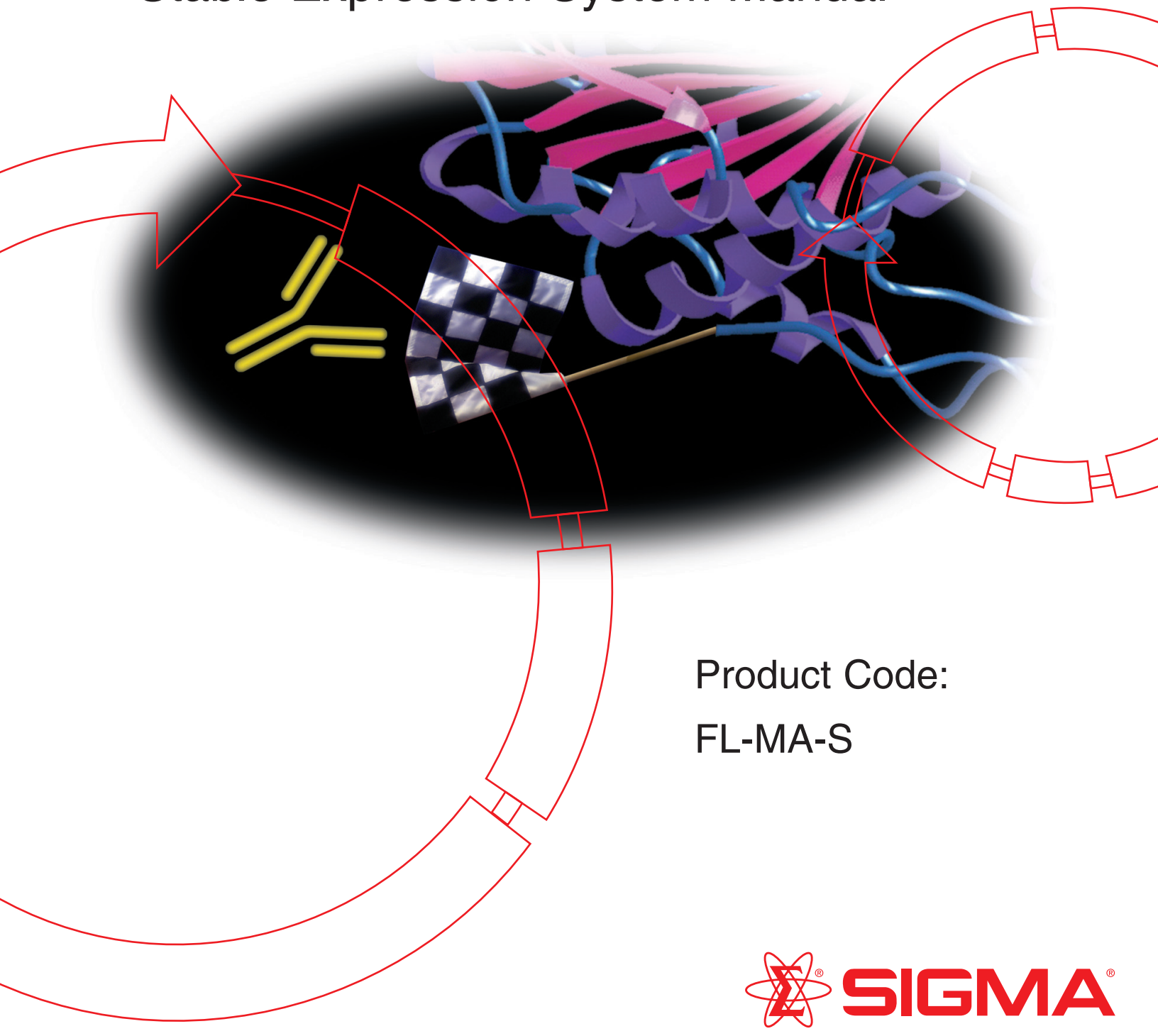
FLAG®

Mammalian Amino-Terminal Stable Expression System Manual



Product Code:

FL-MA-S

SIGMA®

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Mammalian Amino-Terminal FLAG Stable Expression Kit

Appropriate components have been provided for the stable expression and specific immunodetection of cytoplasmic, secreted, and cell surface amino-terminal FLAG fusion proteins in mammalian cells.

Table 1.

Components of Mammalian Amino-Terminal FLAG Stable Expression Kit

KIT COMPONENT	QUANTITY	STORAGE TEMPERATURE
E 8770 pFLAG-CMV-3™ Expression Vector	20 µg	0 to –20° C
E 1775 pFLAG-CMV-4™ Expression Vector	20 µg	0 to –20° C
C 3972 pFLAG-CMV-3-BAP™ Control Plasmid	20 µg	0 to –20° C
C 4722 pFLAG-CMV-4-BAP™ Control Plasmid	20 µg	0 to –20° C
P 5475 C-CMV-24 Reverse Sequencing Primer	1 µg	0 to –20° C
P 5350 N-CMV-30 Forward Sequencing Primer	1 µg	0 to –20° C
F 3165 ANTI-FLAG M2 Monoclonal Antibody	200 µg	0 to –20° C
F 4042 ANTI-FLAG M5 Monoclonal Antibody	200 µg	0 to –20° C
A 2220 ANTI-FLAG M2 Affinity Gel - Freezer Safe	1 ml	0 to –20° C
P 5975 Met-FLAG-BAP Control Protein	100 µg	0 to –20° C
F 3290 FLAG Peptide	4 mg	2 - 8° C (-20° C Following Resuspension)

THE FLAG SYSTEM

The FLAG system is based on the FLAG marker octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). The FLAG sequence is hydrophilic (Figure 1) and the last 5 amino acids (Asp-Asp-Asp-Asp-Lys) represent the target sequence of the protease enterokinase.[1] FLAG fusion proteins are created by cloning the coding sequence of a protein of interest adjacent to the FLAG coding sequence in a FLAG expression vector, followed by expression in the appropriate host cell type.

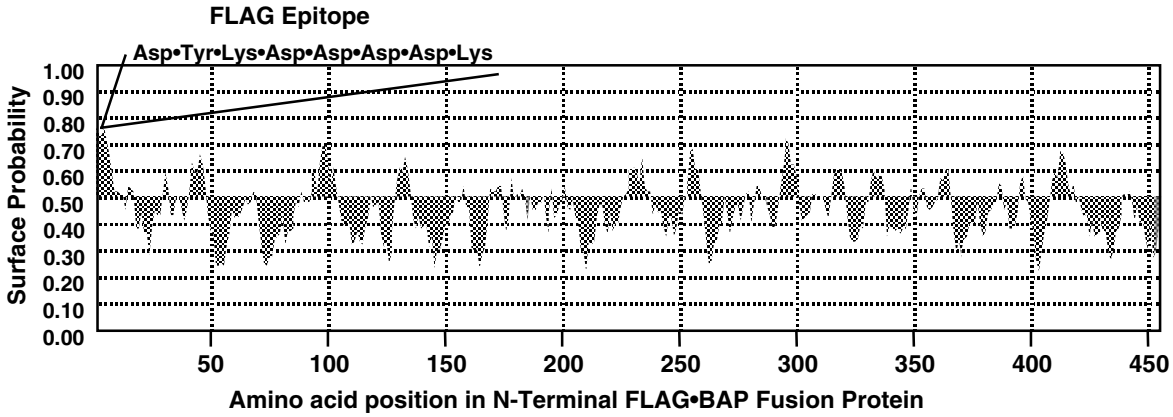
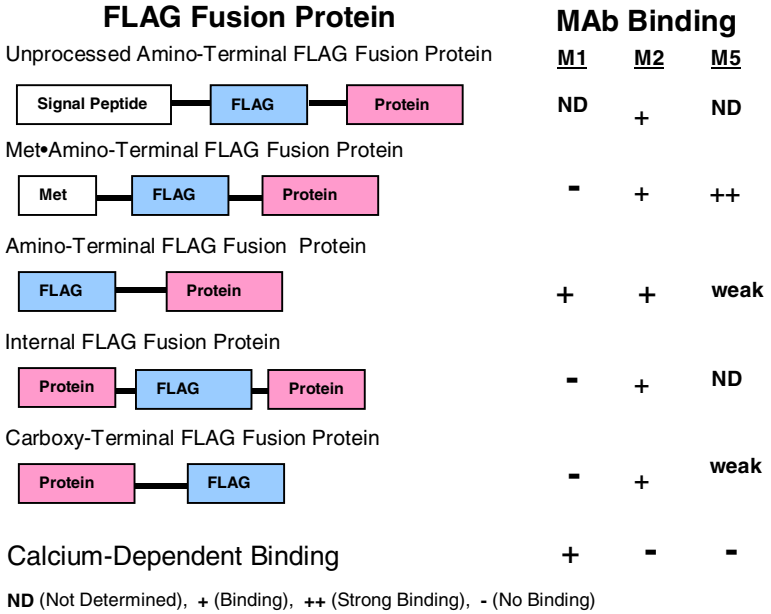


Figure 1. FLAG is a small, hydrophilic peptide with a high surface probability. MacVector plots of surface probability for an amino-terminal FLAG-BAP fusion protein. Surface probability is shown on the y axis and the amino acid position in the protein sequence is shown on the x axis. The FLAG peptide corresponds to the first eight amino acids of the protein (Shown in expanded view in the figure). FLAG also has a high hydrophilicity as calculated on the Hopp-Wood scale.

The kit contains two of three monoclonal antibodies that specifically bind the FLAG epitope: ANTI-FLAG® M2 (IgG_1), and M5 (IgG_1). They differ in their ability to recognize the FLAG epitope in different locations within the FLAG fusion protein and in their requirement for calcium as shown in Figure 2:

Figure 2.



ANTI-FLAG® M2 Monoclonal Antibody

Binding of the ANTI-FLAG M2 is not calcium-dependent and a free amino group is not required. This means that the M2 antibody is capable of binding the FLAG epitope when preceded by a methionine, or when the FLAG marker is placed at the C-terminus of a recombinant protein. In some cases, the M2 antibody will bind the FLAG epitope at internal locations within a protein. However, it is recommended that the site chosen for insertion of the epitope have a high surface probability.

Although the binding of the M2 antibody is not calcium-dependent, mild elution from M2 affinity columns may be accomplished by competition with FLAG peptide. The M2 antibody is recommended for detection and purification of N-terminal Met-FLAG, N-terminal FLAG, and C-terminal FLAG fusion proteins.

ANTI-FLAG M5 Monoclonal Antibody

The ANTI-FLAG M5 monoclonal antibody was raised against the sequence Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys and has a high relative affinity for N-terminal Met-FLAG fusion proteins. Binding is not calcium-dependent. N-terminal Met-FLAG fusion proteins are created by placing an ATG translational start codon immediately before the FLAG coding sequence. When transfected into an appropriate host the N-terminal Met-FLAG fusion protein will be expressed in the cytoplasm of the cell. The binding properties of the M5 antibody to shorter versions of the FLAG sequence have not been determined.

Due to its higher relative affinity for N-terminal Met-FLAG fusion proteins, the M5 antibody is the antibody of choice for immunodetection of these FLAG fusion proteins. M5 antibody coupled to affinity gel is not available.

M5 is the preferred antibody for immunodetection of cytoplasmically expressed N-terminal Met-FLAG fusion proteins expressed in mammalian, yeast, and *Drosophila* cells.

The M5 antibody is not recommended for detection of FLAG fusion proteins expressed in *Escherichia coli.*

ANTI-FLAG M2 Affinity Gel

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 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.

N-TERMINAL FLAG EXPRESSION VECTORS

PFLAG-CMV-3 and pFLAG-CMV-4 Expression Vectors

pFLAG-CMV-3 and -4 are 6.3 kb derivatives of pCMV5[2] and are designed for transient or stable expression of N-terminal FLAG® fusion proteins in mammalian cells. pFLAG-CMV-3 with the preprotrypsin (PPT) leader sequence[3] results in secretion of the FLAG fusion protein, while pFLAG-CMV-4 lacks the PPT leader sequence and therefore results in intracellular expression of the FLAG fusion protein. The multiple cloning sites of both vectors are completely compatible to facilitate transferring open reading frames between the two vectors.

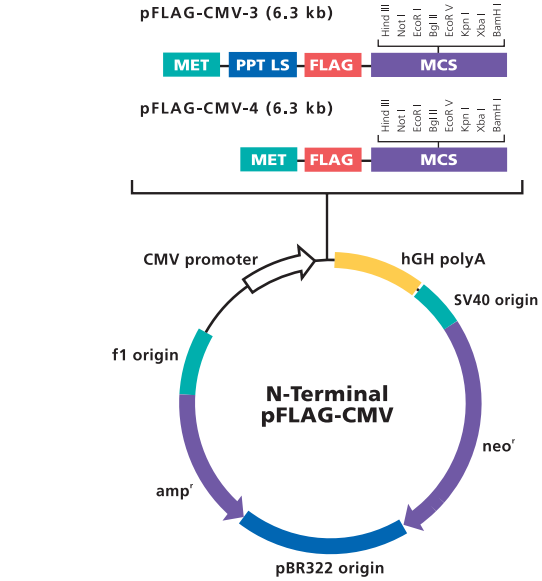
The strong promoter-regulatory region (CMV promoter) of the human cyomegalovirus[4] drives transcription of FLAG-fusion constructs. The aminoglycoside phosphotranferase II gene[5] (Neo) confers resistance to aminoglycosides like Geneticin[6] (G418), allowing for selection of stable transfectants. In addition, both vectors contain the E. coli origin of replication and ampicillin resistance gene and can therefore be propagated in *E. coli*. Efficiency of replication and genomic integration is optimal when using an SV40 T antigen-expressing host such as COS cells.

Table 2.

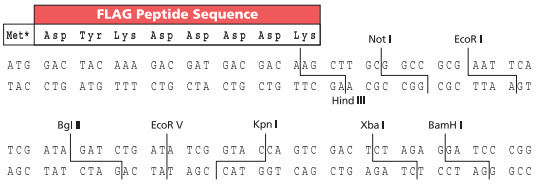
pFLAG-CMV-3 and CMV-4 Features

Feature	Map Position	Map Position
	pFLAG-CMV-3	pFLAG-CMV-4
CMV promoter	166 - 916	166 - 916
CMV-30 seq. primer	825 - 854	825 - 854
Translational initiation	942 - 944	942 - 944
Preprotrypsin leader	945 - 986	N/A
FLAG	987 – 1010	945 – 968
MCS	1008 – 1070	966 – 1028
HGH polyA	1075 – 1694	1033 – 1652
CMV-24 seq. primer	1132 – 1155	1090 – 1113
SV40 origin	1713 – 2052	1671 – 2009
Neo	2088 – 2879	2045 – 2836
SV40 polyA	3526 – 3624	3483 – 3581
PBR322 origin	4543– 4662	4500 – 4619
Ampicillin resistance	4867 – 5727	4824 – 5864
f_1 origin	5862 – 6331	5819 – 6271

Figure 3



Multiple Cloning Site
(pFLAG-CMV-3* and pFLAG-CMV-4)



*For pFLAG-CMV-3, the Met-preprotrypsin leader sequence (PPT LS) precedes the FLAG coding sequence.

PFLAG-CMV-3-BAP and –4-BAP Control Plasmids

These control plasmids were prepared by inserting the bacterial alkaline phosphatase gene (BAP) in pFLAG-CMV-3, and pFLAG-CMV-4, respectively. The control plasmids are provided as an aid in validating the FLAG expression system for your particular application.

N-CMV-30 Forward Sequencing Primer

A chemically synthesized, 30 base, single strand oligodeoxyribonucleotide designed for double strand DNA sequencing of FLAG fusion proteins at the N-terminus of the pFLAG-CMV expression vectors.

DNA Sequence:
5' - AAT-GTC-GTA-ATA-ACC-CCG-CCC-CGT-TGA-CGC- 3'

Map Position:
Map position nucleotides 825-854 of the positive, coding DNA strand of the pFLAG-CMV 3 and 4 vectors.

C-CMV-24 Reverse Sequencing Primer

A chemically synthesized, 24 base, single-strand oligodeoxyribonucleotide designed for double-strand or single-strand DNA sequencing at the C-terminus of inserts in the pFLAG-CMV expression vectors.

DNA Sequence:
5' - TAT-TAG-GAC-AAG-GCT-GGT-GGG-CAC- 3'

Map Position: Map position nucleotides 1132-1155 of the positive, coding DNA strand of the pFLAG-CMV 3 and 1090-1113 for the pFLAG-CMV4 vector.

General Strategy for Cloning and Expression

The general strategy consists of these steps:

1. Correctly prepare and insert an open reading frame of choice into the multiple cloning site of the pFLAG-CMV expression vector (see Tables 3 and 4).

2. Transform *E. coli.* host, and screen ampicillin resistant colonies with mini-preps to identify and isolate positive clones.

3. Confirm the DNA sequence of the fusion junction with the N-CMV-30 and C-CMV-24 primers.

4. Transfect COS cells or other mammalian cells with pFLAG-CMV 3 or 4 construct and select for stable transfectants.

5. Isolate and/or detect FLAG fusion protein using ANTI-FLAG antibodies or resin.

Table 3.

Translational Phase of the First Base of Each Restriction Site in the MCS of the N-Terminal pFLAG Vectors *

Restriction site	PFLAG-CMV-3 PFLAG-CMV-4	pFLAG-CMV-1 pFLAG-CMV-2	pFLAG-ATS pFLAG-MAC	YEpFLAG-1
Hind III (A'AGCTT)	1	1	1	-
Not I (GC'GGCCGC)	1	1	-	1
Eco RI (G'AATTC	3	3	3	1
Cla I (AT'CGAT)	-	3	-	1
Bgl II (A'GATCT)	3	3	3	1
Eco RV (GAT'ATC)	3	3	-	-
Kpn I (GGTAC'C)	3	3	3	-
Asp718 (G'GTACC)	3	3	3	-
Sal I (G'TCGAC)	-	1	-	1
Xbal (T'CTAGA)	1	1	3	-
Bam HI (G'GATCC)	1	1	-	1
Sma I (G'GATCC)	-	2	2	1

Table 4.

Translational Phase Relative to the FLAG Sequence of the First Base of each Restriction Site in the MCS of the C-Terminal pFLAG Vectors*

Restriction site	CMV 5 A	CMV 5 B	CMV 5 C	PFLAG-CTS PFLAG-CTC
Eco RI (G'AATTC	1	3	2	1
Hind III (A'AGCTT)	1	3	2	2
Not I (GC'GGCCGC)	1	3	2	-
Bgl II (A'GATCT)	3	2	1	1
Pst I (CTGCA'G)	3	2	1	-
Eco RV (GAT'ATC)	3	2	1	-
Sal I (G'TCGAC)	1	3	2	1
Bam HI (G'GATCC)	1	3	2	-
Kpn I (GGTAC'C)	1	3	2	1
Xho I (C'TCGAG)	-	-	-	2

*Note: the first base of each restriction site in the MCS of pFLAG-CMV-5.1, a new C-Terminal pFLAG transient expression vector, is in the same translational phase as pFLAG-CMV-2 with respect to the FLAG sequence.

STABLE TRANSFECTION

The pFLAG-CMV-3 and pFLAG-CMV-4 expression vectors encode the aminoglycoside phosphotransferase II gene (Neo) which confers resistance to aminoglycosides such as geneticin (G418), allowing for selection of stable transfectants. Stable transfectants can be generated by lipofection using the appropriate selection from the ESCORT product line for the cell type utilized. See respective product insert for transfection procedure.

Isolation of stable transfectants requires cell lines able to grow as an isolated colony. This can be determined by plating ~100 cells in a 100 mm dish, changing non-selective medium every 4 days. Colonies will appear in 10 to 12 days if formation is possible.

Selection conditions are dependent on the characteristic reponse of a specific cell line to the selecting agent. This reponse may also vary from lot to lot of antibiotic and require reassessment with each lot. It is recommended that the optimal concentration for killing untransfected cells response be determined by plating cells in media containing varying concentrations of G418 in the range of 0 to 1000 microgram/ml[6], changing medium every 4 to 12 days. (See Geneticin product insert for detailed procedure to determine appropriate concentration of antibiotic.)

Immunostaining of Cultured Mammalian Cells Expressing FLAG-Tagged Fusion Protein Using ANTI-FLAG Monoclonal Antibody

At day 1-2 post-transfection and at subsequent timepoints, efficiency of transfection and/or expression can be visualized with duplicate plates using the following procedure**:**

1. Wash 35 mm plates once with 5 ml 1XTBS (T5912).

2. Fix cells with 1 ml methanol:acetone (1:1, volume: volume) and wash four times with 2 ml 1XTBS.

3. Incubate fixed cells with M2:HRP-conjugate (1 ml/ plate) at room temperature for 30 minutes.

4. Wash plates 5 times with 2 ml 1XTBS.

5. Add 30% hydrogen peroxide solution and o-dianisidine at final concentrations of 0.015 percentage and 100 µg/ml, respectively, in 1XTBS (2 ml/ plate). An incubation time of ~ 1-15 minutes is required before staining is initially observed. The reaction is stopped by replacing substrate with 1XTBS.

6. Unstained cells can be counterstained by removing 1XTBS and staining cells with Mayer's hemotoxylin solution:water (1:1, volume:volume, 1ml/plate) for 30-90 seconds at room temperature, depending on cell type. Wash cells with water and temporarily place under 1XTBS.

7. Cells can be photographed and further preserved by removing 1XTBS and adding Crystal Mount to each plate. Follow manufacturer's recommended procedure.

Immunoprecipitation of FLAG Fusion Proteins using the ANTI-FLAG M2 Affinity Gel

Isolation of FLAG-tagged proteins from culture medium (secreted by cells transfected with a pFLAG-CMV-3 construct) or from lysates (extracts of cells transfected with either a pFLAG-CMV-3 construct or pFLAG-CMV-4 construct) can be accomplished with the following immunoprecipitation method using ANTI-FLAG M2 Affinity Gel. This method is recommended for the purification of small amounts of FLAG tagged proteins. (Note: Use of the ANTI-FLAG M2 Affinity Gel is covered in the Appendix)

General notes:

- 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.

- 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 in the Appendix for additional chemicals.

1. 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).

   a. 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.

   b. 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.

   c. 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.

d. 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.

e. 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.

f. 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.

g. Centrifuge the resin for 5 seconds at 10,600 X g. Remove the supernatants with a narrow-end pipette tip.

h. 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.

2. 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.

a. Elution with 3X FLAG peptide
   i. Prepare 3X FLAG elution solution. Dissolve 3X FLAG peptide (Product No. F4799) 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).

   ii. Add 100 µl 3X FLAG elution solution to each sample and control resin.

   iii. Incubate the samples and controls with gentle shaking for 30 minutes at 2-8°C.

   iv. 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.

b. 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.**

i. Add 100 µl 0.1 M glycine HCl pH 3.5 buffer to each sample and control resin.

ii. Incubate the samples and controls with gentle shaking for 5 minutes at room temperature.

iii. 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.

c. Elution with SDS-PAGE Sample Buffer

General notes:

- 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 kD 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.

- 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.

i. 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.

ii. Boil the samples and controls for 3 minutes. Allow to cool to room temperature: do not place on ice.

iii. 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.

Western Blot Method for Detecting FLAG Fusion Proteins using ANTI-FLAG M2 Antibody

Procedure

1. Separate FLAG-tagged proteins from sample lysates using a standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protocol. Load 2.5 to 10 µg total lysate protein per lane.

2. Transfer proteins from the gel to an Immobilon-P™ or other polyvinylidene difluoride (PVDF) membrane. Nitrocellulose membranes can be used but typically result in less sensitivity.

3. Wash the blot in at least 0.5 ml/cm^2 Milli-Q™ water for 2-3 minutes with mild agitation.

4. Block the blot with at least 0.5 ml/cm^2 of TBS with 3% nonfat dry milk (Product No. T8793 or 50 mM Tris, 0.138 M NaCl, 2.7 mM KCl, pH 8.0, containing 30 mg/ml nonfat dry milk) for 30 minutes at room temperature with agitation (about 50-60 rpm).

5. Remove the blocking agent and wash once with 0.5 ml/cm^2 TBS (Product No. T6664).

6. Add ANTI-FLAG M2 antibody to a final concentration of 10 µg/ml* to the blot in at least 0.5 ml/cm^2 of TBS with 3% nonfat dry milk and incubate at room temperature for 30 minutes.

   *Note: Using less M2 ANTI-FLAG antibody may help to reduce background and cross-reactivity. See the Troubleshooting Guide.

7. Remove the ANTI-FLAG M2 antibody solution and wash once with at least 0.5 ml/cm^2 TBS.

8. Add rabbit anti-mouse IgG, peroxidase conjugate (Product No. A9044 or equivalent) to at least 0.5 ml/cm^2 of TBS with 3% nonfat dry milk. Use the concentrations listed in the table below. These concentrations are recommended starting concentrations for the antibodies used in the Western blot. Incubate the blots with shaking at room temperature for 30 minutes.

   Table 5.

ANTI-FLAG M2 primary Antibody (µg/ml)	Substrate	Secondary antibody Concentration
0.5–10	ECL+™	1:80,000
0.5–10	ECL™	1:10,000

9. Wash the blot eight times for a total of 20 minutes in 50 mM Tris, 0.138 M NaCl, 2.7 mM KCl, pH 8.0, plus 0.05% Tween 20 (TBS-Tween 20, Product No. T9039).

10. Develop the blots with the appropriate substrate for 5 minutes.

2. Expose BioMax™ light film to the blot. Exposure times range from 30 seconds to 10 minutes. It is best to do a quick exposure of 10 to 30 seconds to determine what exposure time is needed. If the signal is too intense even at the short exposure times let the signal decay from 1 to 8 hours or longer if necessary and then re-expose the film.

REFERENCES

1. Hopp *et al.* A short Polypeptide Marker Sequence Useful for Recombinant Protein Identification. *Bio/Technology,* **6,**1204-1210. (1988).

2. Andersson *et al.* Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264,** 8222-8229. (1989).

3. Stevenson *et al.* Sequence organisation and transcriptional regulation of the mouse elastase II and trypsin genes. *Nucl. Acids. Res.* **21,** 8307-8330. (1986)

4. Thomsen *et al.* Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. *PNAS* **81,** 659-663. (1984).

5. C.B. Brewer, *Methods of Cell Biology* **43**: 233-245 (1994).

6. Jiminez, A. and Davies, J., *Nature* **287**, 869-871 (1980).

Appendix

Preparation and Sequencing of pFLAG-CMV DNA Templates with the C-CMV-24 and N-CMV-30 Sequencing Primers

(Note: for sequencing single-stranded DNA with the C-CMV-24 primer, proceed to Priming pFLAG-CMV DNA Template with N-CMV-30 or C-CMV-24 Sequencing Primers, below)

Preparation of Denatured DNA Template:

The following protocol is designed to allow sufficient template for 2 sets of 4 sequencing reactions: 2G, 2A, 2T and 2C reactions.

1. Denature 7 micrograms of highly purified DNA in 70 µl of 1x TE by adding 3µl (1/25 volume) of 5M NaOH at 37°C for 5 minutes.

2. Precipitate denatured pFLAG-CMV supercoiled DNA for 30 minutes at room temperature by adding 150 µL (2 volumes) of premixed potassium acetate and isopropanol made as follows: 1 volume 3M potassium /5M acetate*: 3 volumes isopropanol.
   *(Recipe: 60 ml of 5M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml water).

3. Collect precipitated and denatured pFLAG-CMV DNA by centrifugation at 10,000xg for 5 min.

4. Wash the pFLAG-CMV DNA pellet with 1 ml ethanol and dry.

5. Resuspend pFLAG-CMV DNA in 20 µl of 1x TE.

(The final concentration should be 0.35 µg/µl).

(You can store the denatured pFLAG-CMV DNA template at -20°C at this point if desired).

Priming pFLAG-CMV DNA Template with N-CMV-30 or C-CMV-24 Sequencing Primer:

The following protocol uses 10 µl of the preceding preparation of denatured pFLAG-CMV DNA template. This is sufficient for 1 set of 4 sequencing reactions (1G, 1A, 1T and 1C reaction).

1. Dilute the sequencing primer from an initial concentration of 5 pmol/µl to a final concentration of 1.67 pmol/ µl by removing a 3 µl aliquot of sequencing primer from the vial and adding 6 µl of 1X TE to a final volume of 9 µl.

2. Add 2-3 µl (3.5 to 5 pmoles) of sequencing primer to 10 µl (1 pmol) of denatured pFLAG-CMV DNA template. Heat the 12-13 µl of primer: pFLAG-CMV DNA template mixture in an appropriate volume of sequencing buffer to make the buffer 1X (at 70°C for 2 minutes).

3. Slow cool the mixture by placing at room temperature for about 20 minutes. Distribute the primed pFLAG-CMV DNA template to 4 tubes to be used in the G, A, T and C DNA sequencing reactions.

4. The primer: pFLAG-CMV template is now ready for supercoil sequencing of the DNA sequence corresponding to the N-terminal FLAG fusion junction.

5. The DNA sequence corresponding to the fusion junction will be >143 bases away from the 3' end of the FLAG-CMV 30 sequencing primer using pCMV-FLAG-1 template and >98 bases away using pCMV-FLAG-2 template reading from the bottom of your autoradiogram.

6. In N-terminal FLAG vectors, the DNA sequence corresponding to the FLAG fusion junction will be >58 bases away from the 3' end of the FLAG-CMV 24 sequencing primer reading from the bottom of your autoradiogram. In C-terminal vectors, the DNA sequence corresponding to the FLAG fusion junction will be >91 bases away from the 3' end of the FLAG-CMV 24 sequencing primer reading from the bottom of your autoradiogram.

Preparation of pFLAG-CMV Phagemids and ss-DNA Template:

1. Plate *E. coli* cells carrying your ORF in the pFLAG-CMV expression vector on M9 medium + 50 µg / ml Ampicillin. This medium selects for the F' phenotype necessary for single strand production.

2. Grow cells overnight at 37°C.

3. Add 2 ml of 2x YT containing 250 µg/ml Ampicillin to the surface of the plate. Suspend bacteria.

4. Add 1 ml of the cell suspension to 10 ml of 2x YT containing 250 µg/ml Ampicillin. Grow until OD_{600} is between 0.4 - 0.6.

5. Dilute the culture 1/50 in 50 ml 2x YT + 250 µg/ml Ampicillin in a 500 ml flask. Shake at 37°C for 30 minutes. Take an OD_{600} to estimate the number of cells/ml.

6. Add M13K07 Helper phage to a Multiplicity of Infection (MOI) of 20. After 30 minutes growth, add 70 µl of 50 mg/ml kanamycin. Continue shaking for 6 hours. Longer growth periods may result in deletions within the DNA.

7. Pellet cells at 17,000xg for 30 minutes. Recover supernatant and filter. For each ml of supernatant add 0.25 ml of 20% polyethylene glycol, 3.5 M ammonium acetate (Note: use molecular biology grade PEG, Cat. No. P5413). Mix well by inversion and place on ice for 2 - 48 hours.

8. Centrifuge as in step **7.** Decant supernatant. A small white pellet should be visible.

9. Resuspend in 300 - 700 µl of TE by vortexing. Transfer to a microfuge tube.

10. Add an equal volume of freshly distilled phenol. Vortex 1-2 minutes. Spin and recover the aqueous phase without disturbing the white interface.

11. Extract with an equal volume of 1:1 phenol/chloroform (Chloroform is 24:1 $CHCl_3$: isoamyl alcohol). Continue extractions 4 - 6 times until a slight white interface is seen.

12. Extract with an equal volume of 24:1 $CHCl_3$: isoamyl alcohol. Remove upper aqueous phase.

13. Ethanol precipitate the aqueous phase by adding 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of ethanol. Precipitate overnight at -20°C or 30 minutes at -70°C. Wash pellet with 70% ethanol. Air dry or dry down in a speed vac.

14. Resuspend DNA in 20 - 40 µl distilled, deionized water.

The single-stranded DNA is ready for priming and sequencing.

Use of the ANTI-FLAG M2 Affinity Gel, A 2220

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. B3553, or CelLytic B II, Product No. B3678
- Appropriate column or centrifuge tubes
- Sodium chloride, Product No. S3014
- Trizma® base, Product No T6066
- Protease inhibitor cocktail for use with mammalian cells and tissue extracts, Product No. P8340

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

1. 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. B3553) or CelLytic B II (Product No. B3678) 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

      i. Grow the cells (about 1 liter or less) under conditions that induce production of FLAG fusion proteins.

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

      iii. Decant the media from the cell paste.

      iv. 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.

      v. 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.

vi. 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.

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

viii. 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

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

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

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

iv. 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.

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

vii. 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. P8340) to the lysis buffer (10 µl per 1 ml lysis buffer), especially if the lysate is to be stored for further use.

i. Wash cells.

- 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).

- For cells in suspension:
  Collect the cells into an appropriate centrifuge conical test tube. Centrifuge for 5 minutes at 420Xg. 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).

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

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

iv. Centrifuge the cell lysate for 10 minutes at 12,000 X g.

v. 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.

2. 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.

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

b. 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.

c. 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.

d. Immediately transfer the suspension to the column.

e. 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.

f. 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.

g. 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.**

h. 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.

Table 6. Reagent Compatibility Table For A2220 ANTI-FLAG Affinity Gel

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.