Technical Bulletin

ANTI-FLAG® M2 Magnetic Beads

M8823

Product Description

ANTI-FLAG® M2 Magnetic Beads consist of a mouse-derived, ANTI-FLAG® M2 monoclonal antibody that has been attached to superparamagnetic iron-impregnated 4% agarose beads with an average diameter of 50 µm. The M2 antibody binds to fusion proteins that contain the FLAG® peptide sequence.[1] The M2 antibody recognizes the FLAG® octapeptide sequence (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) at the N-terminus, Met-N-terminus, or C-terminus locations of a FLAG®-tagged fusion protein in mammalian and bacterial extracts.

The ANTI-FLAG® M2 Magnetic Beads are useful for detection and capture of FLAG®-tagged fusion proteins by commonly used immunoprecipitation procedures. The magnetic properties allow for rapid separation of the beads from a suspension, to facilitate experimental procedures, such as repetitive washings or processing of multiple samples performed in multiwell plates. Several theses[3-4] and dissertations[5-46] have cited use of this M8823 product in their research protocols.

Reagent

The ANTI-FLAG® M2 Magnetic Bead resin is supplied as a 50% suspension in 50% glycerol with 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4, and 0.02% (w/v) sodium azide.

Binding capacity: 0.6 mg of FLAG® fusion protein per 1 mL of packed magnetic beads.

Specificity: ≥ 90% specificity towards FLAG® fusion proteins from mammalian and bacterial cell extracts.

Equipment Required but Not Provided

Magnetic Separators for:

- Microcentrifuge tubes (Cat. No. M1167)
- Tissue culture flasks
- Centrifuge tubes

Magnet for 96-well tissue culture plates (Cat. No. SHM05)

Magnetic plate for standard sized well plates, T-25 through T-75 tissue culture flasks, and up to 5 cm dishes (Cat. No. SHM04)

Do not use a magnetic stirring system. A magnetic stirring system will destroy the resin beads.

Storage/Stability

The ANTI-FLAG® M2 Magnetic Beads ship on wet ice. Storage at –20 °C is recommended. The product is supplied in a 50% glycerol solution with preservative and is stable for 2 years at –20 °C. After use, the resin should be cleaned and stored in 50% glycerol with TBS or PBS that contains preservative, to protect the product. **Freezing the magnetic beads in the absence of 50% glycerol will irreversibly damage the bead structure.**

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.

There are many different procedures for performing small-scale affinity capture experiments. The following procedures are written for a single sample.

For batch-wise purification, 100 µL of the resin bead suspension per reaction (~50 µL of packed gel) is recommended. For use in a 96-well plate format, 10 µL of packed gel is recommended per well. The amount of resin bead can be varied, depending on the amount of target protein in the sample and the type of magnetic separator utilized.

Part I. Sample Preparation

Adjust the pH of the protein extract to a value between pH 7-8. It is also useful to adjust the salt concentration with NaCl or KCl to ≥ 0.15 M in the protein extract, to help mitigate non-specific protein binding to the resin.

The FLAG® fusion protein extract must be clarified to remove any insoluble material. A large amount of insoluble material may require centrifugation (10,000-20,000 × *g* for 15 minutes) for removal. The protein extract should also be filtered through a 0.45 or 0.22 µm filter, to remove any remaining cell debris and particulates that may interfere with protein binding.

Part II. Binding Procedures

To purify FLAG® fusion proteins, the resin can be used in either a batch or a 96-well plate format. For larger lysate volumes, the batch format is recommended to capture the target protein quickly from a large volume of extract. For purifying smaller samples, the FLAG® fusion protein can be immunoprecipitated.

Batch Format for Absorption of FLAG® Fusion Proteins with ANTI-FLAG® M2 Magnetic Beads

This procedure provides a quick and efficient way to purify FLAG® fusion proteins from a dilute solution.

The ANTI-FLAG® M2 Magnetic Beads are stored in 50% glycerol with buffer. The glycerol must be removed just prior to use, and the resin equilibrated with buffer (Steps 1-5). The equilibration can be done at room temperature or at 2-8 °C. Remove only the volume of resin that is necessary for purification (see Table 1).

Table 1. Binding Capacity of Beads

Packed Gel Volume (µL)	Binding Capacity (µg)
20	~ 12
50	~ 30
100	~ 60
200	~ 120

Do not allow the resin to remain in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) buffer for extended periods of time (> 24 hours) unless an antimicrobial agent (such as 0.02% sodium azide) is added to the buffer.

1. Thoroughly resuspend the resin by gentle inversion. Make sure the bottle of ANTI-FLAG® M2 Magnetic Beads is a uniform suspension. Remove an appropriate volume for use (see Table 1).
2. Transfer resin to an appropriately sized tube. Equilibrate beads by resuspending with 5 packed gel volumes of TBS (50 mM Tris HCl, 150 mM NaCl, pH 7.4). Mix thoroughly. Place tube in the appropriate magnetic separator to collect the beads. Remove and discard the storage buffer/TBS mixture.
3. Equilibrate beads by resuspending with an additional 5 packed gel volumes of TBS (50 mM Tris HCl, 150 mM NaCl, pH 7.4). Mix thoroughly.
4. Place tube in the appropriate magnetic separator to collect the beads. Remove and discard TBS buffer.
5. Repeat Steps 3 and 4 once. Allow a small amount of buffer to remain on the top of the beads.
6. Incubate the protein extract (see Sample Preparation) with the equilibrated beads (Step 5) for ~1 hour at room temperature with gentle mixing to capture the FLAG® fusion proteins. Mixing should be done on either a rotating device or a platform shaker. **Do not use a magnetic stirring system. A magnetic stirring system will destroy the resin beads.**
7. Once the binding step is complete, collect the magnetic beads by placing the tube in the appropriate magnetic separator. Remove the supernatant.
8. Wash the resin beads with TBS to remove nonspecifically bound proteins. Washing should be done with 20 packed gel volumes of TBS, performed in three sequential bead washings.

   Note: The washing process can be monitored by measuring the absorbance of the supernatant at 280 nm. Continue washing the resin until the absorbance difference between the wash solution aspirated from the beads and the wash solution (TBS) blank is < 0.05.
9. The FLAG® fusion proteins can be eluted from the magnetic beads either by a low pH method, or by competition with the FLAG® peptide.

- Elution under low pH conditions with glycine: Elute the bound FLAG® fusion protein from the magnetic beads with 10 packed gel volumes of 0.1 M glycine HCl, pH 3.0, collecting 1 packed gel volume of eluate in a vial containing 15-25 µL of 1 M Tris, pH 8.0. Do not leave eluate in the glycine HCl solution for longer than 20 minutes. Re-equilibrate the resin to neutral pH as soon as possible after elution.
- Elution by competition with the FLAG® Peptide: Elute the bound FLAG® fusion protein by competitive elution with 5 packed gel volumes of a solution containing the FLAG® peptide (100 µg/mL, Cat. No. F3290) in TBS.

10. Cleaning the Magnetic Beads: It is recommended to clean the beads immediately after use by washing with 3 packed gel volumes of 0.1 M glycine HCl, pH 3.0. The beads should be immediately re-equilibrated in TBS until the effluent is at neutral pH.

11. Storing the Magnetic Beads: After cleaning the beads, collect the magnetic beads by placing the tube in the appropriate magnetic separator, and remove the buffer. The beads may be stored as a 50% suspension in 50% glycerol with TBS or PBS containing 0.02% sodium azide. Store the beads at 2-8 °C or –20 °C without draining.

Format for Immunoprecipitation of FLAG® Fusion Proteins using ANTI-FLAG® M2 Magnetic Beads

This procedure is recommended for the purification of small amounts of FLAG® fusion proteins.

Note: For antigens and protein:protein complexes that require a special lysis buffer with a different percentage of detergent, it is suggested to pre-test the resin before use. The ANTI-FLAG® M2 resin bead is resistant to various detergents up to the following concentrations:

- 5.0% TWEEN® 20
- 5.0% Triton™ X-100
- 0.1% IGEPAL® CA-630
- 0.1% CHAPS
- 0.2% digitonin

The ANTI-FLAG® M2 resin bead can be used with 1.0 M NaCl or 1.0 M urea. See the Reagent Compatibility Table for use with additional reagents.

The following procedure is an example of a single immunoprecipitation (IP) reaction. For multiple IP reactions, calculate the volume of reagents needed according to the number of samples to be processed. For IP reactions, it is recommended to use 40 µL of the 50% bead suspension per reaction (~20 µL of packed gel volume). Smaller amounts of resin (~10 µL of packed gel volume, which binds >1 µg FLAG® fusion protein) can be used.

Note: Two control reactions are recommended for the procedure. The first control is IP with FLAG-BAP™ fusion protein (positive control). The second is a reagent blank with no protein (negative control).

1. Thoroughly resuspend the resin by gentle inversion. Make sure the bottle of ANTI-FLAG® M2 Magnetic Beads is a uniform suspension. Remove an appropriate volume for use (see Table 1). To lessen damage to beads, it is recommended to cut the end of the pipette tip.
2. Place tube in the appropriate magnetic separator to collect the beads. Aspirate and discard storage buffer.
3. Wash the packed gel twice with 10 packed gel volumes of TBS. Be sure most of the wash buffer is removed and no resin is discarded.

   Note: For multiple IP samples, wash the total volume of resin needed for all samples together. After washing, resuspend the resin in TBS and divide the resin according to the number of samples tested. Place tube in the appropriate magnetic separator to collect the beads. Remove and discard TBS.
4. Add 200 µL-1000 µL of cell lysate to the washed resin beads. If necessary, bring the final volume to 1 mL by adding lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton™ X-100). The volume of cell lysate to use depends on the expression level of FLAG® fusion protein in the transfected cells. For the positive control, add 1 mL of TBS and 4 µL of 50 ng/µL FLAG-BAP™ fusion protein (~200 ng) to the washed resin beads. For the negative control, add 1 mL of lysis buffer only with **no** protein. 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 detection, use 1 µg of FLAG-BAP™ fusion protein.
5. Agitate or shake (a roller shaker is recommended) gently all samples and controls for 2 hours. To increase the binding efficiency, the binding step may be extended overnight.
6. Place tubes in the appropriate magnetic separator to collect the beads. Remove the supernatant with a narrow-end pipette tip.
7. Wash the resin 3 times with a total of 20 packed gel volumes of TBS. Make sure to remove all the supernatant with a Hamilton® syringe or equivalent device.
8. Elution of the FLAG® fusion proteins: Three elution methods are recommended according to protein characteristics or downstream usage.
   A. Elution under native conditions by competition with 3X FLAG® Peptide. The elution efficiency is very high using this method.
   B. Elution under acidic conditions with 0.1 M glycine HCl, pH 3.0. This is a fast and efficient elution method. Neutralization of the eluted protein with wash buffer may help preserve its activity.
   C. Elution with sample buffer for gel electrophoresis and immunoblotting.

Elution with 3X FLAG® Peptide

1. Prepare 3X FLAG® elution solution. Dissolve 3X FLAG® peptide (Cat. No. F4799) in 0.5 M Tris-HCl, pH 7.5, with 1 M NaCl at a concentration of 25 µg/µL. Dilute 5-fold with water to prepare a 3X FLAG® stock solution at 5 µg/µL of 3X FLAG® peptide. For elution, add 3 µL of this 5 µg/µL 3X FLAG® peptide stock solution to 100 µL of TBS (150 ng/µL final concentration).
2. Add 5 packed gel volumes of 3X FLAG® elution solution to each sample and control resin.
3. Incubate the samples and controls with gently shaking or on a rotator for 30 minutes at 2-8 °C.
4. Place tube in the appropriate magnetic separator to collect the beads. Transfer the supernatants to fresh tubes using a Hamilton® syringe or equivalent device. Be careful not to transfer any resin.
5. Repeat Steps 1-4, pooling eluates in the same tube.
6. For immediate use, store the combined eluates at 2-8 °C. Store at –20 °C for long-term storage.
7. For cleaning and storage of used resin, see Batch Format Procedure, Steps 10 and 11.

Elution with 0.1 M Glycine HCl, pH 3.0

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

1. Add 5 packed gel volumes of 0.1 M glycine HCl buffer, pH 3.0, to each sample and control resin.
2. Incubate the samples and controls with gentle shaking or on a rotator for 5 minutes at room temperature.
3. Place tube in the appropriate magnetic separator to collect the beads. Transfer the supernatants to fresh tubes containing 10 µL of 0.5 M Tris-HCl (pH 7.4) with 1.5 M NaCl, using a Hamilton® syringe or equivalent device. Be careful not to transfer any resin.
4. Repeat Steps 1-3, pooling eluates in same tube.
5. For immediate use, store the combined eluates at 2-8 °C. Store at -20 °C for long term storage.
6. For cleaning and storage of used resin, see the "Batch Format for Absorption" procedure, Steps 10 and 11.

Elution with SDS-PAGE Sample Buffer

The procedure should be performed at room temperature. Sample buffer should be at room temperature before use.

To minimize denaturation and elution of the M2 antibody, no reducing agents, such as DTT or 2-mercaptoethanol, should be included in the sample buffer. Addition of reducing agents will result in the dissociation of the light and heavy chains of the immobilized M2 antibody into 25 and 50 kDa bands.

If reducing conditions are absolutely necessary, a reducing agent may be added. 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.

Note: Elution of the bound FLAG® fusion protein as a SDS-PAGE sample causes damage to the ANTI-FLAG® M2 Magnetic Beads. The beads cannot be used again, as the SDS in the sample buffer denatures the M2 antibody. Boiling also damages the bead structure.

a. Add 20 µL of 2× sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, and 0.004% bromophenol blue) to each sample and control.
b. Boil the sample and control tubes for 3 minutes.
c. Place tubes in the appropriate magnetic separator to collect the beads.
d. Transfer the supernatants to fresh tubes with a Hamilton® syringe or a narrow-end Pasteur pipette.

The samples and controls are ready for loading on SDS-PAGE and immunoblotting using ANTI-FLAG® or specific antibodies against the 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® sequence 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 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 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 nonspecifically to the ANTI-FLAG® monoclonal antibody, the resin beads, or the microcentrifuge tubes	• Pre-clear lysate with Mouse IgG-Agarose (Cat. No. A0919) to remove nonspecific 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. • Increase 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 nonspecific trapping of denatured proteins from the lysate during the initial centrifugation of the affinity resin complexes.

Reagent Compatibility Table

Reagent	Effect	Comments
Chaotropic agents (such as guanidine HCl or urea)	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 the FLAG® fusion proteins. • If necessary, low concentrations of urea (1 M or less) can be used.
Reducing agents (such as 2-mercapto-ethanol, DTT, or DTE)	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.
TWEEN® 20, 5% or less	Reduces non-specific protein binding to the resin bead	May be used up to recommended concentration of 5%, but do not exceed.
Triton™ X-100, 5% or less	Reduces non-specific protein binding to the resin bead	May be used up to recommended concentration of 5%, but do not exceed.
IGEPAL® CA-630, 0.1% or less	Reduces non-specific protein binding to the resin bead	May be used up to recommended concentration of 0.1%, but do not exceed.
CHAPS, 0.1% or less	Reduces non-specific protein binding to the resin bead	May be used up to recommended concentration of 0.1%, but do not exceed.
Digitonin, 0.2% or less	Reduces non-specific protein binding to the resin bead	May be used up to recommended concentration of 0.2%, but do not exceed.
Sodium chloride, 1.0 M or less	Reduces non-specific protein binding to the resin bead by reducing ionic interactions	May be used up to recommended concentration of 1.0 M, but do not exceed.
Sodium dodecyl sulfate (SDS)	Denatures the immobilized M2 antibody	• **Do not** use any reagent that contains sodium dodecyl sulfate in the loading and washing buffers, since SDS will denature the M2 antibody on the resin bead and destroy its ability to bind the FLAG® fusion proteins. • SDS in the sample buffer is useful for the removal of proteins for immunoprecipitation, **but the resin bead cannot be reused**.
0.1 M glycine HCl, pH 3.5	Elutes FLAG® protein from the resin bead	Do not leave the column in glycine HCl for longer than 20 minutes. Longer incubation times will begin to denature the M2 antibody.
Deoxycholate	Interferes with M2 binding to FLAG® fusion proteins	**Do not** use any reagent that contains deoxycholate, since deoxycholate will inhibit the M2 antibody from binding to FLAG® fusion proteins.

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