Technical Bulletin

EZview™ Red ANTI-FLAG® M2 Affinity Gel

F2426

Product Description

EZview™ Red ANTI-FLAG® M2 Affinity Gel is a highly visible, red-colored ANTI-FLAG® M2 agarose affinity gel, designed for use in immunoprecipitation (IP) experiments. The affinity resin contains ANTI-FLAG® M2 monoclonal antibody covalently attached to crosslinked 4% agarose beads.

EZview™ Red ANTI-FLAG® M2 Affinity Gel functions to bind FLAG® fusion proteins from cell lysates and other biological samples in the same manner as the standard non-colored ANTI-FLAG® M2 Monoclonal Antibody Agarose Affinity Gel (Cat. No. A2220). FLAG® fusion proteins, bound to the ANTI-FLAG® Affinity Gel, are recovered by centrifugation. The red color gives the affinity gel enhanced visibility, to ease subsequent steps such as repetitive washing, recovery of the affinity resin beads, and recovery of the bound FLAG® fusion proteins. The enhanced visibility results in less tedious sample processing and greater experimental reproducibility, for more accurate quantitation of the proteins of interest.

Several theses[6-7] and dissertations[8-29] cite use of this product in their research protocols.

Reagent

EZview™ Red ANTI-FLAG® M2 Affinity Gel is supplied as a ~50% slurry suspension in phosphate buffered saline (PBS), containing 50% glycerol and 0.0015% (15 ppm) Kathon® CG/IPCII as an antimicrobial preservative.

Binding Specificity: FLAG® octapeptide (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C), at N-terminal, C-terminal, or Met-N-terminal locations of a fusion protein.

Binding capacity: ≥ 0.6 mg per mL of packed resin, testing with a bacterial alkaline phosphatase FLAG® fusion protein.

Equipment and Reagents to be Supplied by User

- Cells to be used for preparation of lysate
- Lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TRITON® X-100), CelLytic™ M (Cat. No. C2978) or CelLytic™ B (Cat. Nos. B7435, B7310, or C8740)
- Tris Buffered Saline (TBS): 50 mM Tris-HCl, 150 mM NaCl, pH 7.4
- Vortex Mixer
- Protease Inhibitor Cocktail (Cat. Nos. P8340 or P2714)
- Pipette tips (200 µL)
- Pipette tips, wide orifice (200 µL)
- Pipette tips (1000 µL)
- Pipette, 200 µL
- Pipette, 1000 µL
- 1.5 mL Microcentrifuge Tubes (e.g. Cat. No. T9661)
- 2× Laemmli Sample Buffer (Cat. No. S3401)
- 3X FLAG Peptide (Cat. No. F4799), or Glycine HCl (e.g. Cat. No. G2879)

Storage/Stability

EZview™ Red ANTI-FLAG® M2 Affinity Gel should be stored in 50% glycerol at –20 °C for maximum stability. The unopened product is stable for one year when stored as indicated. **Do not freeze in the absence of glycerol**.

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Procedure

Note: It is recommended that the entire Technical Bulletin be read before use, especially the Reagent Compatibility Table on Page 6 of this Bulletin. There are many different procedures and variations for performing IP experiments. The investigator should choose the specific method to suit the particular experiment. See Reference 1 for additional information and procedures.[1]

The following generic procedure is intended to be an example or starting point. It may not be appropriate for all situations. The procedure is written for one sample and is appropriate for most mammalian tissue culture cell lines. For multiple IP reactions, calculate the volume of reagents needed according to the number of samples to be processed. For easy performance of IP reactions, use 40 µL of gel suspension per reaction (~20 µL packed gel volume).

Smaller amounts of resin (~10 µL packed gel volume, which bind >1 µg FLAG® fusion protein) can be used.

Manipulations should be carried out on ice or at 2-8 °C, unless otherwise specified.

Immunoprecipitation of FLAG® fusion proteins

1. Carefully mix EZview™ Red ANTI-FLAG® M2 Affinity Gel beads until completely and uniformly suspended. Immediately aliquot 40 µL of the 50% slurry into a clean 1.5 mL microcentrifuge tube on ice. To dispense beads, use a wide orifice pipette tip or cut ~1 mm off the tip of a regular pipette tip, to enlarge the opening and allow unrestricted flow of the bead suspension.
2. Wash/equilibrate beads: Add 500 µL of TBS (50 mM Tris HCl, 150 mM NaCl, pH 7.4) to the tube, vortex, and centrifuge in a microcentrifuge for 30 seconds at 8,200 × *g* (10,000 rpm in an Eppendorf® 5415C microcentrifuge). Carefully remove the supernatant with pipette (or carefully aspirate supernatant) and set tube with the bead pellet on ice.
3. Repeat wash as above. After removing supernatant, set washed bead pellet on ice. **Note**: In case of multiple IP samples, the amount of resin needed for all samples can be washed together. Each wash should be performed with TBS at a volume of at least 20 times the total packed gel volume. The washed resin can then be aliquoted for the desired number of samples.
4. Clarify lysate of denatured protein and cell debris by centrifugation for 10 minutes 8,200 × *g* at 2-8 °C. Add 200-1,000 µL cell lysate supernatant to the washed resin. If necessary, bring the final volume to 1 mL by adding lysis buffer. The volume of cell lysate to be used depends on the expression level of FLAG® fusion protein in the transfected cells.
5. Gently agitate or shake all samples and controls (a roller shaker is recommended) for 1-2 hours at 2-8 °C. The binding step may be extended overnight to ensure maximum binding.
6. Centrifuge in microcentrifuge for 30 seconds at 8,200 × *g*. Place on ice. Aspirate supernatant carefully or remove with a pipette. The supernatant may be saved for analysis, if desired. Place the tube with the bead pellet on ice.
7. Wash the bead pellet by adding 500 µL of TBS. Vortex briefly and incubate with thorough, gentle mixing at 2-8 °C for 5 minutes. Centrifuge in microcentrifuge for 30 seconds at 8,200 × *g*. Aspirate supernatant carefully (or remove with a pipette). Place the tube with the bead pellet on ice.
8. Repeat washes two more times as in Step 7. After removing the final wash supernatant, the bound antigen can be eluted and analyzed as desired (see Analysis of Results, below).

Analysis of Results

Elution of the FLAG® fusion protein

Three elution methods are suggested:

- Elution under native conditions by competition with 3X FLAG® peptide. The elution efficiency is very high using this method.
- Elution under acidic conditions with 0.1 M glycine HCl, pH 3.5. This is a fast and efficient elution method. Immediate neutralization of the eluted protein with wash buffer may help preserve its activity.
- Elution with sample buffer for gel electrophoresis and immunoblotting.

Choice of method will depend on protein characteristics and downstream applications.

Elution with 3X FLAG® peptide

1. Prepare 3X FLAG® elution concentrate by dissolving 3X FLAG® peptide (Cat. No. F4799) in 0.5 M Tris-HCl (pH 7.5) and 1 M NaCl at a concentration of 25 µg/µL. Dilute 5-fold with water to prepare a 3X FLAG® stock solution containing 5 µg/µL 3X FLAG® peptide. For 3X FLAG® elution solution, add 3 µL of 5 µg/µL 3X FLAG® peptide stock solution to 100 µL of TBS (150 ng/µL final concentration).
2. Add 100 µL of 3X FLAG® elution solution (150 ng/µL) to each sample and control resin.
3. Incubate the samples and controls with gentle shaking for 30 minutes at 2-8 °C.
4. Centrifuge the resin for 30 seconds at 8,200 × *g*. Transfer the supernatants to fresh test tubes. Be careful not to transfer any resin**.**
5. For immediate use, store the supernatants at 2-8 °C. Store at –20 °C for long-term storage.

Elution with 0.1 M glycine HCl at pH 3.5

Note: The procedure should be performed at room temperature. **Do not leave the resin in this buffer more than 20 minutes.**

1. Add 100 µL of 0.1 M glycine HCl, pH 3.5, buffer to each sample and control resin.
2. Incubate the samples and controls with gentle shaking for 5 minutes at room temperature.
3. Centrifuge the resin for 5 seconds at 8,200 × *g*. Immediately transfer the supernatants to fresh test tubes containing 10 µL of 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, to neutralize samples. Be careful not to transfer any resin.
4. For immediate use, store the supernatant at 2-8 °C. Store at –20 °C for long-term storage.

Elution with SDS-PAGE Sample Buffer

Notes:

- To minimize the denaturation and elution of the antibody, **no reducing agent should be included in the sample buffer**. Examples of such reducing agents are 2-mercaptoethanol and DTT. Addition of reducing agents will result in dissociation of the heavy and light chains of the immobilized M2 antibody (25 kDa and 50 kDa bands).
- However, if reducing conditions are absolutely necessary, reducing agent may be carefully included. The final concentration of 2-mercaptoethanol or DTT in the 1× sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% (v/v) glycerol, and 0.002% bromophenol blue) should be 5% or 50 mM, respectively.
- SDS in the sample buffer will denature the M2 antibody. Consequently, the EZview™ Red ANTI-FLAG® M2 Affinity Gel beads cannot be reused after treatment with the SDS-PAGE sample buffer.

1. Add 20 µL of 2× Laemmli Sample Buffer (Cat. No. S3401) (125 mM Tris HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, and 0.004% bromophenol blue) to each sample and control.
2. Boil the samples and controls for 5 minutes.
3. Vortex briefly and centrifuge the samples and controls at 8,200 × *g* for 30 seconds. The supernatants may be transferred to fresh tubes or may be directly loaded onto SDS-PAGE for subsequent analysis by staining, autoradiography or immunoblotting, using ANTI-FLAG® antibodies or specific antibodies against the fusion protein.

Enzyme assays

Enzyme assays, such as kinase assays, can be performed by adding assay mixture and substrate directly into the sample tube. The bead pellet first should be equilibrated in assay buffer by pre-washing in assay buffer before the assay.

Troubleshooting

See the Troubleshooting Guide table on Page 5.

Because of the enhanced visibility of the EZview™ Red affinity resin beads, it is easy to see if beads are accidentally removed during wash steps. If this happens, simply return the wash supernatant to the tube, and repeat the centrifugation step to pellet the resin again.

Controls:

- For a positive control, add 1 mL TBS and 4 µL of 50 ng/µL FLAG-BAP™ fusion protein (~200 ng) to a comparable tube of washed resin.
- The amount of FLAG-BAP™ fusion protein to be precipitated depends on the detection method.
- 200 ng of protein is sufficient for an activity assay or for an immunoblot analysis.
- For SDS-PAGE analysis with Coomassie® blue or silver staining, use 1 µg of FLAG-BAP™ fusion protein.
- For a negative control to monitor non-specific background, use a comparable lysate volume from a lysate of cells that do not express the FLAG® fusion protein.
- Also, FLAG® peptide can be used as a competitor in the immunoprecipitation to show specificity for the FLAG® fusion protein.

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Troubleshooting Guide

Problem	Possible Cause	Solution
No signal is observed.	FLAG® fusion protein is not present in the sample.	• Make sure the protein of interest contains the FLAG tag by immunoblot or dot blot analyses. • Prepare fresh lysates. Avoid using frozen lysates. • Use appropriate protease inhibitors in the lysate, or increase their concentrations to prevent degradation of the FLAG® fusion protein.
	Washes are too stringent.	• Reduce the number of washes. • Avoid adding high concentrations of NaCl to the mixture. • Use solutions that contain less or no detergent.
	Incubation times are inadequate.	Increase the incubation times with the affinity resin (from several hours to overnight).
	Interfering substance is present in sample.	• Lysates containing high concentrations of dithiothreitol (DTT), 2-mercaptoethanol, or other reducing agents may destroy antibody function, and must be avoided. • Excessive detergent concentrations may interfere with the antibody-antigen interaction. Detergent levels in buffers may be reduced by dilution.
	Detection system is inadequate.	If Western blotting detection is used: • Check primary and secondary antibodies using proper controls to confirm binding and reactivity. • Verify that the transfer was adequate by staining the membrane with Ponceau S. • Use fresh detection substrate or try a different detection system.
Background is too high.	Proteins bind non-specifically to the ANTI-FLAG® monoclonal antibody, the resin beads, or the microcentrifuge tubes.	• Pre-clear lysate with Mouse IgG-Agarose (Cat. No. A0919) and/or EZview™ Red Protein A Affinity Gel (Cat. No. P6486) to remove non-specific binding proteins. • After suspending beads for the final wash, transfer entire sample to a clean microcentrifuge tube before centrifugation.
	Washes are insufficient.	• Increase the number of washes. • Prolong duration of the washes, incubating each wash for at least 15 minutes. • Increase the salt and/or detergent concentrations in the wash solutions. • Centrifuge at lower speed to avoid non-specific trapping of denatured proteins from the lysate during the initial centrifugation of the affinity resin complexes.

Reagent Compatibility Guide

Reagent	Effect	Comments
Chaotropic agents (such as urea, guanidine HCl)	Denatures the immobilized M2 antibody	**Do not** use any reagent that contains chaotropic agents, since chaotropic agents will denature the M2 antibody on the resin and destroy its ability to bind FLAG® fusion proteins. Low concentrations of urea (1 M or less) can be used.
Reducing agents (such as DTT, DTE, 2-mercapto-ethanol)	Reduces the disulfide bridges holding the M2 antibody chains together	**Do not** use any reagent that contains reducing agents, since reducing agents will reduce the disulfide linkages in the M2 antibody on the resin and destroy its ability to bind the FLAG® fusion proteins.
Deoxycholate	Interferes with M2 binding to FLAG® fusion proteins	**Do not** use any reagent that contains deoxychoate, since deoxycholate will inhibit the M2 antibody from binding to FLAG® fusion proteins.
Sodium dodecyl sulfate (SDS)	Denatures the immobilized M2 antibody	**Do not** use any reagent that contains SDS in the lysis and washing buffers, since SDS will denature the M2 antibody on the resin and destroy its ability to bind FLAG® fusion proteins. (SDS is included in the sample buffer for removal of protein from the affinity resin after immunoprecipitation, but the resin cannot be re-used.)
TWEEN® 20	Reduces non-specific protein binding to the resin	May be used up to a concentration of 5%. Do not exceed.
TRITON® X-100	Reduces non-specific protein binding to the resin	May be used up to a concentration of 5%. Do not exceed.
IGEPAL® CA-630 (NP-40)	Reduces non-specific protein binding to the resin	May be used up to a concentration of 0.1%. Do not exceed.
CHAPS	Reduces non-specific protein binding to the resin	May be used up to a concentration of 0.1%. Do not exceed.
Digitonin	Reduces non-specific protein binding to the resin	May be used up to a concentration of 0.2%. Do not exceed.
Sodium chloride (NaCl)	Reduces non-specific protein binding to the resin by reducing ionic interactions	May be used up to a concentration of 1.0 M. Do not exceed.
0.1 M glycine HCl, pH 3.5	Elutes FLAG® fusion protein from the resin	Do not leave the affinity resin in glycine-HCl for longer than 20 minutes. Longer incubation times will begin to denature the M2 antibody.

Related Products

- Mammalian sequencing primers (e.g. Cat. No. P5350, N-CMV-30 Sequencing Primer)
- ANTI-FLAG® M1 Monoclonal Antibody (Cat. No. F3040)
- ANTI-FLAG® M1 Agarose Affinity Gel (Cat. No. A4596)
- ANTI-FLAG® M2 Monoclonal Antibody (Cat. Nos. F3165 and F1804)
- ANTI-FLAG® M2 Affinity Gel (Cat. No. A2220)
- ANTI-FLAG® M5 Monoclonal Antibody (Cat. No. F4042)
- FLAG® Immunoprecipitation Kit (Cat. No. FLAGIPT1)
- Bicinchoninic Acid (BCA) Kit for Protein Determination (Cat. No. BCA1)
- QuantiPro™ BCA Assay Kit (Cat. No. QPBCA)
- FLAG-BAP™ control fusion proteins (Cat. Nos. P7582, P7457, and P5975)
- Protease inhibitor cocktails (for general, bacterial, mammalian, fungal & yeast, plant, tissue culture, and histidine-tagged; Cat. Nos. P2714, P8465, P8340, P8215, P9599, P1860, and P8849)
- Phosphatase inhibitor cocktails (Cat. Nos. P2850, P5726, and P0044)
- FLAG® peptide (Cat. No. F3290)
- 3X FLAG® peptide (Cat. No. F4799)
- ANTI-FLAG® M2-Peroxidase (HRP) Conjugate (Cat. No. A8592)
- ANTI-FLAG® M2-Alkaline Phosphatase Conjugate (Cat. No. A9469)
- Dithiothreitol (DTT) (Cat. No. D9779)
- 2-Mercaptoethanol (Cat. No. M3148)
- Glycine hydrochloride (Cat. Nos. G2879)
- EZview™ Red Protein A Affinity Gel (Cat. No. P6486)

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