

## NEBExpress<sup>®</sup> MBP Fusion and Purification System

NEB #E8201S

1 set

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### The NEBExpress MBP Fusion and Purification System Includes

*The Amylose Resin should be stored at 4°C and all other reagents should be stored at -20°C.*

#### **pMAL-c6T Vector** (200 µg/ml)

His-tagged vector. Supplied in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.

#### **Amylose Resin** (50% v/v)

Supplied pre-swollen in 20% ethanol. Binding capacity > 4 mg/ml bed volume.

#### **TEV Protease** (10,000 U/ml)

Cloned from Tobacco Etch Virus and expressed in *E. coli*. 1 unit of TEV Protease will cleave 2 µg of MBP-fusion protein, MBP6-TEV-Paramyosin ΔSal, to 95% completion in 1 hour at 30°C. Supplied in 50 mM Tris-HCl, 250 mM NaCl, 1 mM TCEP, 1 mM EDTA, 50% glycerol, pH 7.5.

#### **TEV Protease Reaction Buffer** (10X)

500 mM Tris-HCl, 5 mM EDTA, 10 mM DTT, pH 7.5. Dilute to 1X in TEV Protease reactions.

#### **Anti-MBP Monoclonal Antibody**

A murine anti-maltose binding protein antibody, isotope IgG2a. Suggested dilution for Western blotting or ELISA 1:10,000.

#### **MBP6 Protein** (40 µg/ml)

Expressed from pMAL-c6T vector, including an N-terminal hexahistidine tag and amino acid residues coded for by the polylinker up to the HindIII site. MW 45,519 daltons. Supplied in SDS-PAGE Sample Buffer.

**MBP6-TEV-Paramyosin ΔSal (5 mg/ml)**

An MBP fusion protein used as a positive control for TEV Protease cleavage. Supplied in 20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 50% glycerol, pH 7.5. MW 70,103 daltons.

***E. coli* NEB Express (not competent)**

*fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 Δ(mcrC-mrr)114::IS10*.  
Supplied in 50% glycerol. Store short term at -20°C. Store long term (> 30 days) at -80°C.

**Quick Start Guide**

1. Subclone the gene of interest into the pMAL-c6T vector.
2. Grow cells containing the pMAL-c6T fusion plasmid in LB containing ampicillin and 0.2% glucose to an  $A_{600}$  of ~0.5.
3. Induce by adding IPTG to a final concentration of 0.3 mM.
4. Grow for an additional 2 hours at 37°C, or 4 hours at 30°C, or 6–8 hours at room temperature, or overnight at 12–16°C.
5. Harvest the cells and either freeze immediately or resuspend in 25 ml column buffer (CB) per liter of culture (CB, p. 11).
6. Lyse the cells by freeze-thaw followed by sonication.
7. Clarify the lysate by centrifugation at 20,000 x g for 20 minutes.
8. Dilute the supernatant (crude extract) by adding 125 ml cold CB for every 25 ml crude extract.
9. Load the diluted crude extract on a 15 ml amylose column.
10. Wash the column with  $\geq 12$  column volumes of CB.
11. Elute the fusion protein with CB containing 10 mM maltose.



recognition sequence, so that few or no vector-derived residues are attached to the protein of interest, depending on the site used for cloning. In most cases, fusion protein expressed from pMAL-c6T constitutes 20–40% of the total cellular protein. For pMAL-c6T, a band corresponding to the fusion protein can usually be seen by analyzing a small sample of induced cells by SDS-PAGE. 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*. Greater than 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.

## Construction of the Fusion Plasmid

To produce a fusion protein in the pMAL-c6T vector, the gene or open reading frame of interest must be inserted into the pMAL-c6T vector so that it is in the same translational reading frame as the vector's *male* gene. The vector has a multiple cloning site (MCS) containing a restriction site for cloning fragments directly downstream of the sequence encoding the site recognized by TEV Protease. Several other restriction sites are also available for cloning fragments downstream of the primary site, or for directional cloning of a sticky-ended fragment. A subset of these sites is shared with pTYB21 and pKLAC2. If an insert is subcloned using these sites, it can be subcloned into any of the vectors either in parallel or as a subsequent experiment. This simplifies an examination of expression in the IMPACT™ system (NEB #E6901), and in the *Kluyveromyces lactis* Protein Expression Kit (NEB #E1000), respectively. Inserts cloned into the primary site produce a protein of interest that, after cleavage, contains a leucine at its N-terminus following the initial methionine.

## Subcloning Strategies

The MCS of the pMAL-c6T vector is designed to simplify subcloning the gene of interest. One strategy is to insert a fragment with an AlwNI site engineered at the beginning of the gene of interest; the 3' end of the gene can be engineered to contain a restriction site that is shared with the MCS (e.g., SbfI-HF), to facilitate ligation. This creates a fragment that can be inserted into pMAL-c6T that has been digested with AlwNI and a matching restriction enzyme (e.g., SbfI-HF). The fusion protein produced by such a construct, when cleaved by the TEV protease, yields a protein of interest with one extra Leu residue at its N-terminus following the Met start. An alternate strategy can be used in conjunction with the NEBuilder HiFi DNA Assembly Kit by designing PCR primers for the gene of interest and adding sequences that overlap with the pMAL-c6T vector. The NEBuilder web app can aid in the design of the necessary primers. Using this strategy, the fusion protein produced by such a construct, when cleaved by TEV Protease, yields a protein of interest with no additional residues at its N-terminus if your protein of interest starts with Gly, Ser, Met, Cys, Asn, His, Thr, Lys, Asp, Gln or Phe. None of these amino acid residues inhibit TEV Protease cleavage when they are in the P1' position (6).

## Preparing a PCR Fragment for Cloning

The procedure below is for cloning a fragment produced by PCR into the pMAL-c6T vector. It is assumed that the PCR fragment is approximately 1 kb, begins with an AlwNI site, and has a SbfI overhang at the 3' end.

1. Digest 0.5 µg of the pMAL-c6T vector DNA in 20 µl of 1X CutSmart® Buffer (supplied as a 10X stock) with 10 units of AlwNI (NEB #R0514) and 10 units of SbfI-HF (NEB #R3642) at 37°C for 1 hour. Heat inactivate the enzymes by incubating at 65°C for 10 minutes.
2. Check for complete digestion of the pMAL-c6T vector by running 4 µl on an agarose gel. At the same time, run a sample of the PCR fragment to estimate its concentration.
3. Clean up the PCR fragment using the Monarch® PCR & DNA Cleanup Kit (NEB #T1030). Then digest 0.5 µg of the PCR fragment in 20 µl of 1X CutSmart Buffer with 10 units of AlwNI and 10 units of SbfI-HF.
4. Purify the pMAL-c6T vector backbone and the digested PCR fragment by agarose gel using the Monarch DNA Gel Extraction Kit (NEB #T1020).
5. Run a sample of the PCR insert and the vector backbone on a gel to check the concentration.
6. Mix: 20–40 ng digested vector backbone  
20 ng digested insert DNA  
Add H<sub>2</sub>O to bring the volume to 5 µl  
Add 5 µl Instant Sticky-end Ligase Master Mix (2X) (NEB #M0370)  
Mix thoroughly by pipetting up and down  
Place on ice or store at –20°C for subsequent transformation.
7. Transform 2 µl of the assembled product into competent NEB Express cells (NEB #C2523); see transformation protocol on p. 5.

## NEBuilder Assembly of a PCR Fragment

The procedure below is for cloning a fragment produced by PCR with overlapping sequences to a pMAL-c6T vector digested with AlwNI and SbfI-HF using the NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621). It is recommended to read the NEBuilder manual or use the NEBuilder Assembly Tool to create primers with the correct overlapping sequences for assembly.

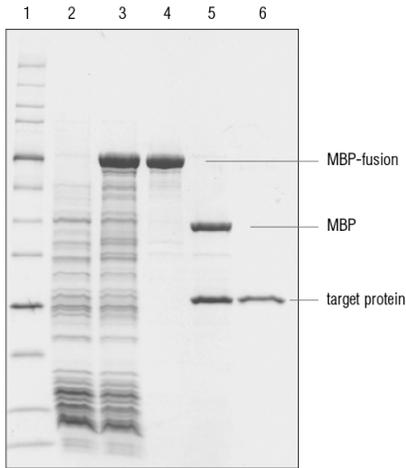
1. Digest 0.5 µg of the pMAL-c6T vector DNA in 20 µl of 1X CutSmart Buffer (supplied as a 10X stock) with 10 units of AlwNI (NEB #R0514) and 10 units of SbfI-HF (NEB #R3642) at 37°C for 1 hour. Heat inactivate the enzymes by incubating at 65°C for 10 minutes.
2. Check for complete digestion of the pMAL-c6T vector by running 4 µl on an agarose gel.
3. Clean up the PCR fragment using the Monarch PCR & DNA Cleanup Kit (NEB #T1030).
4. Purify the pMAL-c6T vector backbone by gel using the Monarch DNA Gel Extraction Kit (NEB #T1020).
5. Run a sample of the PCR insert and the vector backbone on a gel to check the concentration. The recommended DNA molar ratio is vector : insert = 1 : 2
6. Mix: 0.015–0.1 pmol of digested vector backbone  
0.03–0.2 pmol digested insert DNA  
Add deionized H<sub>2</sub>O to bring the volume to 10 µl  
Add 10 µl NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621)  
Mix thoroughly by pipetting up and down
7. Incubate samples in a thermocycler for 15 minutes at 50°C. Following incubation, store samples on ice or at –20°C for subsequent transformation.
8. Transform competent NEB Express cells (NEB #C2523) with 2 µl of the assembled product: see transformation protocol below.

## Transformation Protocol

1. Thaw chemically competent NEB Express cells (NEB #C2523) on ice.
2. Add 2 µl of the chilled assembled product to the competent cells. Mix gently by pipetting up and down. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at 42°C for 30 seconds. Do not mix.
5. Transfer tubes to ice for 2 minutes.
6. Add 950 µl of room-temperature SOC media to the tube.
7. Incubate the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm LB plates containing 100 µg/ml ampicillin to 37°C.
9. Spread 100 µl of the cells onto the LB ampicillin plates. Do not plate on IPTG.
10. Incubate overnight at 37°C.
11. Screen for the presence of inserts in one or more of the following ways:
  - A. Perform colony PCR on several transformants using appropriate primers (see Appendix C item 1.2, p. 13).
  - B. Prepare miniprep DNA (7). Digest with an appropriate restriction endonuclease to determine the presence and orientation of the insert (8).
  - C. SDS-PAGE analysis of transformed cells:
    1. Inoculate several transformants into 5 ml of LB containing 100 µg/ml ampicillin and grow to  $2 \times 10^8$  cells/ml (OD<sub>600</sub> of ~ 0.5).
    2. Split each sample into two 2.5 ml cultures.
    3. Add IPTG to one of the cultures to a final concentration of 0.3 mM, for example add 7.5 µl of a 0.1 M IPTG stock solution. Incubate at 37°C with good aeration for 2 hours.
    4. Withdraw a 0.5 ml sample from each culture. Microcentrifuge for 1 minute, discard the supernatant and resuspend the cells in 100 µl SDS-PAGE sample buffer (NEB #B7703).
    5. Place samples in a boiling water bath for 5 minutes. Analyze 10 µl of each sample by SDS-PAGE along with a protein standard (NEB #P7717) and 15 µl of the supplied MBP6 in SDS-PAGE Sample Buffer. Stain the gel with Coomassie brilliant blue (9).

*For pMAL-c6T constructs, 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 MBP6 (MW 45.5 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 Monoclonal Antibody (Appendix B); even with severe protein degradation, a full-length fusion protein can usually be detected on the Western.*

**Figure 2. Protein expression using the NEBExpress MBP Fusion and Purification System.**



*SDS-polyacrylamide gel electrophoresis of fractions from the affinity purification of MBP6-TEV-Paramyosin  $\Delta$ Sal.*

*Lane 1: Protein Standard. Lane 2: uninduced cells. Lane 3: induced cells. Lane 4: purified fusion protein eluted from amylose column with maltose. Lane 5: purified protein after TEV Protease cleavage. Lane 6: target protein isolated from NEBExpress Ni Resin flow through.*

## Affinity Chromatography

### Small Scale

To test multiple different clones to vary induction conditions for expression of a soluble fusion protein it is often easiest to use Amylose Magnetic Beads (NEB# E8035) to isolate the fusion protein. The binding capacity of these beads is 10  $\mu$ g of fusion protein per mg of amylose magnetic beads. The following protocol is for the isolation of MBP-fusion protein from 200-500  $\mu$ l cell culture supernatant.

#### *MBP Column Binding Buffer*

20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT (optional), pH 7.5.

1. Vortex and thoroughly suspend Amylose magnetic beads.
2. Aliquot 100  $\mu$ l of bead suspension to a sterile microcentrifuge tube.
3. Add 500  $\mu$ l of MBP column buffer and vortex to suspend. Apply magnet for 30 seconds to pull beads to the side of the tube and remove supernatant. Repeat wash.
4. Add 200-500  $\mu$ l of cell culture supernatant to beads.
5. Mix thoroughly and incubate at 4°C with agitation for 1 hour.
6. Apply magnet and remove supernatant.
7. Wash beads three times as in Step 3 above.
8. The purified MBP-fusion protein can now be eluted from the beads or used directly for capture of target proteins.

#### *MBP-Fusion Elution:*

1. Add 50  $\mu$ l of MBP column buffer containing 10 mM maltose (elution buffer) to the bead pellet, vortex and incubate for 10 minutes at 4°C with agitation.
2. Apply magnet and pipet eluted MBP-fusion protein into a clean microcentrifuge tube.
3. Add an additional 50  $\mu$ l of elution buffer to the beads and repeat elution step. Pool elution supernatants.

Note: Efficiency of elution can be checked by eluting any protein that remains bound to the Amylose Magnetic Beads with 50  $\mu$ l of SDS-PAGE gel loading buffer and analyzing 15  $\mu$ l by SDS-PAGE.

### Large Scale

A protocol for purification of a fusion protein from a 1-liter culture is detailed below.

1. Inoculate 1-liter rich broth supplemented with 0.2% glucose & 100  $\mu$ g/ml ampicillin (see Media and Solutions, p. 11) 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 amylose which can degrade the amylose on the affinity resin.*

2. Grow to  $2 \times 10^8$  cells/ml ( $OD_{600} \sim 0.5$ ). 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<sub>2</sub>O (see Media and Solutions). Incubate the cells at 37°C for 2 hours.  
*The length of time and the temperature to use during expression depends on several factors (stability of the protein, host strain, etc.) and variations can be tested to find optimum conditions.*
3. Harvest the cells by centrifugation at 4000 x g for 20 minutes and discard the supernatant. Resuspend the cells in 25 ml Column Buffer (see Media and Solutions).  
*For many unstable proteins, most of the degradation happens during harvesting and cell breakage. Therefore, it is best to harvest the cells quickly and keep them chilled. 25 ml of Column Buffer is based on the expectation of about 4-5 grams cells/liter, i.e., 5 ml for every gram of cells (wet weight).*  
*The EDTA in the lysis buffer is to help inhibit proteases that have a Ca<sup>2+</sup> cofactor. Addition of PMSF (phenyl methylsulfonyl fluoride) and/or other protease inhibitors may help in some cases.*  
*DTT or  $\beta$ -mercaptoethanol can be included to prevent oxidative damage to the fusion protein and 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, p. 11.*
4. Freeze resuspended cells overnight at -20°C.  
*This is a good place to stop in this protocol. The frozen cells can be stored for a week or more at -20°C, depending on the particular fusion.*
5. Thaw the resuspended cells. Do not use lysozyme during lysis, as it can break down the amylose resin. Place metal cup containing the resuspended cells and stir bar in an ice-water bath. Stir cells slowly and sonicate in short pulses of 15 seconds or less. Monitor the release of protein using the Bradford assay (10), adding 5  $\mu$ l of the sonicate to 1 ml Bradford reagent and mixing. Continue sonication until the released protein reaches a maximum (usually about 2 minutes sonication time).
6. Centrifuge the lysate at 20,000 x g for 20 minutes. Save the supernatant (crude extract). Dilute the crude extract 1:5 with Column Buffer.  
*This is another good place to stop the protocol. The crude extract can be stored for a week or more at -20°C, depending on the particular fusion. It is usually more convenient to store the crude extract before diluting with column buffer; dilute it after thawing.*
7. Pour the amylose resin in a 2.5 x 10 cm column. Wash the column with 5 column volumes of Column Buffer.  
*The amount of resin required depends on the amount of fusion protein produced. The resin binds 4 mg/ml bed volume, so a column of about 25 ml should be sufficient for a yield of up to 100 mg fusion protein/liter culture.*
8. Load the diluted crude extract at a flow rate of no more than  $[50 \times (\text{diameter of column in cm})^2]$  ml/hour. This is a maximum of 5 ml/minute for a 2.5 cm column. Slower flow rates may increase MBP-fusion binding to the amylose resin; however, the crude extract should not be incubated with the amylose resin overnight, because native *E. coli* amylases can break down the resin and decrease its binding capacity.
9. Wash with 12 column volumes of Column Buffer at a rate of no more than  $[100 \times (\text{diameter of column in cm})^2]$  ml/hour. This is a maximum of 10 ml/minute for a 2.5 cm column.  
*The column can be washed overnight if it has a safety loop to prevent it from running dry. 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 3 ml fractions (fraction size = 1/5<sup>th</sup> 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 (10).
11. Pool the protein-containing fractions. If necessary, concentrate to ~1 mg/ml in a Vivaspin Centrifugal Concentrator (Sartorius), or the equivalent.

## Regenerating the Amylose Resin Column

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

Water:	3 column volumes
0.1% SDS:	3 column volumes
Water:	1 column volumes
Column Buffer:	3 column volumes
or	
20% Ethanol:	3 column volumes (long term storage)

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.

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. The resin should be stored in 20% ethanol for long term storage.

## Cleavage of the Fusion Protein

MBP and the target protein are fused by a polylinker containing a TEV protease recognition site for easy removal of the MBP-tag. One unit of TEV Protease will cleave approximately 2 µg of fusion protein. Cleavage should be carried out in 1X TEV Protease Reaction Buffer or in Amylose column elution buffer supplemented with DTT to a final concentration of 1 mM. Incubate the reaction mixture for 1 hour at 30°C or overnight at 4°C. Depending on the particular fusion protein, the amount of TEV Protease can be adjusted to get an acceptable rate of cleavage. TEV Protease is highly specific and will not cleave at non-canonical sites. The optimum sequence for the TEV protease cleavage site is Glu-Asn-Leu-Tyr-Phe-Gln-(Gly/Ser). However, it has been reported that other amino acid residues can be used in the P1' position instead of Ser or Gly. These alternative P1' amino acids include Ala, Met, and Cys (6). If the fusion protein sample contains > 2 M urea, > 0.5 M guanidine hydrochloride, > 50 mM imidazole, pH values below 6 or above 9, or cysteine protease inhibitors, then it will be necessary to dialyze the fusion protein before TEV Protease cleavage.

1. If necessary, concentrate the fusion protein to at least 0.5 mg/ml.
2. Perform a pilot experiment with a small portion of your protein. For example:
  - a. Combine 15 µg fusion protein and H<sub>2</sub>O to a volume of 45 µl.
  - b. Add 5 µl of TEV Protease Reaction Buffer (10X) to make a 50 µl total reaction volume.
  - c. Add 1 µl of TEV Protease.
  - d. In a separate tube, combine 5 µg fusion protein, 5 µl TEV Protease Reaction Buffer (10X) and H<sub>2</sub>O to a volume of 50 µl. Do not add TEV Protease (control sample).
  - e. Incubate reaction and control sample for 1, 3, and 8 hours at 30°C (an additional reaction can be made and incubated for 24 hours at 4°C).
  - f. Take 10 µl of the reaction(s) at the indicated times above and add 5 µl SDS-PAGE Sample Buffer (3X). Take 10 µl of the control sample and add 5 µl SDS-PAGE Sample Buffer (3X) after 8 hours (or longest incubation time).
  - g. Incubate the SDS-PAGE samples for 3-5 minutes at 70-100°C and analyze them by SDS-PAGE (9).
3. Scale up the pilot experiment linearly for the amount of the fusion protein to be cleaved. Save at least a small sample of the uncut fusion as a negative control.
4. Check for complete cleavage by SDS-PAGE.
5. TEV Protease and the cleaved MBP contain polyhistidine tags at their N-termini. They can be removed from the cleavage reaction by immobilized metal affinity chromatography, such as Nickel or Cobalt resin, thereby isolating the target protein in the flow through.

## Denaturing the Fusion Protein for Improved TEV Protease Cleavage

If the fusion protein is resistant to TEV Protease cleavage, denaturation and refolding may expose the TEV protease recognition site for improved cleavage.

1. Either dialyze the fusion against 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 for 4 hours, repeat once.

*During refolding, one must 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.*

3. Go to Step 2 or 4 above (pilot experiment), as appropriate.

## **Separating the Protein of Interest from MBP after TEV Protease Cleavage**

Both the MBP-tag and TEV Protease are polyhistidine-tagged for easy removal from the TEV Protease reaction. Loading the digest onto NEBExpress Ni Resin (NEB #S1428) sequesters both the MBP-tag and TEV Protease, thereby isolating the protein of interest in the column flow through.

The binding capacity of NEBExpress Ni Resin is  $\geq 10$  mg/ml. The binding capacity will vary depending on the target protein, binding conditions and the accessibility of the His-tag.

It is recommended to estimate the amount of the His-tagged MBP released by TEV Protease cleavage by first analyzing a sample by SDS-PAGE and comparing its band size and intensity to that of the provided MBP6 protein control.

*Please note that NEBExpress Ni Resin is chemically resistant to DTT. If you are using a resin that is incompatible with DTT, you will need to buffer exchange or modify the TEV Protease reaction conditions accordingly.*

**Binding Buffer** (for use with NEBExpress Ni Resin): 1X TEV Protease Reaction Buffer (or similar): 50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, pH 7.5

### **Resin Preparation**

1. Gently shake the Ni Resin bottle to completely resuspend the slurry and transfer desired amount to a closed column; allow resin to settle.
2. Open column and allow storage buffer to flow through.
3. Equilibrate the resin by adding 5 column volumes (CV) of Binding buffer.

### **Binding**

1. Load the TEV Protease digestion directly onto the equilibrated column and allow it to flow through the resin slowly (flow rate  $\leq 1$  ml/minute). This provides sufficient time for the MBP-tag, TEV Protease, and any remaining MBP-fusion protein to effectively bind to the Ni resin.
2. Collect the flow through containing the protein of interest in a sterile tube.
3. Wash the column with 1 CV of Binding Buffer to maximize the yield of the target protein; collect the flow through.
4. Dialyze or buffer exchange target protein into desired storage buffer.

### **Resin Cleaning**

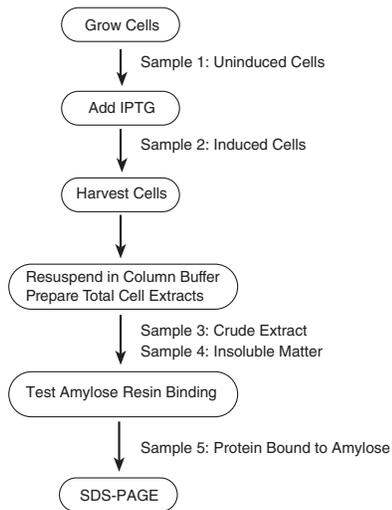
1. Add 1 M NaOH to the column and allow a contact time of 1 to 2 hours
2. Add 1.5 M NaCl to the column and allow a contact time of 10 – 15 minutes followed by 10 CV of water.

### **Resin Storage**

1. For long-term storage, NEBExpress Ni Resin should be stored in 20% ethanol at 2 – 8°C.

## Pilot Experiment

A small-scale experiment is described to determine the behavior of a particular MBP fusion protein. This protocol results in five samples: uninduced and induced cells, a total cell crude extract, a suspension of the insoluble material from the crude extract, and a fraction containing protein that binds to the amylose resin.



1. Inoculate 80 ml rich broth containing 0.2% glucose & ampicillin (see Media and Solutions, p. 11) with 0.8 ml of an overnight culture of cells containing the fusion plasmid.
2. Grow at 37°C with good aeration to  $2 \times 10^8$  cells/ml ( $OD_{600}$  of ~0.5).
3. Take 1 ml sample and microcentrifuge for 2 minutes (sample 1: uninduced cells). Discard supernatant and resuspend the cells in 50  $\mu$ l SDS-PAGE Sample Buffer. Vortex and freeze at -20°C.
4. 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<sub>2</sub>O (see Media and Solutions). Continue incubation at 37°C for 2 hours. Withdraw a 0.5 ml sample and spin down for two minutes (sample 2: induced cells). Discard supernatant and resuspend the cells in 100  $\mu$ l 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.*

5. 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).
6. Freeze the cells in Column Buffer overnight at -20°C. Thaw in cold water.
7. 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 (10), adding 10  $\mu$ l of the sonicate to 1.0 ml Bradford reagent and mixing. Continue sonication until the released protein reaches a maximum (usually about 2 minutes).
8. Centrifuge at 20,000 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  $\mu$ l 3X SDS-PAGE Sample Buffer to 5  $\mu$ l of the crude extract and insoluble matter fractions (samples 3 and 4, respectively).
9. Place ~200  $\mu$ l of the amylose resin in a microfuge tube and spin briefly in a microcentrifuge or follow the small-scale Amylose Magnetic Beads (NEB #E8035) protocol on p. 6. 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  $\mu$ l of Column Buffer. Mix 50  $\mu$ l of crude extract with 50  $\mu$ l of the amylose resin slurry. Incubate for 15 minutes on ice. Microcentrifuge 1 minute, then remove the supernatant and discard. Wash the resin with 1 ml Column Buffer, microcentrifuge 1 minute, discard the supernatant, and resuspend the resin in 50  $\mu$ l 3X SDS-PAGE Sample Buffer (sample 5: protein bound to amylose).

### SDS-PAGE

10. Incubate the samples for 3-5 minutes at 100°C. Microcentrifuge for 1 minute. Load 20  $\mu$ l of the of uninduced cells, induced cells and amylose resin samples (avoid disturbing the pellets), and the entire volume of the remaining samples onto a gel and analyze by SDS-PAGE (9).

- (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 Monoclonal Antibody (NEB #E8032) and, if available, serum directed against the protein of interest (Appendix B).

*Note: 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 (11).*

## Appendix A: Media and Solutions

### Rich Medium + Glucose & Ampicillin:

Per 1 liter: Combine 10 g tryptone, 5 g yeast extract, 5 g NaCl and 2 g glucose. Autoclave; add sterile ampicillin to 100 µg/ml

### 0.1 M IPTG Stock

1.19 g IPTG; add H<sub>2</sub>O to 50 ml; filter sterilize, store at 4°C. Protect from light.

### Column Buffer

PER LITER	FINAL CONCENTRATION
20 ml 1.0 M Tris-HCl, pH 7.5	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 sodium azide
0.7 ml β-mercaptoethanol or 154 mg DTT	10 mM β-ME or 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 affecting the affinity purification. Other buffers that are compatible include MOPS, HEPES and phosphate, at pH values from 6.5 to 8.5. MBP binds to amylose primarily by hydrogen bonding, so higher ionic strength does not decrease its affinity. Nonionic detergents can be used at concentrations of 0.05% or lower, as higher concentrations interfere with the affinity of some fusions.

## Appendix B: Western Protocol

### Materials

Transfer apparatus and associated buffers

Nitrocellulose or PVDF membrane

TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20)

Blocking Buffer (TBST + 5% Nonfat Dry Milk)

Anti-MBP Monoclonal Antibody (NEB #E8032)

Secondary Antibody: Anti-Mouse IgG (H+L) Dylight® 800 4X PEG conjugate (Cell Signaling Technologies, #5257P)

### For a 10 cm x 10 cm gel

- Run the samples on SDS-PAGE - load about 1/5<sup>th</sup> to 1/10<sup>th</sup> the amount that would normally be run for a Coomassie stained gel.
- Transfer protein from the gel to a nitrocellulose or PVDF membrane following the directions of the transfer apparatus manufacturer. Mark the wells of the gel on the filter with a blunt pencil before removing and discarding the gel.
- Rinse the membrane with TBST.
- Incubate the membrane with 25 ml blocking buffer for 1 hour at room temperature (or overnight at 4°C) with gentle shaking.
- Wash the membrane in 25 ml TBST at room temperature with gentle shaking, 3 times for 5 minutes each.
- Add 1 µl of the Anti-MBP Monoclonal Antibody to 10 ml blocking buffer (a 1/10,000 dilution). Cover the membrane with the antibody dilution and incubate for 1 hour at room temperature with gentle shaking.
- Wash the membrane in 25 ml TBST at room temperature with gentle shaking, 3 times for 5 minutes each.
- Make a dilution of an Anti-Mouse IgG Secondary Antibody in 10 ml blocking buffer according to the manufacturer's recommendation and incubate the membrane in the solution for 1 hour in a container that blocks light.

9. Wash the membrane in 25 ml TBST at room temperature with gentle shaking, 3 times for 5 minutes each.
10. Follow the manufacturer's directions for detection.

## **Appendix C: Troubleshooting and Tips**

### **1. Cloning and Transformation**

- 1.1 Strain recommendations
- 1.2 Sequencing primers
- 1.3 Inability to clone
- 1.4 Low yield of DNA in plasmid preps
- 1.5 Obtaining pMAL sequences

### **2. Expression**

- 2.1 Fusion protein is degraded
- 2.2 Fusion protein is insoluble
- 2.3 Fusion protein not expressed
- 2.4 Only MBP-sized protein expressed
- 2.5 Expressing small peptides

### **3. Affinity Purification**

- 3.1 Fusion protein does not bind to amylose column
- 3.2 Amylose resin regeneration
- 3.3 Nonionic detergents and binding
- 3.4 Different buffers and salt concentrations
- 3.5 Degradation of the fusion upon cell lysis
- 3.6 Extra (unexpected) bands upon SDS-PAGE
- 3.7 Batch purification
- 3.8 Purification in the presence of denaturants
- 3.9 Storage of resin at  $-20^{\circ}\text{C}$

### **4. TEV Protease Cleavage**

- 4.1 Control substrates
- 4.2 Removal of TEV Protease
- 4.3 Fusion will not cleave
- 4.4 Molecular weight and calculated pI
- 4.5 Cleavage in glycerol
- 4.6 Cleavage in urea, guanidine hydrochloride and SDS

### **5. Separation of Fusion Protein Domains and Storage**

- 5.1 Separation of MBP and TEV Protease from the protein of interest
- 5.2 Difficulties with removal of maltose by dialysis
- 5.3 How to store proteins

### **6. MBP6**

- 6.1 What is MBP6?
- 6.2 Crystal structure of MBP
- 6.3 How much of MBP is dispensable for binding?
- 6.4  $K_d$ , pI and extinction coefficient for MBP
- 6.5 Origin of the MBP region
- 6.6 Is MBP a monomer?

## 1. Cloning and Transformation

### 1.1 What strain(s) do you recommend as hosts for the pMAL vectors?

The strain we recommend is NEB Express (NEB #C2523). This is an *E. coli* B strain similar to T7 Express and BL21 (DE3), except that it lacks the T7 RNA Polymerase (the pMAL vectors use the *E. coli* RNA Polymerase). NEB Express, T7 Express (NEB #C2566), BL21 (NEB #C2530) and BL21(DE3) (NEB #C2527) all give similar results – the presence of the T7 RNA Polymerase doesn't seem to have any effect. Other successful strains include NEB 10-beta (NEB #C3019), NEB 5-alpha (NEB #C2991) and NEB Turbo (NEB #C2984). We also use other strains in response to a particular problem (for example, see 2.1). One can start with NEB Express, or with whatever competent cells are readily available, and then try another strain if a problem with expression or purification develops.

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

The following sequencing primers can be used (not available from New England Biolabs): malE primer on the 5' side of the insert and the pMAL reverse primer for sequencing from the 3' side.

Forward Primer (24-mer, upstream of MCS)  
5'd(GGTCGTCAGACTGTCGATGAAGCC)3'

Reverse Primer (24-mer, downstream of MCS)  
5'd(TGTCCTACTCAGGAGAGCGTTCAC)3'

The sequences of the pMAL vectors are also available at [www.neb.com](http://www.neb.com). PCR of inserts cloned into the MCS can be performed using the same primer pair.

### 1.3 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 1:30, so if the induced level of the fusion is 40% of the total cellular protein, the uninduced level works out to over 1%. 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).

### 1.4 Why is the yield of pMAL DNA from plasmid preps so low?

The pMAL plasmids are pBR322-low copy number, but for unknown reasons the yield from plasmid preps is often lower than what can be obtained from pBR322. However, modification of the standard alkaline lysis protocol can increase the yield: increasing the volume of the buffers by 1.5-fold doubles the yield of plasmid (i.e., for a 500 ml culture, resuspend the cell pellet in 15 ml instead of the standard 10 ml, and increase the denaturing and neutralizing buffer amounts proportionately).

### 1.5 How can I obtain the sequence of the pMAL-c6T vector?

The pMAL sequence is available in the Tools and Resources > Interactive Tools section of the New England Biolabs web site at <https://www.neb.com/tools-and-resources/interactive-tools/dna-sequences-and-maps-tool>

## 2. Expression

### 2.1 When I analyze my fusion protein expression by Western blot using the Anti-MBP Monoclonal Antibody, only a small fraction of the protein is full-length, while most of it migrates close to the MBP6\* 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 such as NEB Express, which is Lon- and OmpT-. Additionally, try adding a protease inhibitor cocktail to the lysis buffer.

### 2.2 My fusion protein is insoluble; is there anything I can do to express it 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 must also 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:

Reviews on methods to make correctly folded protein in *E. coli*:  
Georgiou, G. et al. (1996) *Current Opinion in Biotechnology* 7:190–197.  
Sahdev S. et al. (2008) *Mol. Cell Biochem* 307:249–264

Reviews on refolding:  
Qoronfleh, M.W. et al. (2007) *Protein Expr. Purif.* 55, 209–224.  
Jungbauer, A. and Kaar, W. (2007) *J. Biotechnol.* 128, 587–596.  
Jungbauer, A. et al. (2004) *Curr. Opin. Biotechnol.* 15, 487–494.  
Vallejo, L.F. and Rinas, U. (2004) *Microb. Cell Fact* 3, 11–22.

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. 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 (12). 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 MBP6\*.*

There are two likely explanations for this result. If the protein of interest is in the wrong translational reading frame, an MBP6-sized band will be produced by translational termination at the first in-frame stop codon. If the protein of interest is very unstable, an MBP6-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 Monoclonal Antibody (NEB #E8032). If proteolysis is occurring, at least a small amount of full-length fusion can almost always be detected. DNA sequencing of the fusion junction will confirm a reading frame problem. If the problem is proteolysis, you might want to try NEB Express (NEB #C2523) which is a protease deficient strain lacking the Lon and OmpT proteases.

2.5 *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?*

The MBP system can be used to express short peptides. However, 40 mg of MBP yields ~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?*

An 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 (prolonged treatment with lysozyme 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. Alternatively, because the MBP possesses an N-terminal hexahistidine tag, it is possible to purify the MBP-fusion via Immobilized Metal Affinity Chromatography.

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-20 to 0.05% typically restores about 80% of the binding.

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

Yes, HEPES, MOPS, and phosphate buffers (at pH values ranging from 6.5 to 8.5) can be used 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 (13-15). The best strategy in either case is to use a host which is deficient in the offending protease(s) and a protease inhibitor cocktail during lysis (see 2.1).

3.6 *When I run my purified fusion protein on SDS-PAGE, why do I see multiple bands instead of a single band of the expected MW?*

There are two likely explanations for this result. The first is that the fusion protein is unstable, which most often leads to degradation *in vivo* (see 3.5). In this case, one would expect to see bands between the size of MBP (45.5 kDa) and the size expected for the full-length fusion, since fragments smaller than MBP would not bind to the affinity column. An exception would be if the fusion protein breaks down at the junction between MBP and the protein of interest, and the protein of interest oligomerizes. In this situation, the protein of interest may bind to the fusion protein, and therefore a band the size of the protein of interest can appear even if it is smaller than MBP. The second explanation is that the protein of interest is binding non-specifically to other *E. coli* proteins, e.g., it has a surface that binds other proteins by electrostatic or hydrophobic interactions. In this case, modifications to the column buffer can sometimes be used to help wash the interacting proteins away. Electrostatic interactions can be weakened by including up to 1 M NaCl in the column buffer, and hydrophobic interactions can be weakened by lowering the salt to 25–50 mM NaCl and including 5% ethanol or acetonitrile in the column buffer. Non-ionic detergents can also be used to weaken hydrophobic interactions, but they can interfere with the affinity of certain fusion proteins.

3.7 *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 may not be critical for loading the column by the batch method.

3.8 *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.

3.9 *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. TEV Protease Cleavage

4.1 *Are there any control substrates for TEV Protease?*

The NEBExpress MBP Fusion and Purification System comes with an MBP6-TEV-Paramyosin-ΔSal fusion as a positive control for TEV Protease cleavage.

4.2 *How can TEV Protease be removed from the reaction after cleavage?*

TEV Protease contains a polyhistidine tag at its N-terminus and can be removed from the reaction by immobilized metal affinity chromatography, such as NEBExpress Ni-NTA Magnetic Beads (NEB #S1423), NEBExpress Ni Spin Columns (NEB #S1427), or NEBExpress Ni Resin (NEB #S1428). The Ni-NTA Magnetic Beads will require dialysis to remove the DTT present in the TEV Protease reaction buffer; whereas, TEV Protease digests can be directly loaded onto NEBExpress Ni Spin Columns or NEBExpress Ni Resin since these two resin formats are chemically resistant to DTT.

4.3 *My protein cleaves very poorly with TEV Protease. Is there anything I can do to improve cleavage?*

In these cases, the fusion protein may be folded in a way that the TEV site is inaccessible. In theory, anything that perturbs the structure might uncover the site, such as denaturing and refolding (see “Denaturing the Fusion Protein”, p. 8) or addition of a chaotropic reagent (up to 2 M urea, up to 3 M guanidine HCl, or up to 0.5% SDS). One approach that often helps is to add amino acid residues to the N-terminus of the protein; either by cloning into one of the downstream sites in the polylinker, or by adding codons to the insert (e.g., adding four alanine codons before the start of the gene). Be aware that with this strategy, the extra residues remain at the N-terminus after TEV Protease cleavage.

4.4 *What is the molecular weight and pI of the TEV Protease?*

The molecular weight of TEV Protease is 27,900 daltons. The pI of TEV Protease is ~8.5.

4.5 *What is maximum concentration of glycerol that TEV Protease can tolerate during cleavage?*

TEV Protease retains ~50% activity in solutions containing 50% glycerol.

4.6 *Is the rate of TEV Protease cleavage affected by urea, guanidine hydrochloride and/or SDS?*

The activity of TEV Protease on an MBP-fusion protein in the presence of these denaturants has been reported by C. Sun et al. (16) as follows:

Urea: In up to 2 M urea near complete cleavage is detected. There is some inhibition of TEV Protease cleavage in urea concentrations of 4 M.

Guanidine HCl: 1 M guanidine HCl yields slight inhibition of TEV Protease cleavage; however, some TEV Protease activity is still observed in the presence of 3 M guanidine HCl.

SDS: TEV Protease is unaffected by concentrations of SDS below 0.5%. In the presence of 1% SDS, TEV Protease retains most, but not all, of its activity.

## 5. Separation of Fusion Protein Domains and Storage

### 5.1 How do I separate MBP and TEV Protease from the protein of interest?

Both MBP and TEV Protease contain polyhistidine tags that can be used to remove them from the protein of interest. The TEV Protease digestion can be directly loaded onto NEBExpress Ni Spin Columns (NEB #S1427) or NEBExpress Ni resin (NEB #S1428), since these resin formats are chemically resistant to the DTT in the TEV Protease reaction buffer. If using NEBExpress Ni-NTA Magnetic Beads (NEB #S1423) or another immobilized metal affinity chromatography format, we recommend dialyzing the TEV Protease digestion into a compatible buffer prior to IMAC.

### 5.2 I want to rebind MBP to the amylose column, but 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 (17). We have determined empirically that binding the fusion to a chromatography resin and then washing away the maltose is much more effective. Standard chromatography (e.g., DEAE) is a preferred separation step, since it can separate the TEV Protease and MBP from the protein of interest if immobilized metal affinity chromatography is not an option. 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. Alternatively, we recommend utilizing the polyhistidine tags present on both MBP and TEV protease with immobilized metal affinity chromatography to separate them from the protein of interest following digestion with TEV Protease (see 5.1).

### 5.3 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 will denature many proteins.

## 6. MBP6

### 6.1 What is MBP6? Is it different from wild-type MBP produced from *E. coli*?

MBP6 is the protein produced from pMAL-c6T that utilizes the naturally occurring stop codon following the SbfI recognition sequence. It differs from wild-type MBP by the addition of a methionine at the N-terminus (as do all fusions made in pMAL-c vectors), the mutations that increase the affinity of MBP for the amylose resin, the deletion of the last four residues of wild-type MBP, and the addition of the residues encoded by the spacer and the TEV protease site.

### 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 (17)  
Sharff, A. J. et al. (1992) *Biochem.* 31, 10657–10663 (18)

### 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 MBP6?

The  $K_d$  of MBP for maltose is 3.5  $\mu$ M; for maltotriose, 0.16  $\mu$ M (19). The extinction coefficient of MBP6 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 Hinf I fragment of the *E. coli malB* region. The Hinf 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 MBP6 a monomer or a higher oligomer?

MBP6 is a monomer with a molecular weight of 45.5 kDa.

## Molecular Weights of pMAL Proteins:

MBP6:	45,519 daltons
MBP6-TEV-Paramyosin ΔSal:	70,103 daltons
TEV Protease:	27,900 daltons

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## Kit Components

### NEB #E8201S Table of Components

NEB #	PRODUCT	VOLUME
N0378S	pMAL-c6T Vector	10 µg
E8021S	Amylose Resin	15 ml
P8112S	TEV Protease	1,000 units
B8035S	TEV Protease Reaction Buffer (10X)	1 ml
E8032A	Anti-MBP Monoclonal Antibody	25 µl
E1020S	MBP6 Protein	10 µg
E1021S	MBP6-TEV-Paramyosin ΔSal	100 µg
E4131S	<i>E. coli</i> NEB Express	0.2 ml cells

### COMPANION PRODUCTS

NEB #	PRODUCT	VOLUME
R0514S/L	AlwNI	500/2,500 units
R3642S/L	SbfI-HF	500/2,500 units
T1030S/L	Monarch PCR & DNA Cleanup Kit (5 µg)	50/250 preps
T1020S/L	Monarch DNA Gel Extraction Kit	50/250 preps
M0370S/L	Instant Sticky-end Ligase Master Mix	50/250 reactions
E2621S/L/X	NEBuilder HiFi DNA Assembly Master Mix	10/50/250 reactions
B7703S	Blue Loading Buffer Pack	8 ml
P7717S/L	Unstained Protein Standard, Broad Range (10-200 kDa)	150/750 gel lanes
E8022S/L	Amylose Resin High Flow	15/100 ml

E8035S	Amylose Magnetic Beads	25 mg
E8037S	Anti-MBP Magnetic Beads	10 mg
S1423S/L	NEBExpress Ni-NTA Magnetic Beads	1/5 ml
S1427S/L	NEBExpress Ni Spin Columns	10/25 columns
S1428S	NEBExpress Ni Resin	25 ml bed volume
C2523I/H	NEBExpress Competent <i>E. coli</i> (High Efficiency)	6 x 0.2 ml/20 x 0.05 ml
C3019I/H	NEB 10-beta Competent <i>E. coli</i> (High Efficiency)	6 x 0.2 ml/20 x 0.05 ml

## Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	
2.0	Place pMAL-c6T Polylinker on Figure 1.	1/21

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