3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-521-8956 • (314) 771-5765
Fax 800-325-5052 • (314) 771-5757
sigma-aldrich.com

ProductInformation

ANTI-FLAG® M2 Affinity Gel Freezer-Safe

Product No. **A 2220**
Store at 0 to –20 °C
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TECHNICAL BULLETIN

Product Description

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

ANTI-FLAG M2 affinity gel is supplied as a 50% suspension in 50% glycerol with 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4, containing 0.02% (w/v) sodium azide (PBS/A). **It is recommended that the entire technical bulletin be read before use, especially the reagent compatibility table.**

Binding Specificity:

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

Reagents and Equipment Required but not Provided
(Sigma Product Numbers have been given where appropriate)

- Cells expressing FLAG fusion protein
- Appropriate centrifuge
- CelLytic B, Product No. B 3553, or CelLytic B II, Product No. B 3678
- Appropriate column or centrifuge tubes
- Sodium chloride, Product No. S 3014
- Trizma® base, Product No T 6066
- Protease inhibitor cocktail for use with mammalian cells and tissue extracts, Product No. P 8340

Precautions and Disclaimer

For laboratory use only. Not for drug, household or other uses.

Storage/Stability

This resin is stable for at least 6 months if unopened. 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.

Preparation Instructions

I. Extract Preparation

The exact procedure must be empirically determined by the researcher, since the conditions may vary for different proteins and different extraction procedures. Typical methods for purifying FLAG fusion proteins from crude *E. coli* extracts are shown below. It is recommended that the CelLytic B (Product No. B 3553) or CelLytic B II (Product No. B 3678) products be used for cell lysis. CelLytic B can also be used for mammalian cells, or use Procedure C below. Detailed information is included the data sheets for the CelLytic products.

A. Recommended procedure for *E. coli*

1. Grow the cells (about 1 liter or less) under conditions that induce production of FLAG fusion proteins.
2. Harvest the cells by centrifugation at 5,000 X *g* for 30 minutes at 2-8 °C.
3. Decant the media 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 CelLytic B per g frozen cell paste or 5 ml of CelLytic B II per g frozen cell paste.
6. Resuspend the cells in the CelLytic B reagent with a pipet. Mix vigorously on a stir plate for 15 minutes to fully extract the cells.

7. Remove the cell debris by centrifuging for 15 minutes at 21,000 X *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.

B. Alternate (sonication) procedure for *E. coli*

1. Grow the cells (usually 1 liter) under conditions that induce production of FLAG fusion proteins.

2. Harvest the cells by centrifugation at 5,000 X *g* for 30 minutes at 2-8 °C. Discard the media.

3. Resuspend the cells in TBS (50 mM Tris HCl, 150 mM NaCl, pH 7.4).

4. Sonicate the resuspended cells for 2-5 minutes on ice to disrupt the cell wall. The cells should be kept on ice to reduce the amount of heating due to sonication.

5. Remove the cell debris by centrifuging for 15 minutes at 21,000 X *g*.

6. After centrifugation, decant the supernatant into a clean container and dispose of the cell pellet. The solution should be clear with no insoluble particles.

C. Recommended procedure for mammalian cells

For 70-90% confluent 100 mm dish (10^6-10^7 cells), use 1 ml lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). If the expression level of the FLAG-tagged protein is relatively low, lyse the cells with a reduced volume of lysis buffer. It is highly recommended to add protease inhibitor cocktail (Product No. P 8340) to the lysis buffer (10 µl per 1 ml lysis buffer), especially if the lysate is to be stored for further use.

1. Wash cells.
   a. For adherent cells.
      Remove the growth media from the cells to be assayed. Rinse the cells twice with PBS (10 mM phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4, at 25 °C) buffer, being careful not to dislodge any of the cells. Discard PBS. Add lysis buffer (10^6-10^7 cells/ml).
   b. For cells in suspension.
      Collect the cells into an appropriate centrifuge conical test tube. Centrifuge for 5 minutes at 420 X g. Decant the supernatant and discard. Wash the cells twice by re-suspending the cell pellet with PBS and centrifuge for 5 minutes at 420 X g. Decant the supernatant and discard. Resuspend the cell pellet in lysis buffer (10^6-10^7 cells/ml).

2. Incubate the cells 15-30 minutes on a shaker.

3. For adherent cells only, scrape and collect cells. For cells in suspension, proceed to step 4.

4. Centrifuge the cell lysate for 10 minutes at 12,000 X *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.

II. Resin Preparation

The ANTI-FLAG M2 affinity resin 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. Take only the amount of resin that is necessary for the purification to be done. Thoroughly resuspend the resin. The matrix may then be poured into a clean chromatography column using standard techniques.

1. Place the empty chromatography column on a firm support.

2. Rinse the empty column twice with TBS (50 mM Tris HCl, 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 with 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 crack 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. **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.

Note: Do not allow the resin to remain in TBS buffer for extended periods of time (>24 hours) unless an anti-microbial agent (e.g. 0.02% sodium azide) is added to the buffer.

Procedure

There are several different ways the ANTI-FLAG M2 affinity gel may be used. For purification of FLAG fusion proteins, the resin can be used in either a column or batch format. The column format works well when there is not a substantial difference between the volume of material to load onto the column and the amount of resin being used. If you are using 1-3 ml of resin, a column will work well if the volume of material to be loaded is only about 100 ml. For larger volumes, 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 culture) is being purified, the FLAG-tagged protein can be immunoprecipitated.

I. Column Chromatography

Pre-equilibrate the column and buffers, and 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. Viscous samples should be sonicated or treated with nuclease to reduce viscosity. FLAG•BAP positive control proteins can be used to verify the functionality of the gel.

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 on p. 8 for more detail.

A. 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 sodium chloride 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 to 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.

B. 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 at pH 3.5 into vials containing 15-25 µl of 1 M Tris, pH 8.0. Do not leave the column in glycine HCl for longer than 20 minutes. Re-equilibrate to neutral pH as soon as possible after elution.

C. 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 (Product No. F3290) in TBS.

D. 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 HCl, pH 3.5. The column should be immediately re-equilibrated in TBS until the effluent is at neutral pH.

E. 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.

II. Batch Absorption of FLAG-Tagged Proteins using ANTI-FLAG M2 Affinity Gel

This method provides a quick and efficient way to purify FLAG-tagged 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 sample to between pH 7 and 8. It is also useful to have a salt (sodium or potassium chloride) concentration of at least 0.15 M to prevent large amount of proteins binding nonspecifically to the resin.

2. The FLAG-tagged protein extract must be clarified to remove any insoluble material. A large amount of insoluble material may require centrifugation (approximately 10,000-20,000 X 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.

3. The ANTI-FLAG M2 affinity gel must be equilibrated before use. See ”Resin Preparation” under Preparation Instructions.

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 about 1 hour with gentle mixing to capture the FLAG-tagged 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 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 X g for 5 minutes) or by filtration, either in a empty column or on a Buchner funnel.

7. Wash the resin with TBS to remove all of the non-specific 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", sections B and C above.

9. The resin can be recycled and stored as described under "Column Chromatography", section D above.

III. Immunoprecipitation of FLAG Fusion Proteins

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

General notes:

1. 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 sample and elution buffers. Perform all centrifugations at 2-8 °C with pre-cooled rotors.

2. 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 the 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 on p.8 for additional chemicals.

A. 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, Sigma recommends the use of 40 µl gel suspension per reaction (~20 µl packed gel volume). Smaller amounts of resin (~10 µl packed gel volume, which binds >1 µg FLAG-tagged protein) can be used.
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 resin in its suspension buffer to a fresh test tube to allow a homogenous dispersion of the resin. For resin transfer, use a clean, plastic pipette tip with the end enlarged to allow the resin to be transferred.

2. Centrifuge the resin for 5 seconds at 10,600 X *g* (10,000 RPM, Eppendorf 5417R microcentrifuge). 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 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 Step:
   In order to remove any traces of an unbound ANTI-FLAG antibody from the resin suspension, wash the resin with 0.5 ml 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, and follow with three washes consisting of 0.5 ml TBS each.

5. Add 200-1000 µl cell lysate to the washed resin. If necessary, bring the final volume to 1 ml by adding lysis buffer (50 mM Tris 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-tagged protein in the transfected cells.

   For the positive control, add 1 ml 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 5 seconds at 10,600 X g. Remove the supernatants with a narrow-end pipette tip.

8. Wash the resin three times with 0.5 ml TBS. Make sure all the supernatant is removed by using a Hamilton syringe or equivalent device.

B. Elution of the FLAG-fusion protein

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

   - Protein elution under native condition by a 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. Equilibration of the eluted protein with wash buffer may help preserve its activity.
   - Elution with sample buffer for gel electrophoresis and immunoblotting.

   1. Elution with 3x FLAG peptide
      a. Prepare 3x FLAG elution solution. Dissolve 3x FLAG peptide (Product No. F 4799) in 0.5 M Tris HCl, pH 7.5, 1 M NaCl at a concentration of 25 µg/µl. Dilute 1:4 with water to prepare a 3x FLAG stock solution containing 5 µg/µl 3x FLAG peptide. For elution, add 3 µl of 5 µg/µl 3x FLAG peptide stock solution to 100 µl of TBS (150 ng/µl final concentration).

      b. Add 100 µl 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 5 seconds at 10,600 X g. Transfer the supernatants to fresh test tubes using a Hamilton syringe or equivalent device. Be careful not to transfer any resin**.**

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

   2. 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.**

      a. Add 100 µl 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 5 seconds at 10,600 X g. Transfer the supernatants to fresh test tubes containing 10 µl of 0.5 M Tris HCl, pH 7.4, 1.5 M NaCl, using a Hamilton syringe or equivalent device. Be careful not to transfer any resin

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

3. Elution with SDS-PAGE Sample Buffer
Note: The procedure should be preformed 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 (i.e. 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 1X sample buffer (62.5 mM Tris HCl, pH 6.8, 2% SDS, 10% (v/v) glycerol, 0.002 % bromphenol blue) should be 5% or 50 mM, respectively.

Note that the SDS in the sample buffer will denature the M2 antibody, and the ANTI-FLAG M2 affinity gel cannot be reused after treatement with the SDS-PAGE sample buffer.

a. Add 20 µl 2x sample buffer (125 mM Tris HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.004 % bromphenol blue) to each sample and control.

b. Boil the samples and controls for 3 minutes.

c. Centrifuge the samples and controls at 10,600 X g for 5 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 tagged protein.

Reagent Compatibility Table

Reagent	Effect	Comments
Chaotropic agents (e.g. 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 tagged proteins. Low concentrations of urea (1 M or less) can be used.
Reducing agents (e.g. 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 it will reduce the disulfide linkages in the M2 antibody on the resin and destroy its ability to bind the FLAG tagged proteins.
Tween 20, 5% or less	Reduces non-specific 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 non-specific 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 non-specific 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 non-specific 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 non-specific 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 non-specific 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 (SDS)	Denatures the immobilized M2 antibody	**Do not** use any reagent that contains this detergent in the loading and washing buffers since it will denature the M2 antibody on the resin and destroy its ability to bind the FLAG tagged proteins. It is included in the sample buffer for removal of protein for immunoprecipition 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.

Related Products

- Mammalian FLAG expression kits, Product No. FL-MA, FL-MA-S and FL-MC
- Mammalian expression vectors, Product No. E7273, E7398, E8770, E1775, E3762, E2275, and E2400
- Mammalian sequencing primers, Product No. P5350 and P5475
- ANTI-FLAG M1 monoclonal antibody, Product No. F3040
- ANTI-FLAG M1 antibody affinity resin, Product No. A1080
- ANTI-FLAG M2, monoclonal antibody, Product No. F3165
- ANTI-FLAG M2 affinity gel, not freezer safe, Product No. A1205
- ANTI-FLAG M5, monoclonal antibody, Product No. F4042
- FLAG peptide, Product No. F3290
- 3X FLAG peptide, Product No. F4799
- FLAG-BAP control fusion proteins, Product No. P7582, P7457, and P5975
- Dithiothreitol (DTT), Product No. D9779, or 2-Mercaptoethanol, Product No. M7154
- Glycerol, Product No. G5516
- Deoxyribonuclease I (DNase I), Product No. D4527
- Sodium azide, Product No. S8032
- Glycine, Product No. G4392
- FLAG Immunoprecipition Kit, Product No. FLAGIPT-1
- ANTI-FLAG M2-peroxidase conjugate, Product No. A8592
- ANTI-FLAG M2-alkaline phosphatase conjugate, Product No. A9469
- Protease and phosphatase inhibitor cocktails, Product No. P2714, P8465, P8340, P8215, P9599, P2850, and P5726

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