Technical Bulletin

FLAG® M Purification Kit

For mammalian expression systems

CELLMM2

Product Description

Epitope-tagged proteins can be affinity-purified using highly specific antibodies that are raised against their epitope. This is useful for the isolation of recombinant proteins or protein complexes. The use of such antibodies facilitates subsequent biochemical and immunological analysis.

The FLAG® epitope system relies on the FLAG® octapeptide (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C; DYKDDDDK), which allows FLAG® fusion proteins to retain their original conformation and function. The hydrophilic character of FLAG® increases the likelihood that it will be located on the surface of the fusion protein where it is accessible to antibodies.

The kit allows rapid and efficient affinity purification of active FLAG® fusion proteins. It can be also used for immunoprecipitation. The affinity purification is performed with ANTI-FLAG®-M2 affinity gel, which is a highly specific monoclonal antibody covalently attached to agarose resin. The use of affinity resin allows efficient binding of FLAG® fusion proteins without the need for preliminary steps and calibrations. The affinity-bound FLAG® fusion proteins can be efficiently eluted from the resin by acidic conditions or by competition with 3X FLAG® peptide. The eluted proteins can be analyzed for their activity, size, post-translational modifications, interactions, and other properties.[1-5]

The FLAG® M Purification Kit includes the CelLytic™ M Cell Lysis Reagent. CelLytic™ M enables efficient and rapid cell lysis, and solubilization of proteins for cells in suspension and adherent cells such that adherent cells do not require scraping from culture dishes.

Several publications cite use of the CELLMM2 product in their research protocols.[6-10]

Storage/Stability

The kit is shipped on wet ice and stored at -20 °C. Upon first thawing, the buffers may be stored at 2-8 °C. For recommended storage of the 3X FLAG® peptide (Cat. No. F4799) and the Amino-terminal FLAG-BAP™ fusion protein (Cat. No. P7582), see the Preparation Instructions.

CelLytic™ M Cell Lysis Reagent (Cat. No. C2978) may appear cloudy after an extended period of storage. Product performance is unaffected, and it may be used, as is, without further filtration or clarification.

Reagents

Sufficient reagents are provided for 3-5 affinity purifications through a 1 mL affinity purification column.

- CelLytic™ M (Cat. No. C2978): 120 mL
- 10× Wash Buffer [Component Number W0390; 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl]: 60 mL
- Elution Buffer [Component Number E6150; 0.1 M Glycine (pH 3.5)]: 60 mL
- 3X FLAG® Peptide (Cat. No. SAE0194): 4 mg
- ANTI-FLAG® M2-Agarose Affinity Gel (Cat. No. A2220): 1 mL
- Amino-terminal FLAG-BAP™ Fusion Protein (Component Number I7582): 40 µg
- Polypropylene chromatography column (Cat. No. C2103): 5 each

Reagents and Equipment Required

(But not provided)

- Test tubes
- Hamilton® syringe (such as Cat. No. 24539) or a Pasteur pipette (such as Cat. No. S6268)
- Shaker
- Centrifuge
- Microcentrifuge, Eppendorf® 5417R or equivalent
- Dulbecco's Phosphate Buffered Saline (PBS), such as Cat. No. D8537
- Protease inhibitor cocktail (such as Cat. No. P8340; see Related Products for examples of other inhibitor cocktail products)
- SIGMA*FAST*™ pNPP substrate tablets set (such as Cat. No. N1891), or pNPP liquid substrate system (such as Cat. No. N7653)

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.

Preparation Instructions

- Defrost solutions: Be sure all kit solutions are defrosted and are mixed to a homogenous form.
- 5 μg/μL 3X FLAG® peptide solution: The 3X FLAG® peptide (N-Met-Asp-Tyr-Lys-Asp-His-Asp-Gly-Asp-Tyr-Lys-Asp-His-Asp-Ile-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) is acidic. To dissolve it properly, add 160 μL of 10× Wash Buffer to 4 mg of 3X FLAG® peptide. When the peptide is completely dissolved, add 640 μL of distilled water to the sample. Mix well and store aliquots at -20 °C.
- Dilute FLAG-BAP™ fusion protein: For control reactions, dilute a portion of the FLAG-BAP™ fusion protein solution to a concentration of 50 ng/μL with 1× Wash Buffer. The diluted solution is stable at −20 °C for about two months.

Procedures

General notes

- See the Certificate of Analysis for A2220 for the lot-specific binding capacity of the ANTI-FLAG® M2 affinity resin.
- 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 should be equilibrated with 1× Wash Buffer. The equilibration can be performed either at room temperature or at 2-8 °C.
- To check reagent compatibility with the ANTI-FLAG® M2 affinity gel, see the Reagent Compatibility Table.
- For an immunoprecipitation procedure, see the Technical Bulletin for the FLAG® Immunoprecipitation Kit (Cat. No. FLAGIPT1) on the Web site. Use the CelLytic™ M reagent component of this kit as a lysis buffer.

Cell Lysis

Notes for cell lysis

- The volume of CelLytic™ M reagent to add to the cells varies with the cell size and protein concentration required. In general, 125 μL of CelLytic™ M are recommended for 10^6-10^7 cells. For adherent cells, the plate size will dictate the amount of buffer covering the plate surface. Suggested working volumes are: 500-1000 μL for a 10 cm plate. 200-400 μL for a 3.5 cm plate.
- In general, to avoid protein degradation, it is recommended that Protease Inhibitor Cocktail be added to the CelLytic™ M reagent.
- Lysate preservation requires low temperatures. Therefore, store the lysate at -70 °C for long-term storage.

1. Wash cells and treat with cell lysis buffer.
   a. For adherent cells:
      - Remove the growth medium from the cells to be assayed.
      - Rinse the cells once with PBS buffer, being careful not to dislodge any of the cells.
      - Discard PBS.
      - Add CelLytic™ M reagent.

   b. For cells in suspension:
      - Collect the cells into an appropriate conical centrifuge tube.
      - Centrifuge for 5 minutes at 450 × *g.*
      - Decant the supernatant and discard.
      - Wash the cells once by resuspending the cell pellet with PBS and centrifuge for 5 minutes at 450 × *g*.
      - Decant supernatant and discard.
      - Resuspend the cell pellet in CelLytic™ M reagent.
2. Incubate the cells for 15 minutes on a shaker.
3. Collect cell lysate.
   - For adherent cells: collect cells.
   - For cells in suspension: skip to Step 4.
4. Centrifuge the lysed cells for 10 minutes at 12,000-20,000 × *g* to pellet the cellular debris.
5. Remove the protein-containing supernatant to a chilled test tube.
   - For immediate use, keep on ice.
   - Otherwise, store the protein as a frozen solution.

Affinity Purification (column)

General notes on protein purification

FLAG® fusion protein can be affinity-purified either on a column or by batch format. The column format works well when there is no substantial difference between the volume of material to load onto the column and the amount of resin being used. For larger volumes, the batch format is recommended to capture quickly the target protein from a large volume of extract.

If you work with a lysate volume up to ~ 6 mL and you do not have equipment for buffer loading under a controlled flow, then for best results, you can use the column closed at its two ends and work according to the batch format.

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 Protease Inhibitor Cocktail to the elution solution.

Cellular debris and particulate matter can clog the column and must be removed prior to purification, especially if the sample has been defrosted.

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 µm filter to remove any remaining cells and particulates.

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.

Resin preparation

1. Place the chromatography column on a firm support.
2. Rinse the empty column with 0.5 mL of 1× Wash Buffer. Allow the buffer to drain from the column and leave residual Wash Buffer in the column to aid in packing the resin.
3. Thoroughly suspend the resin by gentle inversion. Make sure the ANTI-FLAG® M2 affinity gel is a uniform suspension of gel beads. Remove the required amount of resin 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 1× Wash Buffer. 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 will not crack when excess solution is drained under normal circumstances, but do not let the gel bed run dry.
7. Load three column volumes of Elution Buffer. Let the buffer drain completely. Avoid disrupting the gel bed while loading. Do not leave the column in loading buffer longer than 20 minutes. This step is a mock elution for removal of residual impurities off the column. Wash the resin with five column volumes of 1× Wash Buffer, or until the eluent is at a neutral pH to equilibrate the resin for use. Do not let the bed run dry. Allow a small amount of buffer to remain on top of the column. Do not allow the resin to remain in 1× Wash Buffer for extended periods of time (>24 hours) unless an antimicrobial agent (such as sodium azide) is added to the buffer.

Column chromatography

1. 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. In cases when the FLAG® fusion protein is not completely bound (depending on the specific protein and on the loading flow rate), multiple passes over the column will improve the binding efficiency.
2. Usually the sample loading step requires a slow flow to allow binding of the fusion protein to the affinity resin. If the sample volume is up to ~ 6 mL, it can be loaded in a batch mode by incubation of the resin and sample solution in the column, under a gentle rotation.
3. Wash the column with 10-20 column volumes of 1× Wash Buffer. This should remove any proteins that are not bound to the M2 antibody. Allow the column to drain completely.

Elution of FLAG® Fusion Proteins

Select one of the two following elution procedures:

1. Acid Elution with Glycine:
    - Elute the bound FLAG® fusion protein from the column with six 1 mL aliquots of Elution Buffer into vials which contain 50-100 µL of 10× Wash Buffer/1 mL of eluent or 15-25 µL of 1 M Tris (pH 8).
    - Do not leave the column in Elution Buffer for longer than 20 minutes.
    - Re-equilibrate column to neutral pH as soon as possible after elution.
2. Competition with 3X FLAG® Peptide: Elute the bound FLAG® fusion protein by competitive elution with five one-column volumes of a solution containing 150-200 ng/µL of 3X FLAG® peptide (Cat. No. F4799) in 1× Wash Buffer.

Notes

- The elution can be performed stepwise by a sequential addition and collection of a defined volume of Elution Buffer.
- When the elution step requires an extended incubation of the resin with the Elution Buffer or 3X FLAG® peptide, incubate under a gentle rotation.
- Do not leave the column in Elution Buffer for longer than 20 minutes.
- When elution efficiency is low, the concentration of 3X FLAG® in suspension can be increased, or alternatively, a salt can be added to the Elution Buffer.

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

This method provides a quick and efficient way to purity 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. Re-suspend the equilibrated resin (see Resin preparation) in 1× Wash Buffer and add to the protein extract.
2. 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, as this will destroy the resin beads.** This step can be performed at 2-8 °C or at room temperature. The incubation time can range from 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.
3. 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.
4. Wash the resin with 1× Wash Buffer 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 further protein elutes. The eluted protein from the resin can be monitored by measuring the absorbance of the eluent at 280 nm. Continue washing the resin until the absorbance of the wash solution from the column is < 0.05 vs. a wash solution blank.
5. The FLAG® proteins can be eluted from the resin either by low pH (Elution Buffer) or by competition with the 3X FLAG® peptide. Follow the elution steps under Elution section.
6. The resin can be recycled and stored as per the Recycling the Column and the Storing the Column sections.

Recycling the Column

It is recommended that the column be regenerated immediately after use by washing with three column volumes of elution buffer. The column should be immediately re-equilibrated in 1× Wash Buffer until the eluent is at neutral pH.

Storing the Column

Wash the column with ten column volumes of 1× Wash Buffer containing 50% glycerol and 0.02% sodium azide. Then add another 5 mL of buffered glycerol containing 0.02% sodium azide. Store at 2-8 °C or -20 °C without draining.

The number of cycles obtained will be dependent on variables such as sample condition and proteases.

Alkaline Phosphatase analysis

The FLAG-BAP™ protein serves as a functional control for immunoaffinity chromatography and immunoprecipitation with ANTI-FLAG® M2 affinity gel. When using the control protein, we recommend a small-scale detection.

Run the samples and controls on an SDS-PAGE gel. The FLAG-BAP™ fusion protein has a molecular mass of 49.3 kDa. It migrates as a 45-55 kDa band by SDS-PAGE depending on the electrophoresis conditions.

Immunoblot the gel with ANTI-FLAG® or Anti-Bacterial Alkaline Phosphatase (BAP) antibodies. Alternatively, stain the gel with a staining method such as silver staining or Coomassie® blue.

An alternative option is to detect the BAP presence by enzymatic activity. SIGMA*FAST*™ pNPP substrate tablets set (Cat. Nos. N1891 or N2770) or pNPP liquid substrate system (Cat. No. N7653) are recommended for the detection of BAP activity.

References

1. Brizzard, B.L. *et al.*, *BioTechniques*, **16(4)**, 730-734 (1994).
2. Knappik, A., and Plückthun, A., *BioTechniques*, **17(4)**, 754-761 (1994).
3. Chiang, C.M., and Roeder, R.G., *Pept. Res.*, **6(2)**, 62-64 (1993).
4. Ausubel, F.M. *et al.* (eds.), *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc. (New York City, NY), pp. 10.15.1-10.16.29 (1998).
5. Harlow, E., and Lane, D., *Antibodies, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, (Cold Spring Harbor, NY), pp. 514-517, 541-542, 547-549 (1988).

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Troubleshooting Guide

Reagent	Effect	Comments
Chaotropic agents (such as urea, guanidine HCl)	Denatures the immobilized M2 antibody	**Do not** use any reagent that contains these types of components, since chaotropic agents 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 denature 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	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 SDS will denature the M2 antibody on the resin and destroy its ability to bind the FLAG® fusion proteins. SDS 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 deoxycholate will inhibit the M2 antibody from binding to FLAG® fusion proteins.

References (continued)

6. Jin, G. *et al.*, *EMBO J.*, **30(11)**, 2281-2293 (2011).
7. Jeng, M.Y. *et al.*, *J. Exp. Med.*, **215(1)**, 51-62 (2017).
8. Fu, L. *et al.*, *Nucleic Acids Res.*, **47(7)**, 3568-3579 (2019).
9. Zhang, Z. *et al.*, *Cell Rep.*, **29(1)**, 49-61.e7 (2019).
10. Shi, Y. *et al.*, *FASEB J.*, **34(1)**, 208-221 (2020).

Related Products

- Mammalian FLAG® expression vectors, such as Cat. Nos. E8770, OGS1142, OGS1248, OGS1176, OGS3407, OGS1264, OGS3423, OGS1124, OGS1084, OGS625, OGS624, and OGS618
- Mammalian sequencing primers, such as Cat. No. P5350
- Monoclonal ANTI-FLAG® M1, purified immunoglobulin: Cat. No. F3040
- ANTI-FLAG® M1 Agarose Affinity Gel: Cat. No. A4596
- Monoclonal ANTI-FLAG® M2, purified immunoglobulin: Cat. No. F3165
- Monoclonal ANTI-FLAG® M2, affinity isolated antibody: Cat. No. F1804
- Monoclonal ANTI-FLAG® M5, purified immunoglobulin: Cat. No.F4042
- FLAG® peptide: Cat. No. F3290
- FLAG-BAP control fusion proteins: Cat. Nos. P7582, P7457, and P5975
- Glycerol, such as Cat. No. G5516 (for molecular biology)
- Deoxyribonuclease I (DNase I), such as Cat. No. D4527
- Sodium azide, such as Cat. No. 8032 (BioXtra)
- FLAG® Immunoprecipitation Kit: Cat. No. FLAGIPT1
- ANTI-FLAG® M2-peroxidase conjugate: Cat. No. A8592
- ANTI-FLAG® M2-alkaline phosphatase conjugate: Cat. No. A9469
- Protease and phosphatase inhibitor cocktails, such as Cat. Nos. P8215, P2714, P8465, P8340, P9599, P2850, P5726, P0044, MSSAFE, P0001, PIC0002, PIC0004, PIC0005, PIC0006, PPC2020
- *p*-Nitrophenyl Phosphate (pNPP) liquid substrate system: Cat. No. N7653
- SIGMA*FAST* *p*-Nitrophenyl Phosphate tablets sets: Cat. Nos. N1891 and N2770

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