™ Trypsin In-Gel Digest Kit	PP0100

All other reagents were obtained from Sigma-Aldrich, except Anti-Large T Antigen Antibody (Santa Cruz) and ZipTips™ (Millipore).

*Patent Pending