pMAL™ Protein Fusion and Purification System

Expression and Purification of Proteins from Cloned Genes

Instruction Manual

Catalog #E8000S

NEW ENGLAND BioLabs Inc.

Version 5.01
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Table of Contents

System Components

pMAL™-c2X 10 µg
pMAL™-p2X 10 µg

Supplied in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. Store at –20°C

Amylose Resin 15 ml
Supplied pre-swollen in 20% ethanol. Binding capacity > 3 mg/ml bed volume. Store at 4°C

Factor Xa 50 µg
Purified from bovine plasma; MW 42,400 Daltons (two disulfide-linked chains, MW ~ 30 kDa and ~ 20 kDa). 1 µg of Factor Xa will cleave 50 µg of the test substrate MBP paramyosinΔSal to 95% completion in 8 hours or less at 23°C in 50 µl. Supplied in 20 mM HEPES, 500 mM NaCl, 2 mM $CaCl_2$, 50% glycerol, pH 8.0. Store at –20°C

Anti-MBP Antiserum 25 µl
Rabbit serum prepared using purified maltose-binding protein. Suggested dilution for Western blotting or ELISA 1:10,000. Store at –20°C.

MBP2* 10 µg (40 µg/ml)

Expressed from a derivative of pMAL-c2X, including amino acid residues coded for by the polylinker up to the *Xmn* I site. MW 42,698 Daltons. Supplied in SDS-PAGE Sample Buffer. Store at –20°C.

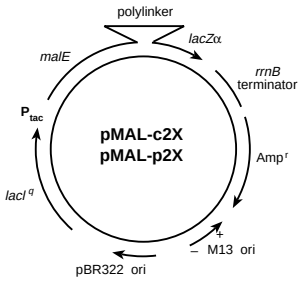
MBP2*-paramyosin ΔSal 100 µg 5 mg/ml

An MBP fusion protein as a positive control for Factor Xa cleavage. Supplied in 20 mM Tris-HCl, 200 mM NaCl, 50% glycerol, pH 7.2. MW 70,216 Daltons. Store at –20°C.

E. coli host TB1

F^- *ara Δ(lac-proAB) [ϕ80dlac Δ(lacZ)M15] rpsL(*Str^R*) thi hsdR*. Supplied frozen in 25% glycerol (not competent). Store at –70°C.

Information presented herein is accurate and reliable to the best of our knowledge and belief, but is not guaranteed to be so. Nothing herein is to be construed as recommending any practice or any product in violation of any patent or in violation of any law or regulation. It is the user's responsibility to determine for himself or herself the suitability of any material and/or procedure for a specific purpose and to adopt such safety precautions as may be necessary.



pMAL-c2X, pMAL-p2X Polylinker

Xmn I *Eco*R I *Bam*H I *Xba* I *Sal* I *Pst* I *Hin*d III

malE...ATC GAG GGA AGG ATT TCA GAA TTC GGA TCC TCT AGA GTC GAC CTG CAG GCA AGC TTG...*lacZα*

Ile Glu Gly Arg

Factor Xa cleavage site

pMAL-c2E, pMAL-p2E Polylinker

Kpn I *Eco*R I *Bam*H I *Xba* I *Sal* I *Pst* I *Hin*d III

malE...GAT GAC GAT GAC AAG GTA CCG GAA TTC GGA TCC TCT AGA GTC GAC CTG CAG GCA AGC TTG...*lacZα*

Asp Asp Asp Asp Lys

Enterokinase cleavage site

pMAL-c2G, pMAL-p2G Polylinker

*Sna*B I *Eco*R I *Bam*H I *Xba* I *Sal* I *Pst* I *Hin*d III

malE...CCG GGT GCG GCA CAC TAC GTA GAA TTC GGA TCC TCT AGA GTC GAC CTG CAG GCA AGC TTG...*lacZα*

Pro Gly Ala Ala **His Tyr**

Genenase I cleavage site

Figure 1. pMAL-2 Vectors. pMAL-c2X (6648 base pairs) has an exact deletion of the *malE* signal sequence. pMAL-p2X (6723 base pairs) includes the *malE* signal sequence. Arrows indicate the direction of transcription. Unique restriction sites are indicated.

Introduction

The pMAL™-2 vectors (Figure 1) provide a method for expressing and purifying a protein produced from a cloned gene or open reading frame. The cloned gene is inserted downstream from the *malE* gene of *E. coli*, which encodes maltose-binding protein (MBP), resulting in the expression of an MBP fusion protein (1,2). The method uses the strong "tac" promoter and the *malE* translation initiation signals to give high-level expression of the cloned sequences (3,4), and a one-step purification of the fusion protein using MBP's affinity for maltose (5). The vectors express the *malE* gene (with or without its signal sequence) fused to the *lacZ*α gene. Restriction sites between *malE* and *lacZ*α are available for inserting the coding sequence of interest. Insertion inactivates the β-galactosidase α-fragment activity of the *malE-lacZ*α fusion, which results in a blue to white color change on Xgal plates when the construction is transformed into an α-complementing host such as TB1 (6) or JM107 (7). When present, the signal peptide on pre-MBP directs fusion proteins to the periplasm. For fusion proteins that can be successfully exported, this allows folding and disulfide bond formation to take place in the periplasm of *E. coli*, as well as allowing purification of the protein from the periplasm (8). The vectors carry the *lacI*^{q} gene, which codes for the Lac repressor. This keeps expression from P_{tac} low in the absence of IPTG induction. The pMAL-2 vectors also contain the sequence coding for the recognition site of a specific protease, located just 5´ to the polylinker insertion sites. This allows MBP to be cleaved from the protein of interest after purification. The pMAL-c2X and pMAL-p2X vectors that are included in the system encode the site for Factor Xa (9, 10). Factor Xa cleaves after its four amino acid recognition sequence, so that few or no vector-derived residues are attached to the

protein of interest, depending on the site used for cloning. pMAL vectors containing sites for alternative proteases are also available (see Figure 1). The vectors pMAL-c2G (NEB #N8068) and pMAL-p2G (NEB #N8069) encode the site for Genenase™I (NEB #P8075), which cleaves following the sequence His-Tyr. The vectors pMAL-c2E (NEB #N8066) and pMAL-p2E (NEB #N8067) encode the site for Enterokinase (NEB #P8070), which cleaves following the sequence Asp-Asp-Asp-Asp-Lys.

In the large majority of cases, fusion protein expressed from a pMAL-c2 plasmid constitutes 20–40% of the total cellular protein, while fusion protein expressed from a pMAL-p2 plasmid constitutes 1–5% of the total cellular protein. For pMAL-c2 vectors, a band corresponding to the fusion protein can usually be seen by running a small sample of induced cells on an SDS-PAGE gel. The yield of fusion protein from the affinity purification ranges up to 200 mg/liter culture, with typical yields in the range of 10–40 mg/liter. The yield varies greatly depending upon the sequences fused to *malE*. In cases where the yield has been compared directly, the pMAL-c2 vectors (no signal sequence) give approximately 10-fold more protein in the affinity purification than pMAL-p2 vectors. The pMAL-p2 vectors are useful for cases where export to the periplasm is desirable, e.g. for purification or disulfide bond formation. 75% of the fusions made so far have worked in the affinity purification. In the cases that have not worked, the fusion binds to the column poorly or not at all, is degraded by *E. coli* proteases, or is insoluble. The pMAL-2 vectors also contain the origin of DNA replication of the *E. coli* bacteriophage M13, which allows the production of single-stranded DNA by infection of cells bearing a pMAL-2 plasmid with a helper phage (11). The single-stranded plasmid DNA can be used for sequencing using a primer available from NEB (see **Sequencing**, page 9), or for oligonucleotide-directed mutagenesis.

Construction of the Fusion Plasmid

To produce a fusion protein in the pMAL-2 vectors, the gene or open reading frame of interest must be inserted into the pMAL-2 vectors so that it is in the same translational reading frame as the vector's *malE* gene. The vectors have a polylinker containing a restriction site for cloning fragments directly downstream of the specific protease site. A number of other restriction sites are also available for cloning fragments downstream of the primary site, or for directional cloning of a blunt/sticky-ended fragment. Inserts cloned into the primary site produce a protein of interest that, after protease cleavage, contains no vector-derived amino acids (9,10). Factor Xa, Genenase I and Enterokinase will not cleave fusion proteins that have a proline immediately following the last residue of their respective sites (the P1´ position), so the first three bases of the insert should not encode Pro when cloning into the primary site. In addition, Factor Xa fails to cleave sites where the P1´ residue is arginine, and Genenase I fails to cleave if the P1´ residue is isoleucine. Several strategies may be employed to create an appropriate fragment to subclone. It is assumed that the sequence of interest includes a translational stop codon at its 3´ end; if not, one should be engineered into the cloning strategy. Alternatively, a linker containing a stop codon (e.g. NEB #S1061S) can be inserted into one of the downstream polylinker sites, or the stop codon present in the *Xba* I site can be shifted into appropriate reading frame (e.g., by filling in the site with Klenow).

Choice of Vector

The pMAL vectors come in six versions, for expressing fusion proteins with one of three different specific protease sites in either the cytoplasm or the periplasm. Choice among the three proteases is determined empirically, since the three dimensional structure of the fusion protein determines susceptibility to cleavage. Choice between the cytoplasmic and periplasmic versions can be guided by the descriptions below.

pMAL-c2 series The *malE* gene on these vectors has a deletion of the signal sequence, leading to cytoplasmic expression of the fusion protein. This vector generally produces more fusion protein than the pMAL-p2 series. Fusion proteins that cannot be exported are more stable in pMAL-c2 vectors.

pMAL-p2 series The signal sequence of the *malE* gene on this vector is intact, potentially allowing fusion proteins to be exported to the periplasm. The pMAL-p2 vectors are the best choice if it is known that the protein of interest does not fold properly in the *E. coli* cytoplasm, or that it requires disulfide bonding to fold correctly (8). The pMAL-p2 vectors may also be the best choice if it is known that the protein of interest is a secreted protein, or is the extracellular domain of a transmembrane protein.

Sequencing

Inserts in the pMAL vectors can be sequenced using primers available from New England Biolabs. The malE Primer (NEB #S1237S) initiates sequence near the 3´ end of malE, 78-81 bases upstream of the primary site in the polylinker. This primer can be used for sequencing single stranded DNA produced using the vector's M13 origin, or for sequencing plasmid DNA. The 3´ end of inserts can be sequenced from the *lacZ*α side using the M13/pUC Sequencing Primers (NEB #'s S1211S, S1212S, or S1224S). The sequences of the pMAL vectors are available from New England Biolabs on our web page at www.neb.com or e-mail a request to info@neb.com.

Creating a Blunt-ended Fragment

Three strategies that can be used to create a blunt-ended fragment to insert into the *Xmn* I site of the pMAL-2X vectors are presented. The same strategies can be used for the pMAL-2G and pMAL-2E vectors by substituting (respectively) *Sna*B I and *Kpn* I/T4 polymerase for *Xmn* I. Where restriction enzymes are used to create an end, the enzyme must be chosen from among those that do not cut within the gene of interest. In all cases, it is desirable to use a restriction enzyme that creates an overhang at the 3´ end of the gene (*Hin*d III in the examples) to direct the orientation of the insert and increase ligation efficiency in the final step. The first codon following the Factor Xa site can be any codon except those coding for proline and arginine. Keep in mind that for strategies that employ oligonucleotides, the fragment (or the oligonucleotides used to make it) must be kinased if the vector is phosphatased after cutting.

Strategy I: Depending on the exact sequence of the gene of interest, one can sometimes create a restriction site at the 5´ end of the gene of interest by oligonucleotide-directed mutagenesis (11,12).

Example:

5´ GACC ATG ACC ATC... ...AAGCTT
3´ CTGG TAC TGG TAG...gene of interest...TTCGAA

↓ mutate to

5´ GACC ATG *T*CC ATC...
3´ CTGG TAC *A*GG TAG...

Creates a *Tth*111 I site GACN/NNGTC
CTGNN/NCAG

↓ Cut with *Tth*111 I

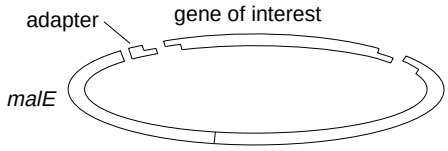
5´ ATG TCC ATC...
3´ AC AGG TAG...

↓ Fill in with DNA Polymerase I, Large Fragment (NEB #M0210) and all four dNTP's, heat inactivate the DNA polymerase, then digest with *Hin*d III

ATG TCC ATC... ...A
TAC AGG TAG...gene of interest...TTCGAA

Cut the pMAL-2X vector with *Xmn* I and *Hin*d III, mix with the prepared fragment, and ligate (see detailed ligation protocol in **Cloning a PCR fragment**, page 14).

Figure 2. Creating a blunt-ended fragment with an adapter



Strategy II: Synthesize an adaptor that recreates the start of the gene, up to a convenient restriction site early in the gene (in this example *Bss* H II) (Figure 2).

Example:

Bss H II *Hin*d III

5´ GACC GAA ACC ATC TTC AGG AGG CGC GCA... gene of ...AAGCTT
3´ CTGG CTT TGG TAG AAG TCC TCC GCG CGT... interest ...TTCGAA

↑1st codon of gene of interest

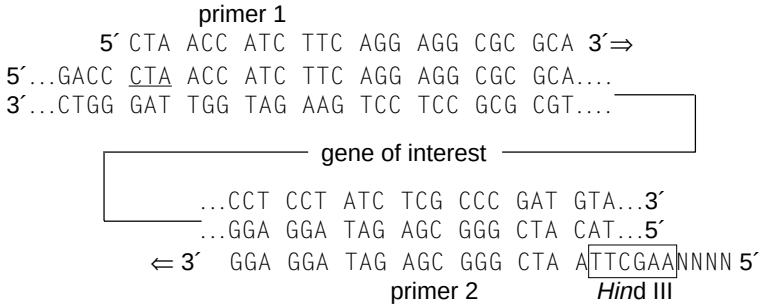
Synthesize the oligonucleotides:

5´ GAA ACC ATC TTC AGG AGG 3´
3´ CTT TGG TAG AAG TCC TCC GCG C 5´

Cut your fragment with *Bss* H II and *Hin*d III, cut the pMAL-2X vector with *Xmn* I and *Hin*d III, mix with annealed oligonucleotides (20X molar excess) and ligate (see detailed ligation protocol in **Cloning a PCR fragment**, page 14).

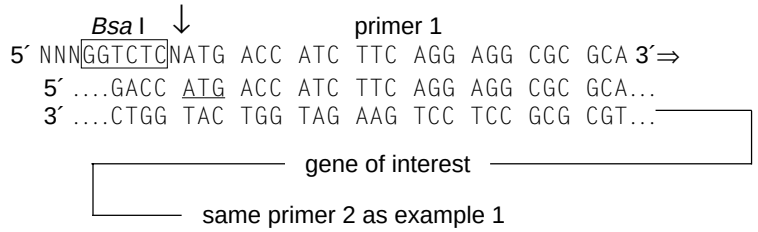
Strategy III: Perform PCR using primers that give a 5´ blunt end and a convenient 3´ sticky end (13). If the gene of interest does not have its own translation stop codon, incorporate one into the downstream PCR primer.

Example 1: Create a blunt end using a 5´ primer that starts with the first codon of the gene of interest (underlined).



Perform PCR, trim ends with T4 polymerase + dNTP's to remove any 3´ overhangs, phenol extract and ethanol precipitate, then cut the PCR fragment with *Hin*d III. Digest the pMAL-2X vector with *Xmn* I and *Hin*d III, mix with the prepared PCR fragment, and ligate (see detailed ligation protocol in **Cloning a PCR fragment**, page 14).

Example 2: Design a primer that incorporates a restriction site for a Type IIS enzyme, 5´ to the first codon of the gene of interest.



Perform PCR, cut with *Bsa* I [GGTCTC(1/5)], fill in with Klenow, heat inactivate the Klenow, then cut with *Hin*d III. Cut the pMAL-2X vector with *Xmn* I and *Hin*d III, mix with the prepared PCR fragment and ligate (see detailed ligation protocol, page 14).

Cloning a PCR Fragment

The procedure below is for cloning a fragment produced by PCR into a pMAL vector. It is assumed that the PCR fragment is approximately 1 kb, begins with a blunt end, and that a stop codon followed by a *Hin*d III site has been incorporated into the 3´ end (see Strategy III, page 12).

1. Streak the TB1 cells on an LB plate. Prepare competent cells (14).
   Any competent cells can be used for the cloning; the cells must contain the lacZΔM15 allele for the α-complementation to occur.
2. Prepare a PCR fragment of the gene of interest as outlined in Strategy III on page 12.
3. Digest 0.5 µg of the pMAL vector DNA in 20 µl of 1X NEBuffer 2 (supplied as a 10X stock) with 10 units of *Xmn* I and 10 units of *Hin*d III at 37°C for 1 hour. Heat inactivate the enzymes by incubating at 65°C for 10 minutes.
4. Check for complete digestion of the pMAL digest by running 4 µl on an agarose gel. At the same time, run a sample of the PCR fragment to estimate its concentration.
5. Digest 0.5 µg of the PCR fragment in 20 µl of 1X NEBuffer 2 with 10 units of *Hin*d III. Heat inactivate the *Hin*d III by incubating at 65°C for 10 minutes.
6. Add EDTA to both digests to a final concentration of 20 mM. Add an equal volume of a 1:1 phenol/chloroform mixture to the restriction digests, mix, and remove the aqueous (top) phase and place in a fresh tube. Repeat with chloroform alone.

Alternatively, the DNA fragments can be isolated from a low melting point agarose gel, or purified using one of the many commercially-available fragment purification kits.

7. Add 10 μg glycogen or tRNA as carrier to both digests, then add 1/9th volume 3 M sodium acetate, mix, and add an equal volume isopropanol. Incubate at room temperature for 10 minutes.

8. Microcentrifuge for 15 minutes. Pour off the supernatant, rinse the pellet with 70% ethanol, and allow to dry.

9. Resuspend each sample in 25 μl of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

   It can be helpful to run another sample of each preparation on an agarose gel to re-estimate the concentration at this point.

10. Mix: 2 μl vector digest (40 ng)
    1 μl insert digest (20 ng)
    Add 14 μl water, then heat the DNA mixture at 45°C for 5 minutes.
    Cool on ice, then add:
    2 μl 10X T4 DNA Ligase Buffer (supplied as a 10X stock)
    1 μl (~400 units) T4 DNA Ligase (NEB #M0202S)
    Incubate at 16°C for 2 hours to overnight.

11. Heat at 65°C for 5 minutes; cool on ice.

12. Mix the ligation reaction with 25 μl competent TB1 (or any *lacZ*α-complementing strain) and incubate on ice for 5 minutes. Heat to 42°C for 2 minutes.

13. Add 0.1 ml LB and incubate at 37°C for 20 minutes. Spread on an LB plate containing 100 μg/ml ampicillin (do not plate on IPTG; see below). Incubate overnight at 37°C. Pick colonies with a sterile toothpick and stab onto a master LB amp plate and an LB amp plate containing 80 μg/ml Xgal and 0.1 mM IPTG. Incubate at 37°C for 8 to 16 hours. Determine the Lac phenotype on the Xgal plate and recover the "white" clones from the corresponding patch on the master plate. It can be helpful to perform a control transformation with about 1 ng of the uncut pMAL vector as well - while most inserts prevent expression of any *lacZ*α fragment, it is possible to get some α-fragment activity in clones with inserts. In this case, a difference in the shade of blue can usually be seen by comparing to transformants containing the vector alone. An alternative way to use the blue-white screen is to replica plate on LB amp and LB amp Xgal IPTG. One can also divide the ligation mixture into two aliquots, and plate one of them on 80 μg/ml Xgal, 0.1 mM IPTG to observe the percentage of clones with inserts, and if it is acceptably high, screen the transformants on the LB amp plate directly by preparing plasmid DNA.

    Note: Because of the strength of the P_{tac} promoter, transformants taken from a plate containing IPTG can contain mutant plasmids that have either 1) lost part or all of the fusion gene, or 2) no longer express it at high levels.

14. Screen for the presence of inserts in one or both of the following ways:
    A. Prepare miniprep DNA (15). Digest with an appropriate restriction endonuclease to determine the presence and orientation of the insert (16).

    B. i) Grow a 5 ml culture in LB amp broth to 2 x 10^8 cells/ml (A_{600} of ~0.5).

ii) Withdraw a 1 ml sample. Microcentrifuge for 2 minutes, discard the supernatant and resuspend the cells in 50 μl protein gel SDS-PAGE Sample Buffer (17).

iii) Add IPTG to the remaining culture to 0.3 mM, for example 15 μl of a 0.1 M stock solution. Incubate at 37°C with good aeration for 2 hours.

iv) Withdraw a 0.5 ml sample. Microcentrifuge for 2 minutes, discard the supernatant and resuspend the cells in 100 μl SDS-PAGE sample buffer.

v) Place samples in a boiling water bath for 5 minutes. Electrophorese 15 μl of each sample on a 10% SDS-PAGE gel along with a set of protein MW standards and 15 μl of the supplied MBP2* in SDS-PAGE Sample Buffer. Stain the gel with Coomassie brilliant blue (17,18).

An induced band should be visible at a position corresponding to the molecular weight of the fusion protein. A band at or around the position of MBP2 (MW 42.5 kDa) indicates either an out of frame fusion or a severe protein degradation problem. These can usually be distinguished by performing a Western blot using the anti-MBP serum (19); even with severe protein degradation, a full length fusion protein can be detected on the Western. The molecular weight of the MBP-β-gal-α fusion is 50.8 kDa.*

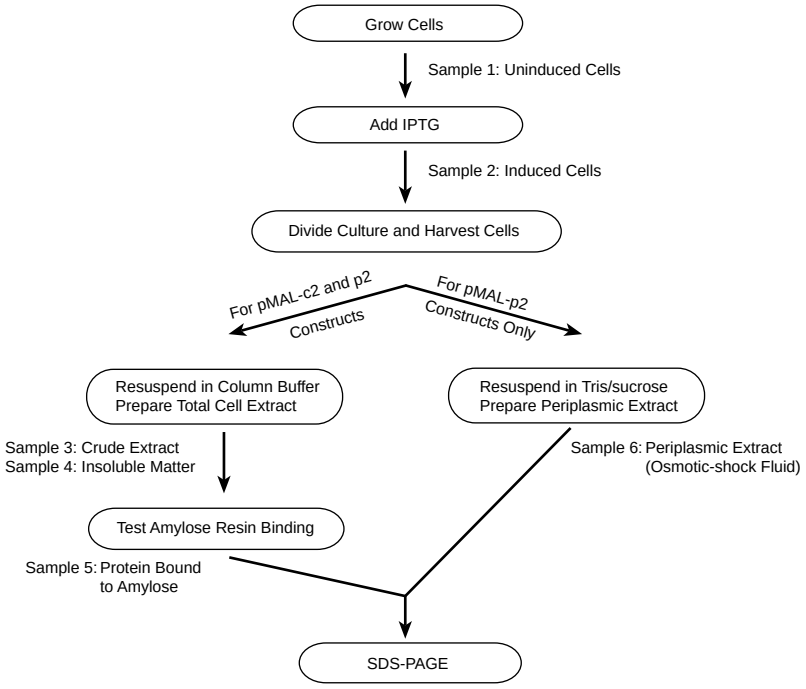


Figure 3. Flow chart for the pilot experiment.

Pilot Experiment

A small scale experiment is described to determine the behavior of a particular MBP fusion protein. This protocol results in five (pMAL-c2 vectors) or six (pMAL-p2 vectors) samples: uninduced and induced cells, a total cell crude extract, a suspension of the insoluble material from the crude extract, a fraction containing protein that binds to the amylose resin, and (for pMAL-p2 constructions) a periplasmic fraction prepared by the cold osmotic shock procedure (20) (Figure 3).

1. Inoculate 80 ml rich broth + glucose & amp (see **Media and Solutions**, page 33) with 0.8 ml of an overnight culture of cells containing the fusion plasmid.
2. Grow at 37°C with good aeration to 2 x 10^8 cells/ml (A_{600} of ~0.5). Take a sample of 1 ml and microcentrifuge for 2 minutes (sample 1: uninduced cells). Discard supernatant and resuspend the cells in 50 µl SDS-PAGE Sample Buffer. Vortex and freeze at –20°C.
3. Add IPTG (isopropylthiogalactoside) to the remaining culture to a final concentration of 0.3 mM, e.g. 0.24 ml of a 0.1 M stock in H_2O (see **Media and Solutions**). Continue incubation at 37°C for 2 hours. Withdraw a 0.5 ml sample and microcentrifuge for two minute (sample 2: induced cells). Discard supernatant and resuspend the cells in 100 µl SDS-PAGE Sample Buffer. Vortex to resuspend cells and freeze at –20°C.

   Additional time points at 1 and 3 hours can be helpful in trying to decide when to harvest the cells for a large scale prep.
4. Divide the remaining culture into two aliquots. Harvest the cells by centrifugation at 4000 x *g* for 10 minutes. Discard the supernatants and resuspend one pellet in 5 ml of Column Buffer (see **Media and**

Solutions), for protocol A. For pMAL-p2 constructions, resuspend the other pellet in 10 ml 30 mM Tris-HCl, 20% sucrose, pH 8.0, for protocol B (8 ml / 0.1 g cells wet weight).

Protocol A (all constructions)

5A. Freeze the cells in Column Buffer in a dry ice-ethanol bath (or overnight at –20°C; –20°C is more effective than –70°C, but takes longer). Thaw in cold water.

6A. Place the cells in an ice-water bath and sonicate in short pulses of 15 seconds or less. Monitor the release of protein using the Bradford assay (21), adding 10 µl of the sonicate to 1.5 ml Bradford reagent and mixing. Continue sonication until the released protein reaches a maximum (usually about 2 minutes); a standard containing 10 µl of 10 mg/ml BSA in 1.5 ml of Bradford reagent can be helpful as an approximate endpoint.

7A. Centrifuge at 9,000 x *g* at 4°C for 20 minutes. Decant the supernatant (crude extract) and save on ice. Resuspend the pellet in 5 ml Column Buffer. This is a suspension of the insoluble matter. Add 5 µl 2x SDS-PAGE Sample Buffer to 5 µl of the crude extract and insoluble matter fractions (samples 3 and 4, respectively).

8A. Place ~200 µl of the amylose resin in a microfuge tube and spin briefly in a microcentrifuge. Remove the supernatant by aspiration and discard. Resuspend the resin in 1.5 ml Column Buffer, then microcentrifuge briefly and discard the supernatant; repeat. Resuspend the resin in 200 µl of Column Buffer. Mix 50 µl of crude extract with 50 µl of the amylose resin slurry. Incubate for 15 minutes on ice. Microcentrifuge 1 minute, then remove the supernatant and discard. Wash the pellet with 1 ml Column Buffer, microcentrifuge 1 minute, and resuspend the resin in 50 µl SDS-PAGE Sample Buffer (sample 5: protein bound to amylose).

Protocol B (pMAL-p2 constructions only)

5B. Add 20 µl 0.5 M EDTA (1 mM final conc.) to the cells in Tris/sucrose and incubate for 5–10 minutes at room temperature with shaking or stirring.

6B. Centrifuge at 8000 x *g* at 4°C for 10 minutes, remove all the supernatant, and resuspend the pellet in 10 ml ice-cold 5 mM $MgSO_4$.

7B. Shake or stir for 10 minutes in an ice-water bath.

8B. Centrifuge as above. The supernatant is the cold osmotic shock fluid. Add 10 µl 2X SDS-PAGE Sample Buffer to 10 µl of the cold osmotic shock fluid (sample 6).

SDS-PAGE (all constructions)

9. Place the samples in a boiling water bath for 5 minutes. Microcentrifuge for 1 minute. Load 20 µl of the of uninduced cells, induced cells and amylose resin samples (avoid disturbing the pellets), and all of the remaining samples, on a 10% SDS-PAGE gel (17).

10. (Optional) Run an identical SDS-PAGE gel(s) after diluting the samples 1:10 in SDS-sample buffer. Prepare a Western blot(s) and develop with anti-MBP serum and, if available, serum directed against the protein of interest (19).

    If the fusion is in the periplasmic fraction, consider using Method II in ***Affinity Chromatography.*** *In this case, another pilot to optimize expression and export may be desirable. If the protein is insoluble, modify the conditions of cell growth to attempt to produce soluble fusion. Two changes that have helped in previous cases are i) changing to a different strain background, and ii) growing the cells at a lower temperature (8).*

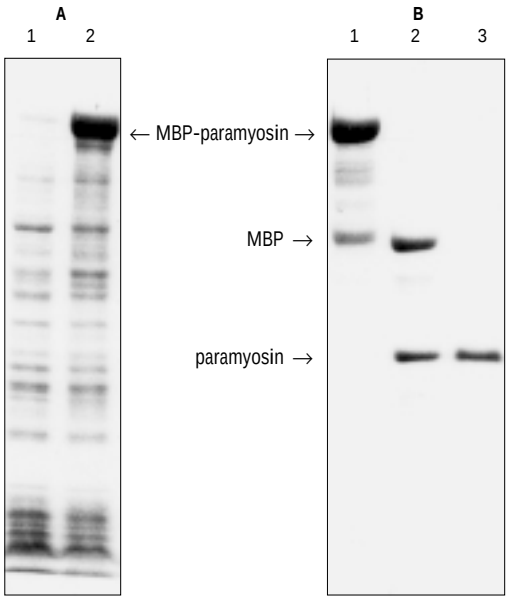


Figure 4. SDS-polyacrylamide gel electrophoresis of fractions from the purification of MBP-paramyosin-ΔSal. A: Lane 1: uninduced cells. Lane 2: induced cells. B: Lane 1: purified protein eluted from amylose column with maltose. Lane 2: purified protein after Factor Xa cleavage. Lane 3: paramyosin fragment in flow-through from second amylose column.

Affinity Chromatography

Two protocols for purification of a fusion protein from a 1 liter culture are detailed below. Method I is designed for purification of a soluble fusion protein expressed in the cytoplasm from a pMAL-c2 vector. However, the crude extract prepared by this method also contains periplasmic proteins, so it can be used to prepare secreted fusion proteins expressed from a pMAL-p2 vector as well. Method II is designed for purification of a secreted fusion protein expressed from a pMAL-p2 vector. It results in a periplasmic fraction that contains many fewer *E. coli* proteins than the total cell crude extract prepared by method I, but the procedure is more difficult to scale up.

Method I

1. Inoculate 1 liter rich broth + glucose & ampicillin (see **Media and Solutions**, page 33) with 10 ml of an overnight culture of cells containing the fusion plasmid.

   Glucose is necessary in the growth medium to repress the maltose genes on the chromosome of the E. coli host, one of which is an amylase which can degrade the amylose on the affinity resin.

2. Grow to 2 x 10^8 cells/ml (A_{600} ~0.5). Add IPTG to a final concentration of 0.3 mM, e.g. 72 mg or 3 ml of a 0.1 M stock in H_2O (see **Media and Solutions**). Incubate the cells at 37°C for 2 hours.

   The period of time and the temperature to use during expression depends on several factors (stability of the protein, host strain, etc.), and variations can be tried to find optimum conditions for expression.

3. Harvest the cells by centrifugation at 4000 x *g* for 20 minutes and discard the supernatant. Resuspend the cells in 50 ml Column Buffer (see **Media and Solutions**).

   For many unstable proteins, most of the degradation happens during harvest and cell breakage. Therefore, it is best to harvest the cells quickly and keep them chilled. 50 ml of Column Buffer is based on the expectation of about 5 grams cells/liter, i.e. 10 ml for every gram of cells (wet weight).

   The EDTA in the lysis buffer is to help inhibit proteases that have a Ca^{++} cofactor. Addition of PMSF (phenyl methylsulfonyl fluoride) and/or other protease inhibitors may help in some cases.

   *DTT or β–mercaptoethanol can be included to prevent interchain disulfide bond formation upon lysis (disulfide bonds usually do not form intracellularly in E. coli). For more about variations and additions to the Column Buffer, see **Media & Solutions**, page 33.*

4. Freeze sample in a dry ice-ethanol bath (or overnight at –20°C; –20°C is more effective than –70°C, but takes longer). Thaw in cold water.

5. Place sample in an ice-water bath and sonicate in short pulses of 15 seconds or less. Monitor the release of protein using the Bradford assay (21), adding 10 µl of the sonicate to 1.5 ml Bradford reagent and mixing. Continue sonication until the released protein reaches a maximum (usually about 2 minutes sonication time); a standard containing 10 µl of 10 mg/ml BSA can be helpful as an approximate endpoint.

6. Centrifuge at 9,000 x *g* for 30 minutes. Save the supernatant (crude extract). Dilute the crude extract 1:5 with Column Buffer.

7. Pour the amylose resin in a 2.5 x 10 cm column. Wash the column with 8 column volumes of Column Buffer.

   The amount of resin depends on the amount of fusion protein produced.

The resin binds about 3 mg/ml bed volume, so a column of about 15 ml should be sufficient for a yield of up to 45 mg fusion protein/liter culture. A 50 ml syringe plugged with silanized glass wool can be substituted for the 2.5 cm column, but the glass wool should cover the bottom of the syringe (not just in the tip) so the column will have an acceptable flow rate.

8. Load the diluted crude extract at a flow rate of [10 x (diameter of column in cm)^2]ml/hour. This is about 1 ml/minute for a 2.5 cm column.

   The dilution of the crude extract is intended to reduce the protein concentration to about 2.5 mg/ml. If your crude extract is less concentrated, don't dilute it as much. In general, 1 g wet weight of cells gives about 50–120 mg protein.

9. Wash with 12 column volumes of Column Buffer.

   The column can be washed overnight, if it has a safety loop to prevent it from running dry (see Figure 5, page 31). In this case, it is better to restart the column with elution buffer (step 10), rather than continuing the wash. Avoid loading the column overnight.

10. Elute the fusion protein with Column Buffer + 10 mM maltose. Collect 10 to 20 fractions of 3 ml each (fraction size = 1/5th column volume). The fusion protein usually starts to elute within the first 5 fractions, and should be easily detected by UV absorbance at 280 nm or the Bradford protein assay.

11. Pool the protein-containing fractions. If necessary, concentrate to about 1 mg/ml in an Amicon Centricon or Centriprep concentrator, an Amicon stirred-cell concentrator, or the equivalent.

Method II

1. Inoculate 1 liter rich broth + glucose & ampicillin with 10 ml of an overnight culture of cells containing the fusion plasmid.
2. Grow to 2 to 4 x 10^8 cells/ml (A_{600} ~0.5). Add IPTG to a final concentration of 0.3 mM, e.g. 72 mg/l or 3 ml of a 0.1 M stock in H_2O. Incubate the cells at 37°C for 2 hours.

   The period of time and the temperature to use during expression depends on several factors (stability of the protein, host strain, etc.), and variations can be tried to find optimum conditions (see ***Pilot Experiment****). In addition, partial induction of exported proteins may lead to higher yields, since protein export in E. coli may not be able to keep up with full level* P_{tac} *expression.*
3. Harvest the cells by centrifugation at 4000 x *g* for 20 minutes and discard the supernatant. Resuspend the cells in 400 ml 30 mM Tris-HCl, 20% sucrose, pH 8.0 (80 ml for each gram of cells wet weight). Add EDTA to 1 mM and incubate for 5–10 minutes at room temperature with shaking or stirring.
4. Centrifuge at 8000 x *g* for 20 minutes at 4°C, remove all the supernatant, and resuspend the pellet in 400 ml of ice-cold 5 mM $MgSO_4$. Shake or stir for 10 minutes in an ice bath.
5. Centrifuge at 8000 x *g* for 20 minutes at 4°C. The supernatant is the cold osmotic shock fluid.
6. Add 8 ml of 1 M Tris-HCl, pH 7.4 to the osmotic shock fluid.
7. Continue from Method I, step 7 (page 24).

Regenerating the Amylose Resin Column

The resin may be reused three to five times when regenerated with the following sequence of washes:

Water: 3 column volume
0.1% SDS: 3 column volumes
Water: 1 column volumes
Column Buffer: 3 column volumes

Please note that although the column can be washed at 4°C, 0.1% SDS will eventually precipitate at that temperature. It is therefore recommended that the SDS solution be stored at room temperature until needed, and rinsed out of the column promptly. The resin may be reused three to five times. Upon repeated use, trace amounts of amylase in the *E. coli* extract decrease the binding capacity of the column. It is recommended that the column be washed promptly after each use.

Cleavage of the Fusion Protein

For Factor Xa and Genenase I, fusion protein cleavage is carried out at a w/w ratio of 1% the amount of fusion protein (e.g., 1 mg Factor Xa for a reaction containing 100 mg fusion protein). The reaction mixture can be incubated for 3 hours to several days, at room temperature or 4°C. Depending on the particular fusion protein, the amount of protease can be adjusted within the range of 0.1–5.0%, to get an acceptable rate of cleavage. For enterokinase cleavage, 1 unit is used for every 50 μg fusion protein. Again, conditions can be adjusted according to the characteristics of a particular fusion. All the proteases will cleave at non-canonical sites in some proteins; for some fusions, there is a correlation between instability of the protein of interest in *E. coli* and cleavage at additional sites (unpublished observations). Presumably this cleavage activity depends on the three dimensional conformation of the fusion protein. For fusions that are resistant to cleavage, two strategies can sometimes help. Inclusion of small amounts of SDS (0.005–0.05%) in the reaction appears to relax the fusion enough to allow for cleavage in some cases (22). The window of SDS concentrations that work can be small, so a pilot titration with different SDS concentrations is necessary. Another strategy that sometimes helps is to denature the fusion to render the protease site accessible to cleavage (see below and reference 9).

1. If necessary, concentrate the fusion protein to at least 1 mg/ml.

   All three proteases will work in the Column Buffer + maltose used to elute the fusion protein. In addition, they will work in a variety of other buffers, with NaCl concentrations from 0 to 500 mM and pH values around 8.

2. Do a pilot experiment with a small portion of your protein.

   Example: Mix 20 μl fusion protein at 1 mg/ml, with either: 1 μl Factor Xa or Genenase I, diluted to 200 μg/ml, or 0.4 U Enterokinase

In a separate tube, place 5 µl fusion protein with no protease (mock digestion). Incubate the tubes at room temperature. At 2, 4, 8, and 24 hours, take 5 µl of the reaction, add 5 µl 2x SDS-PAGE Sample Buffer, and save at 4°C. Prepare a sample of 5 µl fusion protein + 5 µl 2X sample buffer (uncut fusion).

3. Boil the 6 samples for 5 minutes and run on an SDS-PAGE gel (17).
4. Scale the pilot experiment up for the portion of the fusion protein to be cleaved. Save at least a small sample of the uncut fusion as a reference.
5. Check for complete cleavage by SDS-PAGE.

Denaturing the fusion protein

1. Either dialyze the fusion against at least 10 volumes 20 mM Tris-HCl, 6 M guanidine hydrochloride, pH 7.4 for 4 hours, or add guanidine hydrochloride directly to the sample to give a final concentration of 6 M.
2. Dialyze against 100 volumes Column Buffer, 2 times at 4 hours each.

   During refolding, one has to balance between two objectives. For the protease to cleave it must be present before the protein has completely refolded, so removing the denaturant quickly is desirable. However, when the denaturant is removed quickly some proteins will fail to refold properly and precipitate. Stepwise dialysis against buffer containing decreasing amounts of guanidine hydrochloride can prevent precipitation of the fusion protein; halving the guanidine concentration at each step is convenient, but cases where 0.1 M steps are necessary have been reported. However, if the fusion protein is able to refold into a protease-resistant conformation, it may be better to dialyze away the denaturant in one step and take the loss from precipitation in order to maximize the amount of cleavable fusion protein recovered.

   Go to step 2 or 4 above, as appropriate.

Separating the Protein of Interest from MBP After Protease Cleavage

Method I: DEAE-Sepharose™ ion exchange chromatography

This method potentially purifies the target protein away from MBP and the protease, but also provides an additional purification step for removing trace contaminants. A disadvantage is that occasionally the peak containing the protein of interest overlaps with MBP or the protease, resulting in poor separation. The procedure is written for quantities <25 mg, and can be scaled up for larger amounts.

1. Dialyze the fusion protein cleavage mixture vs. 20 mM Tris-HCl, 25 mM NaCl, pH 8.0 (2 or 3 changes of 100 volumes, at least 2 hours each).
2. Wash about 6 ml of DEAE-Sepharose in 20 ml of 10 mM Tris-HCl, 25 mM NaCl, pH 8.0 a couple of times, letting the resin settle and pouring off the supernatant between washes.
3. Pour the resin into a 1 x 10 cm column to give a bed volume of 5 ml (6–7 cm bed height).
4. Wash the column with 15 ml of the same buffer.
5. Load the fusion protein cleavage mixture onto the column. Collect 2.5 ml fractions of the column flow-through.
6. Wash the column with 3–5 column volumes of the same buffer. Continue collecting 2.5 ml fractions.
7. Start a gradient of 25 mM NaCl to 500 mM NaCl (25 ml each) in 20 mM Tris-HCl, pH 8.0 (see Figure 5). Collect 1 ml fractions.

8. Determine which fractions contain protein by measuring A_{280}, or by the Bradford or Lowry method. The MBP elutes as a sharp peak at 100–150 mM NaCl. Factor Xa elutes at about 400 mM NaCl, Genenase I and Enterokinase flow through the column. The target protein may flow through the column, or it may elute during the gradient. Electrophorese the relevant fractions on an SDS-PAGE gel. Pool the fractions containing the target protein free of MBP and concentrate as desired.

   If your protein is not separated from MBP using DEAE-Sepharose chromatography, other chromatography resins can be tried. SP-Sepharose (phosphate buffer at pH 7.0; MBP flows through) and gel filtration have been used successfully.

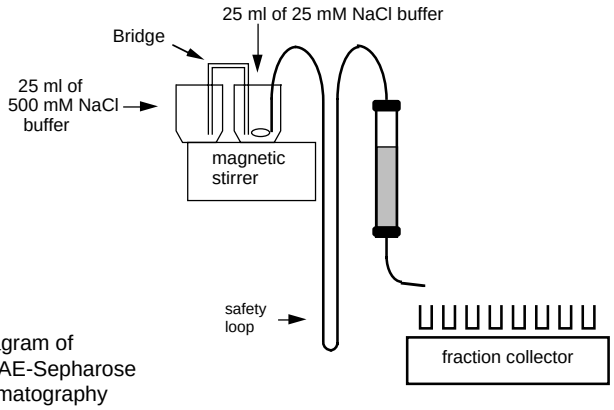


Figure 5. Diagram of setup for DEAE-Sepharose column chromatography

Method II: Removal of maltose by hydroxyapatite chromatography and domain separation by rebinding MBP to amylose

This method requires 2 steps, but since no dialysis is needed and both columns are step-eluted, the procedure is fairly simple. It removes MBP from the cleavage mixture, but not the protease or any other trace contaminants. In addition, any MBP that has been denatured or otherwise damaged will not bind to the amylose column. The procedure must be carried out at room temperature to avoid precipitation of the phosphate buffer. This procedure is written for quantities <25 mg, but can be scaled up for larger amounts.

1. Swell 1 g hydroxyapatite in 20 mM sodium phosphate, 200 mM NaCl, pH 7.2. Allow the resin to settle and pour off excess buffer (removes fine particles). Add fresh buffer and repeat twice.
2. Pour the hydroxyapatite into a 1 x 10 cm column (a disposable polypropylene column such as a BioRad Econo-Pac™ can be used).
3. Load the fusion protein cleavage mixture onto the column.
4. Wash with 80 ml of the same buffer (washes away the maltose).
5. Elute with 0.5 M Na phosphate, pH 7.2 (stock solution in **Media and Solutions**). Collect 2 ml fractions. Assay for protein by A_{280}, Bradford assay or Lowry (21). Most of the protein usually elutes in the first 8 ml.
6. Pour a 15 ml amylose column as described in **Affinity Chromatography**.
7. Load the hydroxyapatite-eluted protein onto the amylose column. Collect the flow-through as 5 ml fractions. Protein in the flow-through should be free of MBP and consist primarily of the target protein. Assay for protein by A_{280}, Bradford assay or Lowry. The protein of interest should flow through the column by the seventh fraction.

Media & Solutions

Rich medium + glucose & ampicillin:
per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose
autoclave; add sterile ampicillin to 100 μg/ml

0.1 M IPTG stock
1.41 g IPTG (isopropyl-β-D-thiogalactoside), hemidioxane adduct, or
1.19 g IPTG; add H_2O to 50 ml; filter sterilize, store at 4°C;
hemidioxane adduct is stable for ~ 6 months, dioxane-free is light sensitive

Column Buffer

PER LITER	FINAL CONCENTRATION
20 ml 1.0 M Tris-HCl, pH 7.4	20 mM Tris-HCl
11.7 g NaCl	200 mM NaCl
2.0 ml 0.5 M EDTA	1 mM EDTA
optional:	
1.0 ml 1M sodium azide	1 mM azide
0.7 ml β-mercaptoethanol	10 mM β-ME
or	or
154 mg DTT	1 mM DTT

Notes on additions or changes to the Column Buffer
The conditions under which MBP fusions will bind to the column are flexible, and the Column Buffer can be modified without adversely effecting the affinity purification. Buffers other than Tris-HCl that are compatible include MOPS, HEPES, and phosphate, at pH values around 7. MBP binds to amylose primarily by hydrogen bonding, so higher ionic strength does not decrease its affinity. Nonionic detergents such as Triton X-100 and Tween 20 have been seen to interfere with the affinity of some fusions, while other fusions are unaffected.

0.5 M sodium phosphate buffer, pH 7.2 (stock)
A) 69.0 g $NaH_2PO_4 \bullet H_2O$, to 1 liter with H_2O
B) 134.0 g $Na_2HPO_4 \bullet 7H_2O$, to 1 liter with H_2O
Mix 117 ml (A) with 383 ml (B). Store at room temperature.

Troubleshooting and Tips

1. Cloning and Transformation

1.1 *What are the differences among the various pMAL vectors?*

The pMAL-c, -cRI and -p are the earliest versions of the pMAL vectors. pMAL-c and pMAL-p have a *Stu* I site in the polylinker for cloning blunt-ended fragments. Because the second half of the *Stu* I site codes for proline, if you clone an *Eco*R I fragment into pMAL-c or pMAL-p, the Factor Xa site reads IEGRP, and RP won't cut with Factor Xa. pMAL-cRI was designed as a short-term solution to fix this problem, by changing the polylinker to code for IEGRI upstream of the *Eco*R I site. The pMAL-c2 and pMAL-p2 vectors are the next generation of pMAL vectors. These vectors avoid the problem with Factor Xa cleavage by using an *Xmn* I site instead of *Stu* I. They also have a spacer between *malE* and the Factor Xa site which allows some fusions to bind more tightly to the amylose resin, and an M13 origin for making single stranded DNA. The third generation of pMAL vectors is distinguished by the addition of vectors that substitute an Enterokinase or Genenase I site for the Factor Xa site. These vectors are called pMAL-c2E and pMAL-p2E (Enterokinase), and pMAL-c2G and pMAL-p2G (Genenase). The Factor Xa versions are now called pMAL-c2X and pMAL-p2X for consistency. This third generation of vectors have a few minor modifications outside the polylinker as well. The *Nde* I site in the pBR322 origin was destroyed by filling in, and an *Nde* I site at the ATG of *malE* was added by site directed mutagenesis. This allows a *malE* fusion to be cut out in order to subclone it, for example into a eukaryotic vector. The *Nco* I site in *malE* was destroyed, as was the *Ava* I site in the M13 origin, making the *Ava* I site upstream of the Factor Xa site unique. In all vectors, the “-c’’ designation refers to cytoplasmic expression, i.e. the signal sequence that directs MBP to the periplasmic space has been

deleted. Vectors that are designated “-p’’ refer to periplasmic expression, and these contain the wild-type *malE* signal sequence.

1.2 *What strain(s) do you recommend as hosts for the pMAL vectors?*

The strain we have used most frequently is TB1, NEB #E4122S, which is JM83 *hsdR*. There is nothing special about it with respect to the pMAL system, but it has given the best results when considering plasmid stability, expression and purification. We have used a number of other strains successfully for certain proteins. We also use other strains in response to a particular problem (for example, see 2.1). One can start with TB1, or one can use whatever competent cells are readily available and then try TB1 or another strain if a problem with expression or purification develops.

1.3 *Can I clone into pMAL vectors just as I would into pUC vectors, i.e. plating my transformants on Xgal/IPTG plates and picking white colonies?*

No. Even though the β-galactosidase α fragment is the same on the pUC and pMAL vectors, the tac promoter on the pMAL vectors is much stronger than the lac promoter on the pUC vectors. If cells bearing a pMAL vector are induced with IPTG, the cells eventually die. The blue-to-white screen is done by replica plating (or picking and stabbing) onto a master amp plate and an amp Xgal plate containing 0.1 mM IPTG.

1.4 *What is the transformation efficiency of the pMAL vectors?*

The pMAL vectors transform about 1/10th as well as pUC and pBR322. High amp concentration (>100 ug/ml) can cause lower efficiencies as well.

1.5 *What primers should I use to sequence the ends of my insert after I clone it into a pMAL vector?*

Use the malE primer (NEB #S1237S) on the 5´ side of the insert. If you want to sequence the 3´ junction use pUC/M13 primers that bind to the lacZα region (e.g. NEB #S1224S).

1.6 *What are some of the possible explanations for an inability to clone an insert into a pMAL vector?*

The most common explanation for this is technical difficulties with the subcloning. Another explanation is that expression of the fusion is toxic to *E. coli*. The tac promoter induction ratio on the pMAL plasmids is about 1:50, so if the induced level of the fusion is 40% of the total cellular protein, the uninduced level works out to 0.8%. This amount of a protein can be toxic, either because of its function (e.g. a protease) or because of its general properties (e.g. very hydrophobic).

1.7 *How can I obtain the sequences of the pMAL vectors?*

The pMAL sequences are available on the New England Biolabs web site at www.neb.com, by e-mail from info@neb.com, and by FAX (call NEB Technical Support, 1-800-632-7799).

1.8 *Which strand of DNA is packaged when cells carrying the pMAL vectors are superinfected with an M13 helper phage such as M13KO7 (NEB #N0315S)?*

The strand corresponding to the antisense strand of the *malE* gene is packaged. This is the strand that is complementary to the polylinker sequence shown in the 2000/01 NEB Catalog on page 276. The malE primer (NEB #S1237S) hybridizes to the strand that is packaged by the helper phage.

2. Expression

2.1 *When we analyze our fusion protein expression by Western blot using the anti-MBP serum, only a small fraction of the protein is full-length, while most of it migrates close to the MBP2* marker.*

It is likely that the fusion protein is degraded, leaving a stable MBP-sized breakdown product. In this case, try using a protease deficient host. A list of strains, which are free with an order or for the price of shipping, can be found on page 51. For cytoplasmic expression, the most protease deficient strain is CAG629 (#E4125S) – it is also, however, the most difficult to work with. ER2508 (#E4127S) and CAG597 (#E4123S) are good alternatives. For periplasmic expression, the most protease deficient strain is CAG597 (#E4123S); KS1000 (#E4128S) and UT5600 (#E4129S) might be worth trying as well. The CAG strains are difficult to transform, and often require electroporation to introduce the fusion plasmid.

2.2 *My fusion protein is insoluble; is there anything I can do to get it expressed as soluble protein?*

Expressing at a lower temperature is the first thing to try. One can go as low as 15°C by moving an incubator into the cold room. Of course, the cells grow very slowly at these temperatures, so grow the culture at 37°C and shift to the low temperature when adding IPTG. One also has to increase the time of induction to compensate for the slower growth – a rule of thumb is 2X for every 7°C. Other references for solubility problems include:

One of the original papers describing how expression at lower temperatures can produce soluble protein:
Bishai, W.R., Rappuoli, R. and Murphy, J.R. (1987) High-level expression

of a proteolytically sensitive diphtheria toxin fragment in *E. coli*. *J. Bact.* 169, 5140–5151.

Same for an exported protein:
Takagi, H. et al. (1988) Control of folding of proteins secreted by a high expression secretion vector, pIN-III-ompA: 16-fold increase in production of active subtilisin E in *E. coli*. *Biotechnology* 6, 948–950.

A method for growing the cells under osmotic stress, which can also help produce soluble protein:
Blackwell, J.R. and Horgan, R. (1991) A novel strategy for production of a highly expressed recombinant protein in an active form. *FEBS Letters* 295, 10–12.

Review on methods to make correctly-folded protein in E. coli:
Georgiou, G. and Valax, P. (1996) Expression of correctly folded proteins in *E. coli*. *Current Opinion in Biotechnology* 7, 190–197.

Reviews on insolubility:
Schein, C.H. (1989) Production of soluble recombinant proteins in bacteria. *Biotechnology* 7, 1141–1149.
Schein, C.H. (1990) Solubility as a function of protein structure and solvent components. *Biotechnology* 8, 308–317.
Wilkinson, D.L. and Harrison, R.G. (1991) Predicting the solubility of recombinant proteins in *E. coli. Biotechnology* 9, 443–448.
Kiefhaber, T. et al. (1991) Protein aggregation *in vitro* and *in vivo*: A quantitative model of the kinetic competition between folding and aggregation. *Biotechnology* 9, 825–829.

Reviews on refolding:
Zardeneta, G. and Horowitz, P.M. (1994) Detergent, liposome, and micelle-assisted protein refolding. *Anal Biochem* 223, 1–6.
Chaudhuri, J.B. (1994) Refolding recombinant proteins: Process

strategies and novel approaches. *Ann NY Acad Sci* 721, 374–385.
Gilbert, H.F. (1994) Protein chaperones and protein folding. *Curr Opin Biotechnol* 5, 534–539.
Schein, C.H. (1991) Optimizing protein folding to the native state in bacteria. *Curr Opin Biotechnol* 2, 746–750.
Kelley, R.F. and Winkler, M.E. (1990) Folding of eukaryotic proteins produced in *E. coli. Genet Eng (NY)* 12, 1–19.
Marston, F.A. and Hartley, D.L. (1990) Solubilization of protein aggregates. *Methods in Enzymology* 182, 264–282.

Extracting from membrane with lauroylsarcosine:
Frankel, S., Sohn, R. and Leinwand, L. (1991) The use of Sarkosyl in generating soluble protein after bacterial expression. *Proc Natl Acad Sci USA*, 88, 1192–1196.

2.3 *When I run my uninduced and induced crude extracts on SDS-PAGE side by side, I don't see an induced band.*

There are a couple of possible explanations. Inserts cloned in a pMAL-p2X vector have about a 8- to 16-fold reduced level of expression when compared to the same insert in a pMAL-c2X vector. This often reduces the amount of expression to the point where there is no visible induced band. In addition, some foreign genes are poorly expressed in *E. coli*, even when fused to a highly expressed carrier gene. Possible explanations are message instability or problems with translation – sometimes it is due to the presence of multiple rare codons in the gene of interest, and in these cases overexpression of the corresponding tRNA can help (23). Even in cases where a band is not visible, one can get yields up to 5 or 6 mg/liter of culture.

2.4 *I've cloned my insert, but after SDS-PAGE the only induced band present is the size of MBP2*.*

There are two likely explanations for this result. If the protein of interest is in the wrong translational reading frame, an MBP2*-sized band will be produced by translational termination at the first in-frame stop codon. If the protein of interest is very unstable, an MBP2*-sized breakdown product is usually produced (MBP is a very stable protein). The best way to distinguish between these possibilities is to run a Western blot using anti-MBP antiserum (NEB #E8030S). If proteolysis is occurring, at least a small amount of full-length fusion can almost always be detected. DNA sequencing of the fusion junction using the malE primer (NEB #S1237S) will confirm a reading frame problem. If the problem is proteolysis, you might want to try one of the protease deficient strains from the list on page 51.

2.5 *What are the possible effects of export (secretion, using a pMAL-p2 vector) on solubility/stability of the fusion?*

Initiating export through the cytoplasmic membrane puts a fusion protein on a different folding pathway – a difference in the solubility or stability of the protein is determined by whether this folding pathway leads to a different 3-dimensional structure for the protein. Some proteins, like MBP itself, can fold properly either in the cytoplasm or when exported to the periplasm. However, the normal folding pathway for some proteins is incompatible with passage through the membrane, the fusion protein gets stuck in the membrane and cannot fold properly; this can lead to degradation (24). Other proteins, especially ones that have multiple disulfide bonds, only fold properly when exported (the *E. coli* cytoplasm is a reducing environment, and the proteins that catalyze disulfide bond formation are present in the periplasm; 25). When this class of protein is

expressed in the cytoplasm, it may fold improperly and become degraded or insoluble.

2.6 *What is the minimum size of a fragment that can be cloned into pMAL and expressed fused to MBP? Can short peptide sequences (about 10 amino acids) be added onto MBP?*

You can use the MBP system to express short peptides. However, for every 40 mg of MBP (42.5 kDa) one gets about 1 mg of a 10 amino acid peptide (1.1 kDa).

3. Affinity Purification

3.1 *Much of my fusion protein flows through the amylose column. Is there anything I can do to improve my fusion's affinity for the amylose column?*

A MBP fusion protein might not stick to the amylose column because of the presence of some factor in the extract that interferes with binding, or because of a low intrinsic affinity. Factors in the crude extract that can interfere with binding include nonionic detergents (see 3.3) and cellular components that are released during alternative methods of lysis (lysozyme/sonication or multiple passes through a French press). In addition, cells grown in LB and similar media have substantial amounts of an amylase that interferes with binding, presumably by either cutting the fusion off the column or by releasing maltose that elutes the fusion from the column. By including glucose in the media, expression of this amylase is repressed and the problem is alleviated. A low intrinsic affinity could be caused by an interaction between the protein of interest and MBP that either blocks or distorts the maltose-binding site. Although this may be inherent in the protein of interest, sometimes the problem can be alleviated by shortening or lengthening the polypeptide that is fused to MBP.

3.2 *How many times can I use the amylose column?*

The most important variable in determining the useful life of the amylose resin is the amount of time it is in contact with trace amounts of amylase present in the crude extract (see 3.1). Under normal conditions (crude extract from 1 liter of cells grown in LB + 0.2% glucose, 15 ml column), the column loses 1–3% of its initial binding capacity each time it is used. If the yield of fusion protein under these conditions is 40 mg, this means that after 3 to 5 runs there would be a decrease in the yield. In practice, we often use a column 8 or 10 times before we notice a significant drop in the yield.

3.3 *What is known about binding in the presence of nonionic detergents?*

Some fusion proteins do not bind efficiently (<5% binding) in the presence of 0.2% Triton X–100 or 0.25% Tween 20, while other fusions are unaffected. For one fusion that does not bind in 0.25% Tween 20, diluting the Tween to 0.05% restores about 80% of the binding.

3.4 *Can I substitute a different buffer and/or salt concentration in the Column Buffer?*

Yes, we have tried HEPES, MOPS, and phosphate buffers (at pH's from 7.0 to 8.5) instead of Tris-HCl in the Column Buffer with similar results. NaCl or KCl concentrations of 25 mM to 1 M are also compatible with the affinity purification.

3.5 *I see my intact fusion protein by SDS-PAGE when I run cells boiled in Sample Buffer, but when I check the crude extract the fusion is degraded.*

For fusions expressed in the cytoplasm, in many cases most of the degradation happens during harvest and lysis. Harvesting promptly and

lysing the cells quickly may help. In other cases, degradation occurs when the fusion protein is exposed to periplasmic or outer membrane proteases (26–28). The best strategy in either case is to use a host which is deficient in the offending protease(s) (see 2.1, and the strain list starting on page 51).

3.6 *Can I perform a batch purification using the amylose resin?*

Yes, batch purification works well, although it is difficult to wash all the nonspecific proteins away as effectively as in a column due to the included volume in the resin. The resin can withstand centrifugation at up to 6000 x g. A good compromise is to load the resin in a batch mode, by incubating with shaking for 2 hours to overnight, then pour it in a column to wash and elute. Dilution of the crude extract is not as critical for loading the column by the batch method.

3.7 *Can MBP fusions be purified in the presence of denaturants like urea or guanidine-HCl?*

No, MBP's affinity to amylose and maltose depends on hydrogen bonds, that in turn are positioned by the three-dimensional structure of the protein. Agents that interfere with hydrogen bonds or the structure of the protein interfere with binding as well.

3.8 *Is the amylose resin damaged by storage at –20°C? When our kit arrived, it was placed at –20°C, but I see that the recommended storage temperature for the amylose resin is 4°C.*

The resin will freeze at –20°C but the performance of the resin is not degraded by one freeze/thaw cycle. After the ethanol is removed, the resin should be stored at 4°C to prevent damage from freezing.

4. Factor Xa Cleavage

4.1 *Factor Xa seems to be cleaving my protein at several sites, even though the protein does not contain any IEGR sequences.*

The specificity of Factor Xa reported in our catalog is as referenced in Nagai and Thøgersen (1987) (10). The basis for this specificity is that the natural Factor Xa sites in prothrombin are IEGR (or sometimes IDGR), and many examples of fusions with IEGR are cut specifically. However, proteins can be cleaved at other basic residues, depending on the context (29–32). A number of the secondary sites (but not all) that have been sequenced show cleavage following gly-arg. We have also seen a correlation between proteins that are unstable in *E. coli* and cleavage at secondary sites with Factor Xa, suggesting that these proteins are in a partially unfolded state. We've tried altering the reaction conditions to increase the specificity, but with no success. Other site-specific proteases, such as Enterokinase and Genenase I, are now available as alternatives to Factor Xa.

4.2 *Are there any control substrates for Factor Xa?*

The Protein Fusion and Purification System comes with an MBP2*-paramyosin-ΔSal fusion as a positive control for Factor Xa cleavage (NEB #E8051S). Sigma also sells a colorimetric substrate, N-benzoyl-ile-glu-gly-arg-p-nitroanilide (Sigma, #B 7020).

4.3 *How can Factor Xa be inactivated?*

The best way is to add dansyl-glu-gly-arg-chloromethyl ketone (Calbiochem, #251700) to a final concentration of 2 μM, and incubate for 1 minute at room temperature. This compound irreversibly inactivates the Factor Xa. It reacts with the active site histidine, so it could conceivably react with other sites on the protein of interest, but this is unlikely at the low concentration used.

4.4 *How can Factor Xa be removed from the reaction mix after cleavage?*

Factor Xa can be removed by passing the reaction mix over a small benzamidine-agarose column (Pharmacia, #17-0568-01 or Sigma, #B 2768). When 50 μg of Factor Xa is passed over a 0.5 ml column, less than 0.2% of the activity flows through.

4.5 *My protein cleaves very poorly with Factor Xa. Is there anything I can do to improve cleavage?*

We presume that, in these cases, the fusion protein folds so that the Factor Xa site is inaccessible. In theory, anything that perturbs the structure might uncover the site. We've tried increasing the temperature, changing buffers and salt conditions, and adding detergents. The only thing that worked was low concentrations of SDS (0.01 to 0.05%)(33). Another researcher found that calcium worked – his protein of interest was a calcium binding protein, supporting the idea that anything that might change the conformation, such as a cofactor or substrate analog, could have a dramatic effect.

4.6 *What is the molecular weight and pI of Factor Xa?*

The molecular weight of Factor Xa is 42,400 Da. It consists of two disulfide-linked chains, 26,700 and 15,700. On our SDS-PAGE gels they run as 30 kDa and 20 kDa. The pI of Factor Xa is around 5.0 (34), and the calculated pI of Factor Xa is 5.09.

4.7 *What is maximum concentration of glycerol that Factor Xa can tolerate during cleavage?*

We have tested Factor Xa cleavage in up to 20% glycerol, where it still cleaves at about half the normal rate.

4.8 *How is the rate of Factor Xa cleavage affected by urea, guanidine hydrochloride and SDS?*

The activity of Factor Xa on the chromogenic substrate Bz-IEGR-pNA in the presence of these denaturants is as follows:
Urea: In 0.25 M urea, Factor Xa cleaves at about 33% its normal rate; at 0.5 M, 25% its normal rate, in 1 M urea, about 10% its normal rate, while in 2 M urea no cleavage is detected.
Guanidine: In 0.25 M guanidine hydrochloride it cleaves at about 15% its normal rate, and in 0.5 M it cleaves at about 5% the normal rate.
SDS: Factor Xa is unaffected by concentrations of SDS below 0.005%. At 0.01% it cleaves at about half its normal rate, and at 0.03% at about one-third normal. At 0.1% and above no cleavage is detected.

4.9 *Can MBP fusions be digested with Factor Xa while bound to the amylose resin?*

Cutting a bound fusion with Factor Xa has been done, (35, unpublished results). It has two problems that make it less than ideal. First, it requires a lot of Factor Xa. With the fusion immobilized, it takes 5% for 24–48 hours to get cleavage roughly equivalent to 1% for 24 hour in solution. The second problem is that during the incubation, some of the MBP falls off the column. This may be because there are trace amounts of amylase bound to the column too, and the amylase liberates enough maltose over time to elute some of the MBP.

5. Separation of Fusion Protein Domains and Storage

5.1 *In order to rebind MBP to the column, the maltose must be removed. Can this be done by dialysis?*

Dialysis does not work very well to remove maltose from maltose-binding

protein. This is a general phenomenon of binding protein/ligand interactions; after the free ligand is gone, ligand that is released from the binding site usually finds another binding site before it encounters the dialysis membrane (36). We have determined empirically that binding the fusion to a chromatography resin and then washing away the maltose is much more effective. We prefer standard chromatography (e.g. DEAE) as the separation step, since it can separate the Factor Xa and MBP from the protein of interest. In case MBP co-elutes with the protein of interest, we include a large volume washing step to remove the maltose before starting the salt gradient. This way, the mixture can be run over an amylose column afterward if necessary.

5.2 *How should I store my protein after it is purified?*

Most proteins can be stored for at least a few days at 4°C without denaturing. For long term storage, one can either freeze at –70°C or dialyze into 50% glycerol and store at –20°C. When storing at –70°C, aliquot the protein so only the portion to be used must be thawed – repeated freeze/thaw cycles denature many proteins.

6. MBP2*

6.1 *What is MBP2*? Is it different from wild-type MBP produced from E.coli?*

MBP2* is the protein produced from pMAL-c2X that has a stop codon linker (NEB #S1061S) cloned into the *Xmn* I site. It differs from wild-type MBP by the addition of a methionine at the amino terminus (as do all fusions made in pMAL-c2 vectors), the deletion of the last four residues of wild-type MBP, and the addition of the residues encoded by the polylinker.

6.2 *Has the crystal structure of MBP been determined?*

The references for the crystal structure of MBP are Spurlino, J.C. et al. (1991) *J. Biol. Chem.* 266, 5202–5219 (37), and Sharff, A.J. et al. (1992) *Biochem.* 31, 10657–10663 (38).

6.3 *How much of MBP is dispensable for binding?*

The exact region of MBP necessary for binding has not been determined, but the structure indicates that most of the protein is necessary. From the structure, it appears that very few, if any, residues could be deleted at the C-terminus (other than the polylinker residues, of course). It is possible that some of the N-terminus could be deleted, but so far this has not been tested.

6.4 *What is the K_d, pI and extinction coefficient for MBP2*?*

The K_d of MBP for maltose is 3.5 μM; for maltotriose, 0.16 μM (39). MBP2*'s extinction coefficient is 1.5 (1 mg/ml, 1 cm path length) and its calculated pI is 4.9.

6.5 *What is the origin of the MBP region of the pMAL vectors?*

The *malE* gene in the pMAL vectors was derived from the *Hin*f I fragment of the *E. coli malB* region. The *Hin*f I fragment lacks the last four amino acids of wild-type *malE*, and, of course, additional amino acids are added as encoded by the polylinker.

6.6 *Is MBP2* a monomer or a higher oligomer*?

MBP2* is a monomer. There is one published report that MBP can dimerize in 10 mM Tris-HCl (40) but we have not been able to reproduce this result with MBP2*. Gel filtration chromatography in both Column Buffer and 10 mM Tris-HCl gives a single peak of about 42 kDa.

Protein Fusion and Purification Strain List

TB1 (#E4122S) F⁻ *ara Δ(lac-proAB) [ϕ80dlac Δ(lacZ)M15] rpsL(Str^R) thi hsdR*
= JM83 *hsdR*

ER2507 (#E4121S) F⁻ *ara-14 leuB6 fhuA2 Δ(argF-lac)U169 lacY1 glnV44 galK2 rpsL20 xyl-5 mtl-5 Δ(malB) zjc::Tn5(Kan^R) Δ(mcrC-mrr)$_{HB101}$*
= PR700 *fhuA* = RR1 *Δ(malB) Δ(lac)U169 pro$^+$ fhuA*

The *malE* gene is included in the *malB* deletion, so this strain does not make any MBP from the chromosome. It does not have the *lacZΔM15* allele, so it cannot be used for α-complementation (no blue-to-white screen on Xgal). This strain can be transformed with high efficiency, similar to RR1 and HB101.

Protease deficient strains:

ER2508 (#E4127S) F⁻ *ara-14 leuB6 fhuA2 Δ(argF-lac)U169 lacY1 lon::miniTn10(Tet^R) glnV44 galK2 rpsL20 xyl-5 mtl-5 Δ(malB) zjc::Tn5(Kan^R) Δ(mcrC-mrr)$_{HB101}$*
= PR745 *fhuA* = RR1 *lon Δ(malB) Δ(lac)U169 pro$^+$ fhuA*

The *malE* gene is included in the *malB* deletion, so this strain does not make any MBP from the chromosome. This strain can be transformed with fairly high efficiency, about 10X down from RR1 and HB101.

CAG626 (#E4124S) F⁻ *lacZ*(am) *pho*(am) *lon trp*(am) *tyrT[supC*(ts)*] rpsL mal*(am)

CAG626 is Eco K r$^+$m$^+$, so your plasmid has to be modified (i.e., come from an m$^+$ strain such as TB1, JM83,

JM107, etc.) in order to get transformants; the transformation frequency is about 100X down from other common strains used for recombinant DNA work, so it helps to use electroporation (41).

CAG597 (#E4123S) F⁻ *lacZ*(am) *pho*(am) *tyrT[supC*(ts)*] trp*(am) *rpsL rpoH*(am)*165 zhg::Tn10 mal*(am)

rpoHam = *htpRam*, codes for the heat-shock sigma factor; this strain has a temperature-sensitive amber suppressor (*tyrTts*), and should be maintained at 30°C. When you induce your expression system (e.g. when you add IPTG), shift the cells to 37° or 42°C. CAG597 is Eco K r^+m^+, so your plasmid has to be modified (i.e., come from an m^+ strain such as TB1, JM83, JM107, etc.) in order to get transformants; the transformation frequency is about 100X down from other common strains used for recombinant DNA work, so it helps to use electroporation (41).

CAG629 (#E4125S) F⁻ *lacZ*(am) *pho*(am) *lon supC*(ts) *trp*(am) *rpsL rpoH*(am*)165 zhg::Tn10 mal*(am)

rpoHam = *htpRam*, codes for the heat-shock sigma factor; this strain has a temperature-sensitive amber suppressor (*tyrTts*), and should be maintained at 30°C. When you induce your expression system (e.g. when you add IPTG), shift the cells to 37° or 42°C. CAG597 is Eco K r^+m^+, so your plasmid has to be modified (i.e., come from an m^+ strain such as TB1, JM83, JM107, etc.) in order to get transformants; the transformation fre-

quency is about 100X down from other common strains used for recombinant DNA work, so it helps to use electroporation (41).

CAG748 (#E4126S) F⁻ *thr::Tn10(Tet^R) dnaJ259 leu fhuA2 lacY Δ(lac)X90 glnV44 thi*

This strain has a mutation in the *dnaJ* gene, which codes for a chaperonin. This defect has been shown to stabilize certain mutant proteins expressed in *E. coli*, e.g. mutants of lambda repressor. (*dnaJ* mutant with linked Tn10); (42, 43).

KS1000 (#E4128S) F´ *lacI^q lac$^+$ pro$^+$/ ara Δ(lac-pro) Δ(tsp)≡ Δ(prc)::Kan^R eda51::Tn10(Tet^R) gyrA rpoB thi-1 argI(am)*

This strain is defective in Prc, a periplasmic protease, which can cleave proteins that are overexpressed in the cytoplasm when the cells are lysed to make a crude extract. The original name of this protease is Tsp (tail specific protease) (26).

UT5600 (#E4129S) F⁻ *ara-14 leuB6 secA6 lacY1 proC14 tsx-67 Δ(ompT-fepC)266 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1*

This strain is deficient in an outer-membrane protease that cleaves between sequential basic amino acids (e.g. arg-arg). It can cleave proteins that are overexpressed in the cytoplasm when the cells are lysed to make a crude extract. (CGSC7092); (27, 28, 44).

Molecular Weights of pMAL Proteins

MBP2*	42,482 Dal
MBP2*-β-gal α fragment made from the pMAL-c2X vector with no insert	50,843 Dal
MBP2*-paramyosinΔSal	70,204 Dal
ParamyosinΔSal domain produced by factor Xa cleavage of MBP2*-paramyosin	27,753 Dal
Factor Xa consists of two disulfide linked chains calculated molecular weights are derived from the sequence, and don't include post-translational modifications	
entire, calculated	42,324 Dal
light chain	15,676 Dal
heavy chain	26,666 Dal
On reducing SDS-PAGE, the heavy chain runs at 30 kDa and the light chain runs at 20 kDa	
Enterokinase	26,262 Dal
Genenase I	27,405 Dal

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