Technical Bulletin

ANTI-FLAG® M2 Affinity Gel

A2220

Storage Temperature −20 °C

Product Description

ANTI-FLAG® M2 Affinity Gel is a purified murine IgG_1 monoclonal antibody covalently attached to agarose by a hydrazide linkage. It is useful for purification or immunoprecipitation of FLAG® fusion proteins. ANTI-FLAG® M2 binding to the FLAG® peptide is not dependent on calcium.

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

Reagent

ANTI-FLAG® M2 Affinity Gel is supplied as a 50% suspension in 50% glycerol with 10 mM sodium phosphate and 150 mM sodium chloride, pH 7.4, containing 0.02% (w/v) sodium azide (PBS/A).

Equipment and Reagents

(Required but not provided)

- Cells expressing FLAG® fusion protein
- Lysis buffer (50 mM Trizma®-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% TRITON® X-100), CelLytic™ M (Cat. No. C2978), or CelLytic™ B (Cat. No. B7435, B7310, or C8740)
- Appropriate centrifuge
- Appropriate column or centrifuge tubes
- Sodium chloride (e.g. Cat. No. S3014)
- Trizma® base (e.g. Cat. No. T6066)
- Protease inhibitor cocktail for use with mammalian cells and tissue extracts (Cat. No. P8340)

Storage/Stability

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. After use, the resin should be cleaned and stored in 50% glycerol with TBS or PBS buffer containing 0.02% sodium azide to protect the product. **Do not freeze in the absence of glycerol.**

Procedure

Note: It is recommended that the entire Technical Bulletin be read before use, especially the Reagent Compatibility Table.

Part I. Cell Lysate Preparation

The researcher must empirically determine the most suitable procedure. Typical methods for purifying FLAG® fusion proteins from crude *E. coli* extracts are provided. It is recommended that the CelLytic™ B Lysis Reagents (Cat. No. B7435, B7310, or C8740) or CelLytic™ B Plus Kit (Cat. No. CB0050 or CB0500) products be used for bacterial cell lysis. CelLytic™ M can be used for mammalian cells.

Recommended procedure for E. coli using CelLytic™ Lysis Reagents

1. Grow the cells (~ 1 liter or less) under conditions that induce production of FLAG® fusion proteins.
2. Harvest the cells by centrifugation at 5,000 × *g* for 30 minutes at 2–8 °C.
3. Decant the medium from the cell paste.
4. Freeze the cell paste using a dry ice/ethanol bath or at −20 °C in a freezer. Cell lysis is enhanced during the slow freezing.
5. Lyse the frozen cells with 10 mL of CelLytic™ B (Cat. No. B7435) per g of frozen cell paste or 5 mL of CelLytic™ B, 2× concentrate (Cat. No. B7310) per g of frozen cell paste.
6. Resuspend the cells in the CelLytic™ B reagent with a pipette. Mix vigorously on a stir plate for 15 minutes to fully extract the protein.

7. Remove the cell debris by centrifuging for 15 minutes at 21,000 × *g*.
8. After centrifugation, decant the supernatant into a fresh container and dispose of the cell pellet. The solution should be clear with no insoluble particles.

Recommended procedure for mammalian cells

For a 70–90% confluent 100 mm dish (10^6–10^7 cells), use 1 mL of lysis buffer (50 mM Trizma®-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% TRITON® X-100). If the expression level of the FLAG® fusion protein is relatively low, lyse the cells with a reduced volume of lysis buffer. It is highly recommended to add a protease inhibitor cocktail (Cat. No. P8340) to the lysis buffer (10 µL per 1 mL of lysis buffer), especially if the lysate is to be stored for further use.

1. Wash adherent or suspension cells if appropriate: *Adherent Cells*: Remove the growth medium from the cells to be analyzed. Rinse the cells twice with PBS (10 mM phosphate, 2.7 mM KCl, and 137 mM NaCl, pH 7.4, at 25 °C) buffer, being careful not to dislodge any of the cells. Discard the PBS. Add lysis buffer (10^6–10^7 cells/mL).

   Cells in Suspension: Collect the cells into an appropriate conical centrifuge tube. Centrifuge for 5 minutes at 420 × *g*. Decant the supernatant and discard. Wash the cells twice by resuspending the cell pellet with PBS and centrifuge for 5 minutes at 420 × *g*. Decant the supernatant and discard. Resuspend the cell pellet in lysis buffer (10^6–10^7 cells/mL).
2. Incubate the cells for 15–30 minutes on a shaker.
3. For adherent cells only, scrape and collect the cells. For cells in suspension, proceed to step 4.
4. Centrifuge the cell lysate for 10 minutes at 12,000 × *g*.
5. Transfer the supernatant to a chilled test tube. For immediate use, keep on ice. If the supernatant is not to be used immediately, store it at –70 °C.

Part II. Resin Preparation

ANTI-FLAG® M2 Affinity Gel is stored in 50% glycerol with buffer. The glycerol must be removed just prior to use, and the resin equilibrated with buffer. The equilibration can be done at room temperature or at 2–8 °C. Remove only the amount of resin necessary for the purification. Thoroughly resuspend the resin. The matrix may then be poured into a clean chromatography column using standard techniques. Do not allow the resin to remain in TBS buffer for extended periods of time (> 24 hours) unless an antimicrobial agent (for example, 0.02% sodium azide) is added to the buffer.

1. Place the empty chromatography column on a firm support.
2. Rinse the empty column twice with TBS (50 mM Trizma®-HCl, with 150 mM NaCl, pH 7.4) or another appropriate buffer. Allow the buffer to drain from the column and leave residual TBS in the column to aid in packing the ANTI-FLAG® M2 Affinity Gel.
3. Thoroughly suspend the resin by gentle inversion. Make sure the bottle of ANTI-FLAG® M2 Affinity Gel is a uniform suspension of gel beads. Remove an appropriate aliquot for use.
4. Immediately transfer the suspension to the column.
5. Allow the gel bed to drain and rinse the pipette used for the resin aliquot with TBS. The 50% glycerol buffer will flow slowly, and the flow rate will increase during the equilibration.
6. Add the rinse to the top of the column and allow to drain again. The gel bed will not form channels when excess solution is drained under normal circumstances, but do not let the gel bed run dry.
7. Wash the gel by loading three sequential column volumes of 0.1 M glycine HCl, pH 3.5. Avoid disturbing the gel bed while loading. Let each aliquot drain completely before adding the next aliquot. **Do not leave the column in glycine HCl for longer than 20 minutes.**
8. Wash the resin with 5 column volumes of TBS to equilibrate the resin for use. Do not let the bed run dry. Allow a small amount of buffer to remain on the top of the column.

Part III. Binding Procedures

For purification of FLAG® fusion proteins, the resin can be used in either a column or batch format. A column using 1–3 mL of resin will work well if the volume of cell lysate to be loaded is only ~ 100 mL. For larger volumes of lysate, the batch format is recommended to quickly capture the target protein from a large volume of extract. If a small sample (1–2 mL of cell lysate) is being purified, the FLAG® fusion protein can be immunoprecipitated.

Column Chromatography

Pre-equilibrate the column and buffers. Perform the purification at room temperature. If there is a problem with proteases, perform column chromatography at 2–8 °C, or add a protease inhibitor cocktail to the elution solution. Cellular debris and particulate matter can clog the column and must be removed prior to purification. Highly viscous samples containing chromosomal DNA or RNA can also clog the column and should be treated with an endonuclease such as Benzonase® (Cat. No. E1014) to reduce viscosity. FLAG-BAP™ positive control proteins can be used to verify the functionality of the gel.

Note: The ANTI-FLAG® M2 Affinity Gel is resistant to many detergents. Do not use reagents that are harmful or potentially harmful to antibodies or proteins in general. See the Reagent Compatibility Table for more detail.

Binding FLAG® Fusion Proteins to the Column

1. Proper binding of FLAG® fusion proteins to the ANTI-FLAG® M2 affinity column requires 0.15 M NaCl and neutral pH.
2. Load the sample onto the column under gravity flow. Fill the column completely several times or attach a column reservoir prior to loading for larger volumes. Depending upon the protein and flow rate, all of the antigen may not bind. Multiple passes over the column will improve the binding efficiency.
3. Wash the column with 10–20 column volumes of TBS. This should remove any proteins that are not bound to the M2 antibody. Allow the column to drain completely.

Select one of the two following procedures for elution.

- Elution of FLAG® Fusion Proteins by Acid Elution with Glycine: Elute the bound FLAG® fusion protein from the column with six 1 mL aliquots of 0.1 M glycine HCl, pH 3.5, into vials containing 15–25 µL of 1 M Trizma®, pH 8.0. Do not leave the column in the glycine HCl solution for longer than 20 minutes. Re-equilibrate to neutral pH as soon as possible after elution.

  OR

- Elution of FLAG® Fusion Proteins by Competition with FLAG® Peptide: Elute the bound FLAG® fusion protein by competitive elution with five one-column volumes of a solution containing 100 µg/mL FLAG® peptide (Cat. No. F3290) in TBS.

Recycling the Column

It is recommended that the column be regenerated immediately after use by washing with three column volumes of 0.1 M glycine HCI, pH 3.5. The column should be immediately re-equilibrated in TBS until the effluent is at neutral pH.

Storing the Column

Wash the column with ten column volumes of 50% glycerol with TBS or PBS buffer containing 0.02% sodium azide, then add another 5 mL of buffered glycerol containing 0.02% sodium azide and store at 2–8 °C or –20 °C without draining. When *E. coli* periplasmic extracts are applied to the column, it may be reused up to 20 times without loss of binding capacity. When *E. coli* crude cell extracts are applied to the column, it may be reused 3 times before loss of binding capacity is observed. The number of cycles observed will be dependent on variables such as sample condition, proteases, etc.

Batch Absorption of FLAG® Fusion Proteins using ANTI-FLAG® M2 Affinity Gel

This method provides a quick and efficient way to purify FLAG® fusion proteins from a dilute solution. It eliminates the time-consuming column chromatography step of placing a large volume of solution through a small amount of resin.

1. Adjust the pH of the protein extract to between pH 7–8. It is also useful to have a salt (sodium or potassium chloride) concentration of at least 0.15 M to reduce the number of proteins nonspecifically binding to the resin.
2. 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 with a 0.45 or 0.22 µm filter to remove any remaining cells and particulates that may clog the column or filter during collection of the resin in step F.
3. The ANTI-FLAG® M2 Affinity Gel must be equilibrated before use. See Procedure, Resin Preparation section.
4. Resuspend the resin in TBS and add to the protein extract.
5. Incubate the protein extract with the ANTI-FLAG® M2 Affinity Gel for ~ 1 hour with gentle mixing to capture the FLAG® fusion proteins. Mixing should be done on either an overhead mixing device or a platform shaker. **Do not** use a magnetic stirring system because this will destroy the resin beads. This step can be done at 2–8 °C or at room temperature. This incubation can go for as short as 30 minutes up to several hours. If the incubation is longer than 3 hours, protease inhibitors and antimicrobial substances should be added to prevent microbial growth and/or proteolysis.
6. Once the binding step is complete, collect the resin from the container. The resin can be collected by centrifugation (1,000 × *g* for 5 minutes) or by filtration, either in an empty column or on a Buchner funnel.
7. Wash the resin with TBS to remove the nonspecific proteins. This may be done in the column format by passing fresh buffer through the column until no more protein elutes off. The protein being eluted from the resin can be monitored by measuring the absorbance of the eluant at 280 nm. Continue washing the resin until the absorbance difference of the wash solution coming off the column is less than 0.05 versus a wash solution blank.
8. The FLAG® proteins can be eluted from the resin either by low pH or by competition with the FLAG® peptide. Follow the elution steps under Column Chromatography, section B.
9. The resin can be recycled and stored as described under Column Chromatography, sections C and D.

Immunoprecipitation of FLAG® Fusion Proteins

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

Note: For antigens and protein:protein complexes requiring a special lysis buffer composed of a different percentage of a detergent, it is recommended to pretest the resin before use. The ANTI-FLAG® M2 Affinity Gel is resistant to many detergents, such as 5.0% TWEEN® 20, 5.0% TRITON® X-100, 0.1% IGEPAL® CA-630, 0.1% CHAPS, and 0.2% digitonin. It can also be used with 1.0 M NaCl or 1.0 M urea. See the Reagent Compatibility Table for additional chemicals.

Perform all steps at 2–8 °C unless the procedure specifies otherwise. Use pre-cooled lysis and wash buffers and equipment. **Do not pre-cool** the cell lysate and elution buffers. Perform all centrifugations at 2–8 °C with pre-cooled rotors.

FLAG® Fusion Protein Immunoprecipitation

The procedure described below is an example of a single immunoprecipitation reaction. For multiple immunoprecipitation reactions, calculate the volume of reagents needed according to the number of samples to be processed. For easy performance of immunoprecipitation reactions, it is recommended to use 40 µL of gel 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 immunoprecipitation with FLAG-BAP™ fusion protein (positive control) and the second is a reagent blank with no protein (negative control).

1. Thoroughly suspend the ANTI-FLAG® M2 Affinity Gel in the vial, in order to make a uniform suspension of the resin. The ratio of suspension to packed gel volume should be 2:1. Immediately transfer 40 µL of the gel suspension to a fresh test tube. For resin transfer, use a clean, plastic pipette tip with the end enlarged to allow the resin to be transferred.

2. Centrifuge the resin at 5,000–8,200 × *g* for 30 seconds. In order to let the resin settle in the tube, wait for 1–2 minutes before handling the samples. Remove the supernatant with a narrow-end pipette tip or a Hamilton™ syringe, being careful not to transfer any resin. Narrow-end pipette tips can be made using forceps to pinch the opening of a plastic pipette tip until it is partially closed.
3. Wash the packed gel twice with 0.5 mL of TBS. Be sure that most of the wash buffer is removed and no resin is discarded. In case of numerous immunoprecipitation samples, wash the resin needed for all samples together. After washing, divide the resin according to the number of samples tested. Each wash should be performed with TBS at a volume equal to 20 times the total packed gel volume.
4. **Optional:** In order to remove any traces of an unbound ANTI-FLAG® antibody from the resin suspension, wash the resin with 0.5 mL of 0.1 M glycine HCl, pH 3.5, before continuing with the binding step. **Do not** leave the resin in glycine HCl for longer than 20 minutes. Discard the supernatant immediately, being careful to remove all supernatant from the resin. Follow with three washes consisting of 0.5 mL of TBS each.
5. Add 200–1,000 µL of cell lysate to the washed resin. If necessary, bring the final volume to 1 mL by adding lysis buffer (50 mM Trizma®-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TRITON™ X-100). The volume of cell lysate to be used 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. For the negative control, add only 1 mL of lysis buffer 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, use 1 µg of FLAG-BAP™ fusion protein.
6. Agitate or shake all samples and controls gently (a roller shaker is recommended) for 2 hours. In order to increase the binding efficiency, the binding step may be extended overnight.
7. Centrifuge the resin for 30 seconds at 5,000–8,200 × *g*. Remove the supernatants with a narrow-end pipette tip.
8. Wash the resin three times with 0.5 mL of TBS. Make sure all the supernatant is removed by using a Hamilton™ syringe or equivalent device.

Elution of the FLAG®-fusion protein

Three elution methods are recommended according to protein characteristics or further usage:

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

Elution with 3X FLAG® peptide

a. Prepare 3X FLAG® elution solution. Dissolve 3X FLAG® peptide (Cat. No. F4799) in 0.5 M Trizma®-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 containing 5 µg/µL of 3X FLAG® peptide. For elution, add 3 µL of 5 µg/µL of 3X FLAG® peptide stock solution to 100 µL of TBS (a final concentration of 150 ng/µl).
b. Add 100 µL of 3X FLAG® elution solution to each sample and control resin.
c. Incubate the samples and controls with gentle shaking for 30 minutes at 2–8 °C.
d. Centrifuge the resin for 30 seconds at 5,000–8,200 × *g*. Transfer the supernatants to fresh test tubes using a Hamilton™ syringe or equivalent device. Be careful not to transfer any resin.
e. 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, pH 3.5

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

a. Add 100 µL of 0.1 M glycine HCl, pH 3.5, buffer to each sample and control resin.
b. Incubate the samples and controls with gentle shaking for 5 minutes at room temperature.

c. Centrifuge the resin for 30 seconds at 5,000–8,200 × *g*. Transfer the supernatants to fresh test tubes containing 10 µL of 0.5 M Trizma®-HCl, pH 7.4, with 1.5 M NaCl, using a Hamilton™ syringe or equivalent device. Be careful not to transfer any resin.

d. For immediate use, store the supernatant at 2–8 °C. Store at –20 °C for long term storage.

Elution with SDS-PAGE Sample Buffer

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

In order to minimize the denaturation and elution of the antibody, no reducing agent (2-mercaptoethanol or DTT) should be included in the sample buffer. The addition of reducing agents will result in the dissociation of the heavy and light chains of the immobilized M2 antibody (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 Trizma®-HCl, pH 6.8, with 2% sodium dodecyl sulfate, 10% (v/v) glycerol, and 0.002% bromphenol blue) should be 5% or 50 mM, respectively.

Note: Sodium dodecyl sulfate in the sample buffer will denature the M2 antibody, and the ANTI-FLAG® M2 Affinity Gel cannot be reused after treatment with the SDS-PAGE sample buffer.

a. Add 20 µL of 2× sample buffer (125 mM Trizma®-HCl, pH 6.8, with 4% sodium dodecyl sulfate, 20% (v/v) glycerol, and 0.004% bromophenol blue) to each sample and control.

b. Boil the samples and controls for 3 minutes.

c. Centrifuge the samples and controls at 5,000–8,200 × *g* for 30 seconds to pellet any undissolved agarose. Transfer the supernatants to fresh test 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.

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.

References

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Reagent Compatibility Table

Reagent	Effect	Comments
Chaotropic agents (for example, urea, guanidine HCl)	Denatures the immobilized M2 antibody	Do not use any reagent that contains these types of components, since it will denature the M2 antibody on the resin and destroy its ability to bind the FLAG® fusion proteins. Low concentrations of urea (1 M or less) can be used.
Reducing agents (such as DTT, DTE, 2-mercaptoethanol)	Reduces the disulfide bridges holding the M2 antibody chains together	Do not use any reagent that contains these types of components, 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 nonspecific protein binding to the resin	May be used up to recommended concentration of 5%, but do not exceed.
TRITON™ X-100, 5% or less	Reduces nonspecific protein binding to the resin	May be used up to recommended concentration of 5%, but do not exceed.
IGEPAL® CA-630, 0.1% or less	Reduces nonspecific protein binding to the resin	May be used up to recommended concentration of 0.1%, but do not exceed.
CHAPS, 0.1% or less	Reduces nonspecific protein binding to the resin	May be used up to recommended concentration of 0.1%, but do not exceed.
Digitonin, 0.2% or less	Reduces nonspecific protein binding to the resin	May be used up to recommended concentration of 0.2%, but do not exceed.
Sodium chloride, 1.0 M or less	Reduces nonspecific protein binding to the resin by reducing ionic interactions	May be used up to recommended concentration of 1.0 M, but do not exceed.
Sodium dodecyl sulfate	Denatures the immobilized M2 antibody	Do not use any reagent that contains this detergent in the loading and washing buffers, since sodium dodecyl sulfate will denature the M2 antibody on the resin and destroy its ability to bind the FLAG® fusion proteins. It is included in the sample buffer for removal of protein for immunoprecipitation, but the resin cannot be reused.
0.1 M glycine HCl, pH 3.5	Elutes FLAG® protein from the resin	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® proteins	Do not use any reagent that contains this detergent, since it will inhibit the M2 antibody from binding to FLAG® fusion proteins.

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 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. 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 nonspecific trapping of denatured proteins from the lysate during the initial centrifugation of the affinity resin complexes.

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