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FAQs for Protein Fusion & Purification (pMAL) System

CLONING AND TRANSFORMATION

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 (NEB #C2566) and BL21 (DE3) (NEB #C2527), except that it lacks the T7 RNA polymerase (the pMAL vectors use the *E.coli* RNA polymerase). Another strain that has been used extensively is TB1 (NEB #E4122), which is JM83 *hsdR*. This strain has given good results when considering plasmid stability, expression and purification. Other successful strains include NEB 5-alpha (NEB #C2991), NEB Turbo (NEB #C2984), and T7 Express (NEB #C2566) Competent *E. coli*. We also use other strains in response to a particular problem. 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.

2. 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 #S1237) on the 5´ side of the insert and the pMAL reverse primer (NEB #S1288) for sequencing from the 3´ side.

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).

4. Why is the yield of pMAL DNA from plasmid preps so low?
The pMAL plasmids are pBR322-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 x 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).

5. How can I obtain the sequences of the pMAL vectors?
The pMAL sequences are available on the DNA Sequences and Maps page.

6. **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 MBP5* 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. NEB Express (NEB #C2523) is Lon- and OmpT-. For cytoplasmic expression, the most protease deficient strain is CAG629 (#E4125) – it is also, however, the most difficult to work with. CAG597 (#E4123) is another good alternative. For periplasmic expression, the most protease deficient strain is CAG597 (#E4123); KS1000 (#E4128) and UT5600 (#E4129) might be worth trying as well. The CAG strains are difficult to transform, and often require electroporation to introduce the fusion plasmid.

7. **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:

   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* 307:249-64

   Reviews on refolding:
   Qoronfleh MW, et al. (2007) *Protein Expr Purif* 55(2):209-24.
   Jungbauer A and Kaar W. (2007) *J Biotechnol* 128(3):587-96
   Jungbauer A, et al. (2004) *Curr Opin Biotechnol.* 15(5):487-94
   Vallejo, LF and Rinas,U (2004) *Microb Cell Fact* 3(1):11-22

8. **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-p5 vectors have about a 8- to 16-fold reduced level of expression when compared to the same insert in a pMAL-c5 vectors. 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 (16). Even in cases where a band is not visible, one can get yields up to 5 or 6 mg/liter of culture.

9. **I've cloned my insert, but after SDS-PAGE the only induced band present is the size of MBP5*.**

   There are two likely explanations for this result. If the protein of interest is in the

wrong translational reading frame, an MBP5-sized band will be produced by translational termination at the first in-frame stop codon. If the protein of interest is very unstable, an MBP5-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 #E8030) or anti-MBP Monoclonal Antibody (NEB # E8032 or E8038). 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 #S1237) 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.

10. **What are the possible effects of export (secretion, using a pMAL-p5 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. In these cases, the fusion protein gets stuck in the membrane and cannot fold properly, which can lead to its degradation (17). 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 (18). When this class of protein is expressed in the cytoplasm, it may fold improperly and become degraded or insoluble.

11. **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).

AFFINITY PURIFICATION

12. **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 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.

13. 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. 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.

14. 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.

15. 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.

16. 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. The best strategy in either case is to use a host which is deficient in the offending protease(s).

17. 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*. In this case, one would expect to see bands between the size of MBP (42.5 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.

18. 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.

19. 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.

FACTOR Xa CLEAVAGE

20. 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 here is as referenced in Nagai and Thøgersen (1987). 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. 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.

21. Are there any control substrates for Factor Xa?
The Protein Fusion and Purification System comes with an MBP5-paramyosin-ΔSal fusion as a positive control for Factor Xa cleavage (NEB #E8052). Sigma also sells a colorimetric substrate, N-benzoyl-ile-glu-gly-arg-p-nitroanilide (Sigma, #B7020).

22. 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.

23. 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 (GE Healthcare, #17-5143-02). When 50 µg of Factor Xa is passed over a 0.5 ml column, less than 0.2% of the activity flows through.

24. 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%). 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. Another 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 latter strategy, the extra residues remain at the N-terminus after Factor Xa cleavage.

25. What is the molecular weight and pI of Factor Xa?
The molecular weight of Factor Xa is 42,400 daltons. 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 (26), and the calculated pI of Factor Xa is 5.09.

26. 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.

27. 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.

28. Can MBP fusions be digested with Factor Xa while bound to the amylase resin?

Cutting a bound fusion with Factor Xa has been done, (27, 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.

SEPARATION OF FUSION PROTEIN DOMAINS AND STORAGE.

29. 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 (28). 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.

30. Are there any alternatives to the protocols in the manual for separating the domains after cleavage?

Some fusion proteins can be eluted from the amylose resin with water, although they may not come off in as sharp a peak as they do with column buffer + 10 mM maltose. This can be useful for re-binding the MBP to an amylose column after factor Xa cleavage. Be aware that some proteins may denature or precipitate in water, however.

Small amounts of MBP can be removed from the protein of interest by incubating with immobilized anti-MBP antibody (NEB#E8037). The capacity of the beads makes this impractical for a large scale preparation.

31. 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.

MBP INFORMATION

32. What is MBP5? Is it different from wild-type MBP produced from _E. coli_?
MBP5 is the protein produced from pMAL-c5X that has a stop codon linker cloned into the XmnI site. It differs from wild-type MBP by the addition of a methionine at the amino 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 protease site.

33. 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 (29), and Sharff, A. J. et al. (1992) _Biochem._ 31,10657–10663 (30).

34. 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.

35. What is the K_d, pI and extinction coefficient for MBP?
The K_d of MBP for maltose is 3.5 µM; for maltotriose, 0.16 µM (31). MBP2*'s extinction coefficient is 1.5 (1 mg/ml, 1 cm path length) and its calculated pI is 4.9.

36. 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.

37. What is the origin of the MBP region of the pMAL vectors?
MBP5 is a monomer. There is one published report that MBP can dimerize in 10 mM Tris-HCl (32) but we have not been able to reproduce this result with MBP5. 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

38. NEB Express (#C2523)

fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1(mcrC-mrr)114::IS10

NEB Express is an *E. coli* B strain similar to T7 Express and BL21(DE3), but without the T7 RNA polymerase (the T7 RNA polymerase is unnecessary since the pMAL vectors use the *E. coli* RNA polymerase). It is naturally deficient in the Lon protease, and mutant in the ompT protease. Available as competent cells.

39. ER2507 (#E4121)

F⁻ *ara-14 leuB6 fhuA2 Δ(argF-lac)U169 lacY1 glnV44 galK2 rpsL20(Str^R) 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. This strain can be transformed with high efficiency, similar to RR1 and HB101.

40. ER2508 (#E4127)

F⁻ *ara-14 leuB6 fhuA2 Δ(argF-lac)U169 lacY1 lon::miniTn10(Tet^R) glnV44 galK2 rpsL20(StrR) 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*

This is a Lon- version of ER2507. 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.

41. CAG626 (#E4124)

F⁻ *lacZ(am) pho(am) lon trp(am) tyrT[supC(ts)] rpsL*(Str^R) *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 (33).

42. CAG597 (#E4123) F⁻ *lacZ(am) pho(am) tyrT[supC(ts)] trp(am)*
rpsL(Str^R) *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 (33).

43. CAG629 (#E4125) F⁻ *lacZ(am) pho(am) lon tyrT[supC(ts)] trp(am)*
rpsL(Str^R)*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.

CAG629 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 (33).

44. PR1031 (#E4126) F⁻ *thr:Tn10*(Tet^R) *dnaJ259 leu fhuA2 lacZ90(oc) lacY*
formerly *glnV44 thi*
CAG748

This strain has a mutation in the *dnaJ* gene, which codes for a chaperone. 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); (34, 35).

45. KS1000   (#E4128)   F´ *lacIq lac+ pro+/ ara Δ(lac-pro) Δ(tsp)≡ Δ(prc)::*Kan^R*eda51::Tn1*0(Tet^R) *gyrA*(Nal^R) *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) (18).

46. UT5600   (#E4129)   F⁻ *ara-14 leuB6 secA6 lacY1 proC14 tsx-67 Δ(ompT-fepC)266 entA403 trpE38 rfbD1 rpsL109*(Str^R) *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); (19, 20, 36).