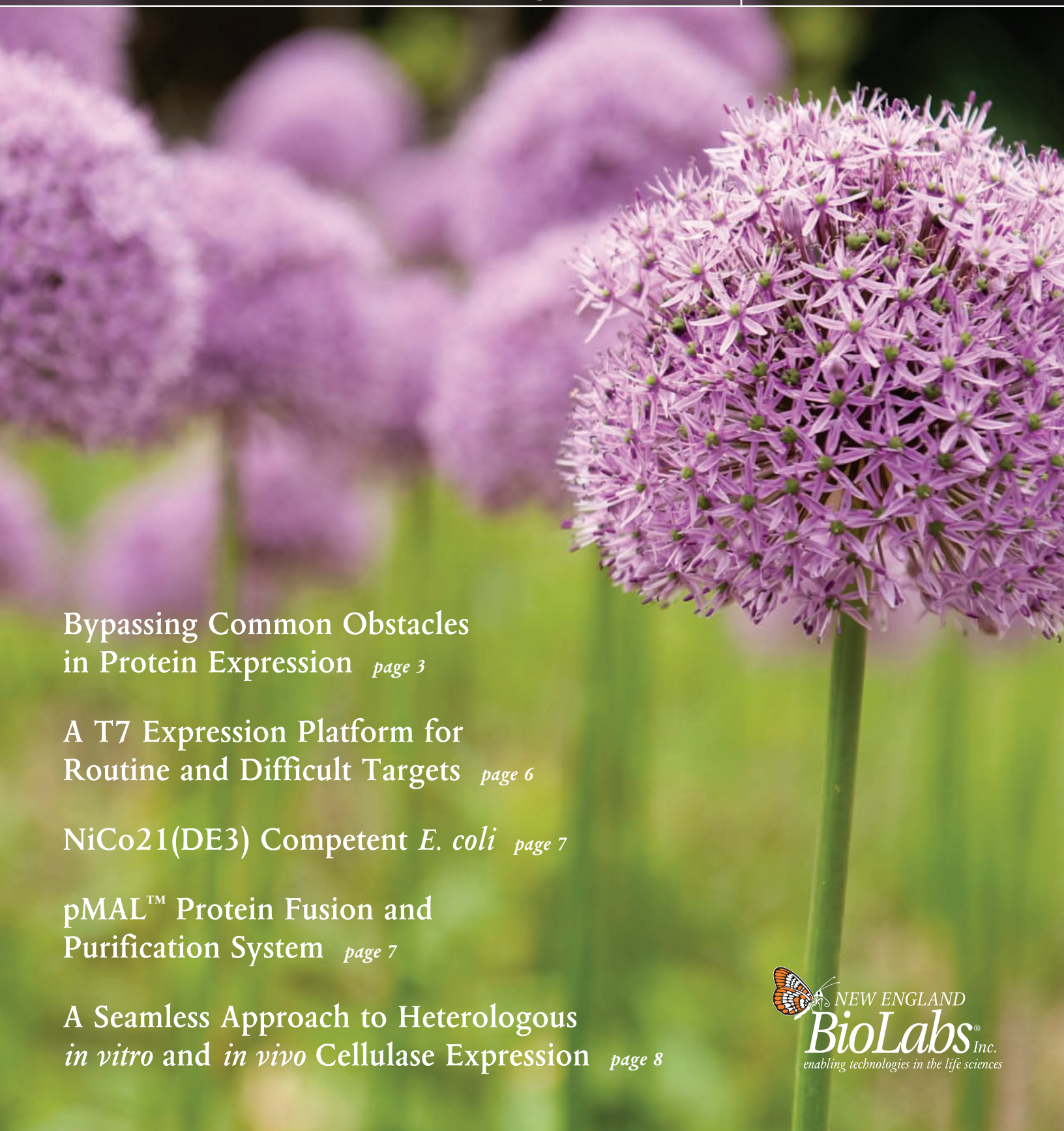
NEB EXPRESSIONS

A scientific update from New England Biolabs | Summer Edition 2011



NEW ENGLAND BioLabs® Inc.
enabling technologies in the life sciences

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Upcoming Tradeshows

Visit the NEB booth at the following meetings:

- **AACC (American Association of Clinical Chemistry)**
  July 24–28, 2011
  Atlanta, GA
  Booth 1308
  http://aacc.org/events/2011am/exhibitors/pages/default.aspx

- **National Association of Scientific Materials Managers**
  July 24–29, 2011
  Minneapolis, MN
  http://naosmm.org/

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A Letter from NEB

Dear Researcher,

Protein expression and purification can be very challenging, and there is no single approach that is appropriate for every protein or every downstream application. Successful expression can be affected by each individual protein's intrinsic ability to fold, its solubility, its toxicity or its need for post-translational modifications. Additionally, the application for which the protein is ultimately to be used may dictate how the protein needs to be expressed or purified. It is therefore important to have a flexible set of tools and methods that can be applied to each unique project.

This edition of NEB Expressions addresses the obstacles that researchers face when expressing challenging proteins and offers suggestions and tools to overcome many of them. NEB supplies several unique competent cell strains and expression systems that aid in the expression of difficult targets, including membrane proteins, proteins with solubility issues and proteins with multiple disulfide bonds. Application notes utilizing several of these tools are found on pages 6 and 8.

Wishing you continued success in your research,

New England Biolabs

Allium flowers as seen on the New England Biolabs campus. Photographed by Kim Indresano.

Proof that the wait was worth while...

Thank you to over 3,000 customers who requested a sample of our new One*Taq*™ DNA Polymerase. We invited customers to share their feedback, and the results are as follows:

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Bypassing Common Obstacles in Protein Expression

All too often, a protein of interest expresses poorly due to toxicity in the host cell, insolubility, or mRNA secondary structure preventing interactions with cellular machinery. Occasionally, the gene of interest is rich in codons that are inconsistent with the host strain's available supply of tRNAs. Uncontrolled basal expression can affect host cell growth and decrease protein yield, while overly robust induction can result in inclusion bodies. Exporting a protein to the *E. coli* periplasm or the inner membrane introduces more complications for targets that must be folded with disulfide bonds or incorporated into a membrane.

NEB has a long history in recombinant protein expression, and has developed a breadth of knowledge that serves as a valuable resource for customers. Often a well-timed piece of advice is enough to fix experimental troubles. But for more difficult problems—or for simply streamlining the process and improving yield—NEB's portfolio of expression products offers a variety of solutions.

Chris Womack, Science Writer, Austin, TX

Choosing a Host Strain

E.coli strains are generally designed for cloning or for protein expression; although some strains are suitable for both purposes. The *endA1* mutation is an important host feature for cloning and propagation of plasmid DNA since mutation of the *endA* gene abolishes Endonuclease I activity resulting in higher quality plasmid preparations. The T7 Express and NEB Express strains offer the option of direct cloning, followed by protein expression; these strains carry the *endA1* mutation and are available as high efficiency competent cells (Table 1). The common expression strain BL21(DE3) is a poor choice for direct cloning, because its Endonuclease I activity may degrade plasmids after isolation, and its high basal T7 expression level may result in clone instability and/or intolerance of toxic proteins. Another host feature to consider during cloning is the *recA1* mutation, which abolishes homologous recombination. Undesired DNA recombination is more likely when the gene contains repeat sequences or if the plasmid clone contains sequences homologous to the host chromosome. Most cloning strains carry a *recA* mutation, while it is generally not necessary for protein expression strains unless the plasmid clone is known to be unstable.

Table 1. T7 Express and NEB Express

STRAIN	NEB #
NEB Express Competent *E. coli*	C2523H/I
NEB Express I^q Competent *E. coli*	C3037H/I
T7 Express Competent *E. coli*	C2566H/I
T7 Express I^q Competent *E. coli*	C3016H/I
T7 Express *lysY* Competent *E. coli*	C3010H/I
T7 Express *lysY*/I^q Competent *E. coli*	C3013H/I
T7 Express Crystal Competent *E. coli*	C3022H/I

Visit www.neb.com/compcelloverview for additional expression strains, including BL21 and BL21(DE3).

With regard to expressed protein quality, researchers should seek expression strains that lack proteases, such as OmpT, which are likely to degrade target proteins during processing. Along with T7 Express and NEB Express, several commercially available strains lack the proteases OmpT and Lon. One caveat, however—Lon may serve as a "quality control" protease important to particular expression scenarios (1). For proteins that show signs of proteolysis, NEB recommends using an OmpT-deficient expression strain and adding protease inhibitor cocktail during processing.

Additionally, strains that lack F´ episomes are preferred as protein expression hosts because many cloning strains carry the *ompP* protease gene on an F´ episome (2). Four expression strains from NEB carry a miniF plasmid, but this single-copy vector lacks the *ompP* gene.

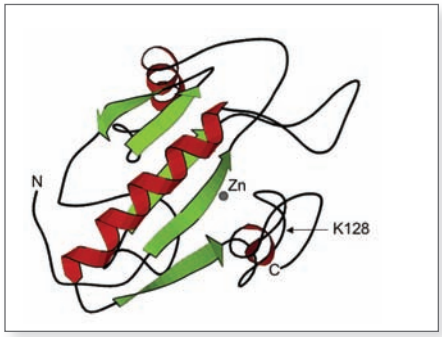
Many other attributes of good host strains, such as $lacI^q$ control of basal expression are addressed below in discussions of gene expression problems.

Solving High Basal Expression in a Lac Promoter System

High uninduced expression of a target protein can seriously hamper a host strain's viability or result in loss of plasmid from a significant share of the cell population.

Many commercial plasmids and host strains can provide regulated expression to reduce or eliminate this problem. For expression plasmids using a variation of the lac promoter, such as P_{lac}, P_{lacUV5}, P_{tac} and P_{trc}, the first step is to be certain that the expression system supplies additional LacI repressor. Most systems include the *lacI* gene on an expression vector, while many host strains feature enhanced LacI production (3).

Figure 1: LysY is a T7 lysozyme variant (K128Y)



LysY lacks amidase activity against the cell wall, yet retains the ability to inhibit T7 RNA polymerase. Cheng et al. Proc. Natl. Acad. Sci., USA (1994) T7 lysozyme structure 2.2 Å resolution. (Residues 6–150, SWISS-Pdb viewer, PDB ID:1LBA)

Additional plasmid encoded *lacI* genes are often not enough to control basal expression; so many systems instead supply the $lacI^q$ gene, whose mutated promoter increases LacI repressor expression ten-fold (4). NEB scientists recommend using a host expression strain harboring the $lacI^q$ gene (e.g., NEB Express I^q, NEB #C3037). Compared to strains lacking $lacI^q$, strains carrying this gene are more easily transformed with lac-promoter plasmids carrying genes that encode toxic proteins (3).

Reducing Basal Expression in the T7 System

The most common protein-expression strain, BL21(DE3), expresses T7 RNA polymerase at a high basal level. So target proteins in this strain—and many of its derivatives—are often expressed before inducer is added.

Control of T7 expression is best provided by hosts that co-express T7 lysozyme, which naturally inhibits T7 RNA Polymerase through a 1:1 protein interaction (5). T7 lysozyme is generally available in plasmids pLysS

and pLysE, as well as in *lysY* host strains. Researchers at NEB recommend switching to a *lysY* or pLysS strain as a first resort if plasmid transformation fails, or if the protein of interest might be toxic to the host.

Plasmids carrying *lysS* or *lysE* produce T7 lysozyme with amidase activity, but at a lower level than pLysE. A freeze-thaw cycle can lyse strains carrying pLysS, so it's important to consider downstream processing when planning T7-system expression. LysY host strains produce a variant T7 lysozyme that lacks amidase activity.

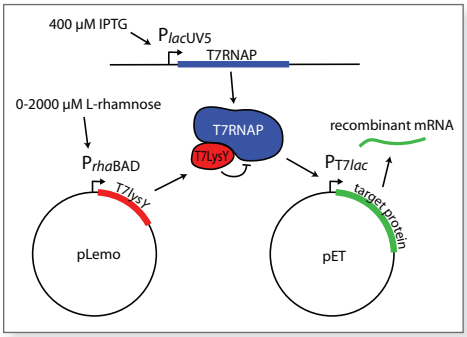
It is important for investigators to take care when using pLysE hosts—the excess amidase activity of pLysE can damage *E. coli* cell walls, which may cause a growth defect (6). NEB researchers have observed culture lysis in experiments with pLysE strains, in which the protein of interest is targeted to the cell envelope.

In DE3 strains, adding 1 percent glucose to the medium can decrease basal expression from the *lacUV5* promoter by lowering the cAMP levels that stimulate it. Switching from glucose to a poor carbon source in final growth cycles can also help maximize IPTG-induced expression (6).

Low-Basal Expression Alternatives

NEB's T7 Express strains use a wild-type lac promoter to express the T7 RNA polymerase from within the lac operon, which results in lower basal production compared to DE3 strains. T7 Express strains also optionally express *lacIq*, *lysY*, or a combination of these control elements.

Figure 2. Tuning protein expression in Lemo21(DE3)



In Lemo21(DE3) T7 RNA polymerase activity can be modulated precisely by its natural inhibitor T7 lysozyme, which is expressed from the extremely well titratable rhaBAD promoter. The combination of PlacUV5 expression of T7 RNA polymerase from the chromosome and rhamnose inducible expression of T7 lysozyme from pLemo guarantees the greatest possible range of target protein expression. Figure courtesy of Xbrane Bioscience AB.

Tunable Expression for Toxic Proteins

In addition to tight promoter control, expressing toxic proteins often requires tunable expression. Keeping expression at a desired moderate level can maximize yields by maintaining the concentration of a toxic target protein just below a host strain's tolerance. Alternatively, tuning expression allows researchers to prevent well-expressed target proteins from creating inclusion bodies.

The PrhaBAD promoter is a key part of many expression systems. For example, the Lemo21(DE3) strain expresses lysY control protein under the PrhaBAD promoter (Fig 2). Finding the right expression level involves running parallel expression trials using L-rhamnose concentrations from 0 µM to 2,000 µM. While most promoters are either "on" or "off," protein production per cell in the Lemo21(DE3) strain is inversely proportional to L-rhamnose concentration.

To express a highly toxic protein, it may be necessary to employ a cell-free expression system, such as the PURExpress *In Vitro* Protein Synthesis Kit (NEB #E6800), which uses only recombinant components, and is free of contaminating nucleases, proteases, and protein-modifying enzymes. For more information on PURExpress, see pages 8 and 10.

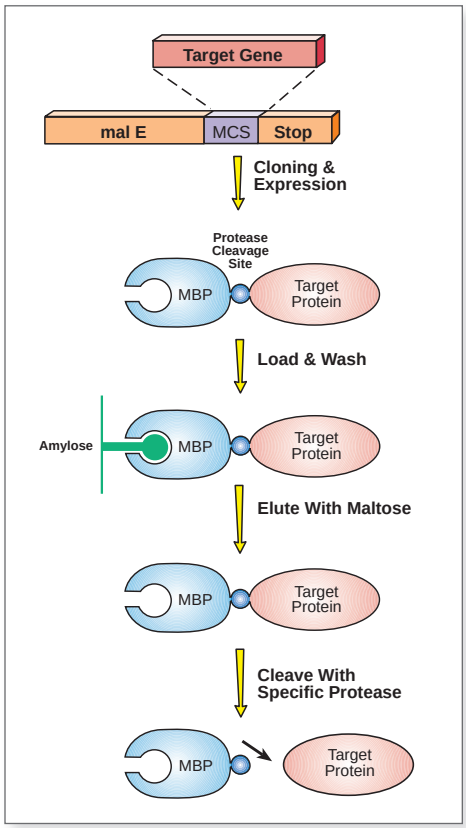
Raising Low-Solubility Protein Yields

Proteins that are insoluble—or nearly insoluble—require approaches beyond tuning expression. Inducing protein expression at a lower temperature, between 15–20°C, can often raise yields of properly folded protein.

Another approach to expressing low-solubility proteins is to fuse them to a "solubility tag" using vectors such as the pMAL Protein Fusion and Purification System (NEB #E8200). The pMAL vectors encode maltose binding protein, a fusion tag that aids in expression and solubility, and allows for simple purification using an amylose column (Fig 3). Removing the MBP fusion requires protease cleavage and additional purification. However many MBP fusion proteins can be readily studied, since they often retain activity in this form.

Some researchers achieve good results by co-expressing low-solubility proteins with chaperonins, such as GroEL, DnaK, and ClpB (7,8). While chaperonin overexpression may improve target protein solubility, some target protein may remain complexed with chaperones. Methods including native PAGE analysis and size exclusion chromatography can reveal oligomeric complexes in expressed protein samples.

Figure 3. Schematic of the pMAL System



The target protein is fused to MBP, enhancing solubility and expression.

Changing Sequence to Improve Expression

During recombinant gene expression experiments, intra-RNA interactions can sometimes prevent optimal translation. Troublesome secondary structure can be a problem in the 5´ untranslated region, the ribosomal binding site and the affinity tag coding sequence. Even the frequency of particular codons in the gene of interest can cause expression problems.

In genes with troublesome secondary structure, it is often possible to improve expression by altering ribosomal binding sites and removing inhibitory secondary structure. Altering ribosomal binding sites for better expression usually means changing their sequences to more closely match the ideal *E. coli* sequence, AGGAGGT. Changing an affinity tag's position, and adding more adenines to the next codon after the ini-

tiation codon (9), may also improve expression in some cases.

Finally, translation can stall in genes whose translation calls for tRNAs that are in low abundance in the host species (10). In this case, consider co-expressing rare tRNAs in the host organism (11) or completely redesign the gene using preferred bacterial codons. The decreasing cost of gene synthesis makes this second option increasingly attractive. However, redesigned genes can become so well-expressed that solubility and inclusion bodies begin to become problems, and it may be necessary to adopt a tunable expression system.

Making the Right Disulfide Bonds

When disulfide bonds are essential for target protein folding or stability, investigators often direct the protein to *E. coli's* oxidative periplasm, where Dsb enzymes can establish the correct bond configuration. Several commercially available vectors include an N-terminal signal sequence for exporting proteins to the periplasm. An example would be the pMAL-p5 vectors (NEB #N8109), whose wild-type MBP gene contains an N-terminal periplasmic localization signal.

Alternatively, NEB's SHuffle strains are excellent options for expressing proteins having complex disulfide bonds. SHuffle strains carry mutations that alter cellular reduction conditions, allowing proper disulfide bond formation in a now-partially oxidizing cytoplasm (Fig 4). SHuffle strains also express disulfide bond isomerase (DsbC) in the cytoplasm, rather than only in the periplasm.

It is also possible to modify cell-free systems to produce proteins with disulfide bonds. In one approach, eliminating DTT from the reaction mixture before translation can properly alter oxidation conditions for bond formation (12). A second approach adds iodoacetamide, a glutathione redox buffer, and a disulfide-bond isomerase (13). For researchers using the PURExpress system, the PURExpress Disulfide Bond Enhancer (NEB #E6820)—a proprietary blend of proteins and buffer components—will assist correct folding of proteins with multiple disulfide bonds.

Improving Membrane-Protein Yields

Membrane proteins are especially difficult to produce in quantity, and targeting them to the *E. coli* inner membrane is often the best expression strategy. However, in unregulated expression systems it is possible for newly synthesized protein to overwhelm the SecYEG translocation machinery. These situations often require a tunable expression system, such as Lemo21(DE3) Competent *E. coli* (NEB #C2528) (14).

As another solution to translocation bottlenecks, NEB researchers have weakened the ribosomal binding site by altering its sequence, which lowered basal expression and enabled a higher yield of a difficult membrane protein (3).

Difficult membrane proteins may also call for a low- to medium-copy plasmid conferring kanamycin or chloramphenicol resistance—rather than ampicillin resistance—to reduce the likelihood of plasmid loss. NEB researchers recommend testing for expression plasmid maintenance at the point of induction by plating cells with and without antibiotic. After each expression experiment, verify that a significant portion of the target membrane protein is integrated into the membrane. If not, express the protein at a lower temperature, perhaps 20–25°C, and with early induction at OD_{600} of 0.35–0.45.

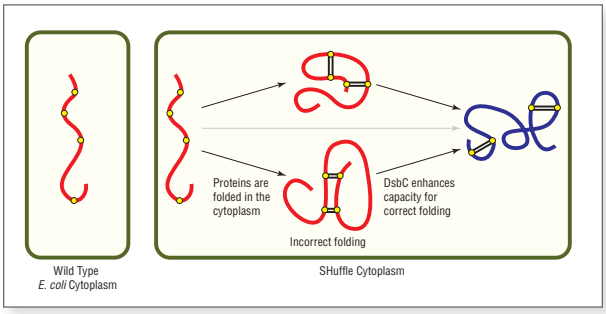
As an alternative to host-cell expression, cell-free translation systems are also viable options for expressing troublesome membrane proteins. Ion-channel proteins, transporters, receptors, and other integral membrane proteins can affect viability by disrupting membranes, or simply by aggregating in the cytoplasm. Either case leads to low yields.

Cell-free expression of membrane proteins often requires additional detergents, synthetic lipids or bilayers similar to those in a target protein's source organism. When aggregation is a problem, cell-free systems allow researchers to investigate adding detergents or lipids to prevent precipitation.

Conclusion

Some proteins present truly intractable expression problems in heterologous hosts. But protein expression techniques are currently experiencing rapid improvement, with new developments in tunable expression, solubility technology, protein targeting, and cell-free systems, greatly improving yields and purity over the expression systems of only a few years ago. NEB aims to continue contributing to the field with innovative products and helpful advice.

Figure 4. Expression of protein with multiple disulfide bonds using SHuffle Competent *E. coli*



Disulfide bond formation in the cytoplasm of wild type E. coli is not favorable, while SHuffle is capable of correctly folding proteins with multiple disulfide bonds in the cytoplasm.

References

1. Link, A. J., et al. (2008) *Protein Sci.* 17, 1857–1863.
2. Hwang, B. Y., et al. (2007) *J Bacteriol.* 189, 522–530.
3. Samuelson, J. C. (2011) Recent Developments in Difficult Protein Expression: A Guide to E. coli Strains, Promoters, and Relevant Host Mutations. *Methods Mol. Biol.* 705:195–209.
4. Calos, M. P. (1978) *Nature,* 274, 762–765.
5. Zhang, X. and Studier, W. F. (1997) *J. Mol. Biol.* 269, 10–27.
6. Pan, S. H. and Malcolm, B. A. (2000) *Biotechniques,* 29, 1234–1237.
7. Amrein, K.E., et al. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 1048–1052.
8. Nishihara, K., et al. (1998) *Appl. Environ. Microbiol.* 64, 1694–1699.
9. Stenstrom, C.M., et al. (2001) *Gene,* 263, 273–284.
10. McNulty, D.E., et al. (2003) *Protein Expr. Purif.* 27, 365–374.
11. Dieci, G., et al. (2000) *Protein Expr. Purif.* 18, 346–354.
12. Kawasaki, T. et al. (2003) *Eur. J. Biochem.* 270, 4780–4786.
13. Yin, G. and Swartz, J.R. (2004) *Biotechnol. Bioeng.* 86, 188–195.
14. Wagner, S., et al. (2008) *Proc. Natl. Acad. Sci. USA* 105, 14371–14376.

Application Note

E. coli Lemo21(DE3) - A T7 RNA Polymerase-based protein overexpression platform for routine and difficult targets

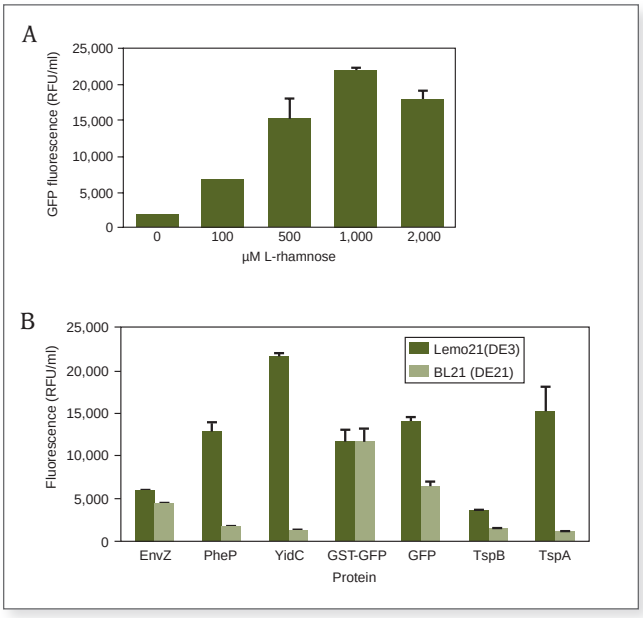
David Vikström – Xbrane Bioscience AB, Mirjam Klepsch – Center for Biomembrane Research (Stockholm University, Stockholm, Sweden), Samuel Wagner – Xbrane Bioscience AB, James C. Samuelson – New England Biolabs and Jan-Willem de Gier – Xbrane Bioscience AB and Center for Biomembrane Research

Introduction

In almost all *E. coli* protein production strains, the overexpression of proteins is driven by T7 RNA polymerase. Finding the optimal conditions for the overexpression of a protein is usually based upon time consuming and laborious screens involving many different strains and culture/induction regimes. Lemo21(DE3) is a T7 strain designed for the expression of challenging proteins, including membrane proteins, toxic proteins and proteins with solubility issues. In Lemo21(DE3), T7 RNA polymerase activity can be modulated precisely by its natural inhibitor T7 lysozyme, which is expressed from the extremely well titratable rhamnose promoter (1). However, Lemo21(DE3) is also well-suited for routine protein expression. The versatility of this strain makes it possible to identify optimal conditions for the overexpression of any protein using one strain and a limited number of culture/induction conditions.

Results

Figure 1. Optimizing protein overexpression in Lemo21(DE3)



A. Optimizing expression of the membrane protein YidC in Lemo21(DE3). To monitor expression levels, green fluorescent protein (GFP) was fused to its C-terminus. Thus, GFP fluorescence may be used as a direct measure of protein overexpression yields in whole cells (2,3,4). Cells were cultured in Luria Bertani medium in the presence of different concentrations of rhamnose.

B. Comparison of protein overexpression in BL21(DE3) and Lemo21(DE3). All proteins were C-terminally fused to GFP. Cells were cultured in Luria Bertani medium and whole cell fluorescence was measured 8 hours after IPTG induction. For expression in Lemo21(DE3), the optimal rhamnose concentration was used. The target proteins are: bacterial membrane proteins EnvZ, PheP and YidC; human membrane proteins Tetraspanin A and B (TspA and TspB); soluble proteins Glutathione S-transferase and GFP alone. For graphical reasons, fluorescence values of TspA and TspB were multiplied by 10, and fluorescence values of GST-GFP and GFP were divided by 10 and 50, respectively.

Summary

The T7 expression strain Lemo21(DE3) allows researchers to sample a wide range of expression levels to find the optimal conditions for each unique target protein. More specifically, Lemo21(DE3) can be used for the expression of routine and difficult targets, eliminating the testing of multiple strains to achieve desired expression levels. This can result in both time and cost savings.

Ordering Information

PRODUCT	NEB #	SIZE
Lemo21(DE3) Competent *E. coli*	C2528H	6 x 0.05 ml/tube

General Protocol

1. Transform the T7 promoter-based expression construct into Lemo21(DE3). Always use fresh transformants (not older than 4-5 days) for overexpression experiments. The Lemo21(DE3) strain contains pLemo, a pACYC184 derivative carrying the *lysY* gene. Accordingly, chloramphenicol (30 µg/ml) is required to maintain pLemo. In most cases, the T7 promoter-based expression vector will be compatible with pLemo.
2. Inoculate a single colony into 1 or 2 ml culture medium with the antibiotics required to maintain both pLemo and the overexpression vector; grow overnight to produce a starter culture. Use medium without glucose for optimal strain performance.
3. To sample different expression levels, set up 10 ml cultures at the beginning of day 2 with various levels of L-rhamnose (e.g., 0, 100, 250, 500, 750, 1,000 and 2,000 µM). Inoculate each 10 ml culture with 0.2 ml of starter culture.
4. Culture at 30°C until OD_{600} reaches 0.4–0.8.
5. Induce with 40 µl of a 100 mM stock of IPTG (final concentration of 400 µM). IPTG should not be varied, only L-rhamnose concentration is varied. Induce for 4 hours to overnight at 30°C.
6. Check for expression after different induction times either by Coomassie stained protein gel, Western Blot or activity assay (Figure 1). Check expression in both the total cell extract (soluble + insoluble) and the soluble fraction only. In the case of over-expressed membrane protein, most of the target should be in the low-speed spin supernatant after cell breakage by French Press, cell disruption or sonication (in combination with EDTA-lysozyme treatment).

   If a significant fraction of the target protein is insoluble (low speed pellet), repeat expression at a temperature lower than 30°C. Membrane protein expression may be improved by early induction (OD_{600} = 0.4) at 20 to 25°C.
7. For large scale, prepare liquid medium with antibiotics and the optimal level of L-rhamnose determined in a small scale trial. Both shake flasks and fermenters can be used for scaling up expression. For shake flasks, use 10 ml of freshly grown culture per liter for inoculation. Incubate at 30°C until OD_{600} reaches 0.4–0.8. Add 400 µM IPTG and express protein using optimal time/temperature determined in a small scale trial.

References
1. Wagner, S., et al. (2008) *Proc. Natl. Acad. Sci.* 23, 14371–14376.
2. Drew, D., et al. (2006) *Nat. Meth.* 3, 303–313.
3. Daley, D.O., et al. (2005) *Science,* 27, 1321–1323.
4. Drew, D., et al. (2005) *Protein Science,* 14, 2011–2017.

NiCo21(DE3) Competent *E. coli*

Affinity purification, including immobilized metal affinity chromatography (IMAC) is an effective method to purify most his-tagged proteins. However, poly-histidine tagged recombinant proteins that are isolated by IMAC are often contaminated with significant amounts of endogenous *E. coli* metal binding proteins. The protein expression strain NiCo21(DE3), a derivative of BL21(DE3), has been engineered to minimize *E. coli* protein contamination of IMAC fractions; GlmS is mutated to eliminate binding to IMAC resins and three other proteins (SlyD, ArnA and Can) are tagged with chitin binding domain (CBD) to enable rapid removal by chitin affinity chromatography. The result is a target protein with higher purity.

Advantages

- Alternative to BL21(DE3) for routine protein expression
- Improved purity of His-tagged proteins
- Economical removal of CBD-tagged *E. coli* metal binding proteins
- Identical growth characteristics as BL21(DE3)
- Deficient in proteases Lon and OmpT
- Resistant to phage T1 (*fhuA2*)

Ordering Information

PRODUCT	NEB #	SIZE
NiCo21(DE3) Competent *E. coli*	C2529H	20 x 0.05 ml

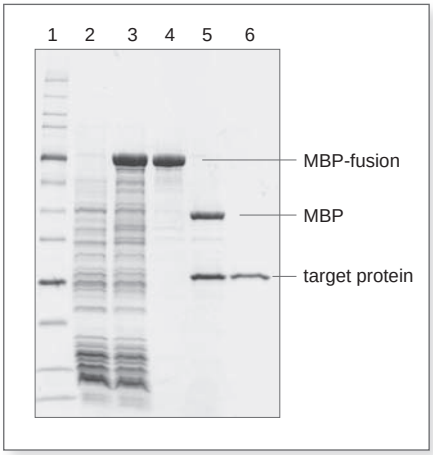
pMAL™ Protein Fusion and Purification System

This system takes advantage of the strong P_{tac} promoter and the translation initiation signals of maltose binding protein (MBP) to enhance expression and solubility levels of a desired protein in *E. coli*. The resulting product is an MBP fusion protein, which is then purified by affinity chromatography. For more information, see page 4.

Advantages

- Reliable expression: substantial yields (up to 100 mg/L) in more than 75% of the cases tested
- Expression in either the cytoplasm or periplasm: periplasmic expression enhances folding of proteins with disulfide bonds
- Fusion to MBP has been shown to enhance the solubility of proteins expressed in *E. coli*
- Gentle elution with maltose: no detergents or harsh denaturants

Protein Expression using pMAL



SDS-polyacrylamide gel electrophoresis of fractions from the purification of MBP-paramyosin-ΔSal. Lane 1: Protein Ladder (NEB #P7703). Lane 2: uninduced cells. Lane 3: induced cells. Lane 4: purified protein eluted from amylose column with maltose. Lane 5: purified protein after Factor Xa cleavage. Lane 6: paramyosin fragment in flow-through from second amylose column.

Ordering Information

PRODUCT	NEB #
pMAL Protein Fusion and Purification System	E8200

Application Note

Heterologous *in vitro* and *in vivo* Cellulase Expression using the PURExpress® *In Vitro* Protein Synthesis Kit, Disulfide Bond Enhancer and SHuffle™ Competent *E. coli*

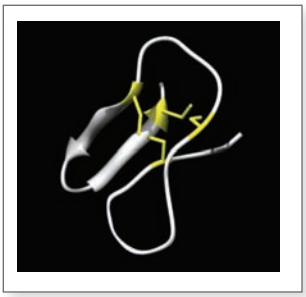
Richard Cohen, Charles Emrich, Nitzan Koppel, Nicholas Toriello and Robert Blazej – Allopartis Biotechnologies, Inc.

Introduction

Advances in industry and medicine have led to the engineering of complex "designer" proteins, such as antibodies in targeted therapeutics and enzymes in process development. The ability to easily generate an almost infinite number of variants at the DNA level has increased the demand for improved protein expression methodologies to fully capture what can be produced genetically. Often, the protein of interest is eukaryotic in origin and may require post-translational modifications specific to its native host or may be toxic to the host cells expressing them. Cell-free protein expression systems have allowed us to step beyond the limits of traditional *in vivo* expression methodologies by decoupling protein expression from host cell viability (1,2,3). Furthermore, the ability to produce complex proteins using cell-free transcription/translation systems uniquely enables high-throughput directed evolution and protein engineering efforts (4,5). Several cell-free protein expression systems have been developed in the last decade with recent advances focusing on special folding or assembly environments (6,7,8). Equally as important is the capability to transition from the *in vitro* system to larger-scale *in vivo* expression, while maintaining activity of the target protein (9,10).

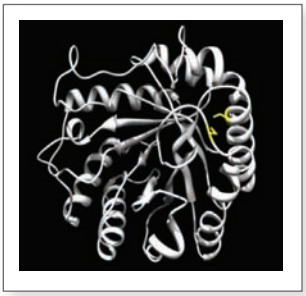
The PURExpress® *In Vitro* Protein Synthesis Kit (NEB #E6800), supplemental PURExpress Disulfide Bond Enhancer (NEB #E6820), and SHuffle® Competent *E. coli* (NEB #C3026) provide a seamless system for *in vitro* to *in vivo* protein expression. PURExpress is a novel cell-free transcription/translation system reconstituted from the purified components necessary for *E. coli* translation. The nuclease-free and protease-free nature of the PURExpress platform preserves the integrity of DNA and RNA templates and results in proteins that are free of modification and degradation. The PURExpress® Disulfide Bond Enhancer (PDBE) is a blend of proteins and buffer components designed to correctly fold target proteins with multiple disulfide bonds produced in PURExpress reactions. These enhancements can increase the yield of soluble and functionally active complex proteins containing disulfide bonds. Target proteins expressed in the PDBE/PURExpress environment can then be transitioned to *in vivo* expression using SHuffle *E. coli* strains. These engineered strains are capable of expressing proteins with increasing disulfide bond complexity in the cytoplasm. SHuffle strains express a disulfide bond isomerase that isomerizes mis-oxidized substrates into their correctly folded state, greatly enhancing the fidelity of disulfide bond formation. When used in conjunction, these three products increase the likelihood of generating fully active, complex proteins with multiple disulfide bonds.

Figure 1. *H. insolens* EG3 carbohydrate-binding module



Structure shows two non-consecutive disulfide bonds (yellow) (Residues 19 to 52, SWISS-MODEL prediction based on template: 1az6).

Figure 2. *H. insolens* EG3 catalytic core



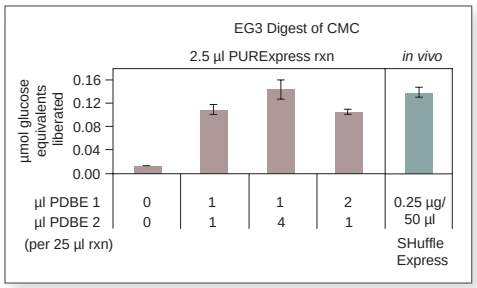
EG3 catalytic core contains a family 5 glycoside hydrolase, contains one disulfide bond (yellow) (Residues 86 to 387, SWISS-MODEL prediction based on template: 1gzj)

Cellulases are glycoside hydrolases that catalyze the cleavage of β-1,4-D-glycosidic linkages in cellulose, a linear polymer of glucose units. Cellulases are often multidomain proteins consisting of a catalytic core, a flexible linker and a carbohydrate-binding module. Bacterial and eukaryotic cellulase mixtures perform key functions in the conversion of lignocellulosic biomass into fermentable sugars for renewable chemical and biofuel production, textile processing, and detergent formulations. EG3 from the fungi *Humicola insolens*, an endoglucanase that creates internal glucan chain scissions, is a multi-domain 42 kDa protein containing a carbohydrate-binding module (CBM1) having two non-consecutive disulfide bonds connected by a flexible linker to a catalytic core (GH5) with one disulfide bond (based on the predicted crystal structure; Figs 1 and 2). This enzyme is used as a detergent cellulase for color brightening, softening and soil removal. The capabilities of the PDBE/PURExpress system with downstream expression in SHuffle *E. coli* strains are well-matched to the expression of this complex fungal protein in a flexible *E. coli*-based system.

Results

Active *Humicola insolens* EG3 was successfully expressed in *in vitro* transcription and translation (IVTT) reactions by using the PURExpress *In Vitro* Synthesis Kit. The addition of NEB's PURExpress Disulfide Bond Enhancer (PDBE) increased enzyme activity 13-fold. SHuffle Competent *E. coli* provide a quick path to milligram quantities of difficult-to-express eukaryotic proteins in *E. coli*. Several growth conditions, including temperature, media, and inoculum volume were investigated for optimal EG3 expression. Using the protocol described on page 9, soluble, active yields of 46 mg/L were achieved from SHuffle cells, significantly greater than titers from BL-21 lines (≥ 0.6 mg/L). Figure 3 presents the relative activities of EG3 expressed *in vitro* with varying amounts of PDBE supplementation and *in vivo* by using SHuffle cells.

Figure 3. The effect of PDBE supplementation in *in vitro* expression and the activity relative to 0.25 µg *in vivo*-expressed EG3.



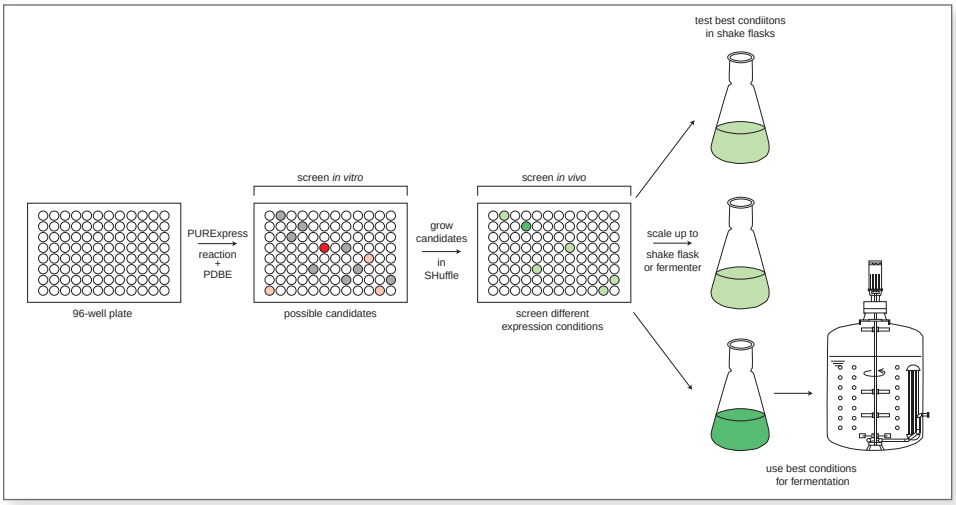
Summary

The PURExpress *In Vitro* Protein Synthesis Kit supplemented with PURExpress Disulfide Bond Enhancer was used to express active *Humicola insolens* EG3, a multi-domain fungal cellulase containing three disulfide bonds, from a synthetic gene construct. Useful quantities of EG3 were produced directly from the *in vitro* expression reaction. Direct scale-up to milligram quantities was made possible by transferring the gene construct into SHuffle Competent *E. coli*.

These tools can be utilized in a general workflow where the high throughput screening and selection of variants of a complex protein of interest can be done in a cell-free environment. The successful variant can then be expressed *in vivo* by using a specialized *E. coli* strain that also facilitates the required protein folding needs. In this case, the formation of complex disulfide bonds with the correct pairing was crucial to successful expression of active EG3 both *in vitro* and *in vivo* expression environments.

Thanks to Corinna Tuckey at New England Biolabs, Inc. for editorial contribution.

Figure 4. Workflow illustration of *in vitro* protein expression and screening followed by *in vivo* expression and scale up.



Ordering Information

PRODUCT	NEB #	SIZE
PURExpress® *In Vitro* Protein Synthesis Kit	E6800S	10 reactions (25 µl vol)
PURExpress® Disulfide Bond Enhancer	E6820S	50 reactions
SHuffle™ T7 Competent *E. coli*	C3026H	6 x 0.05 ml/tube

References

1. Stiege W. and Erdmann V.A. (1995) *J. Biotechnol.* 41, 81-90.
2. Jermutus L., Ryabova L.A. and Plückthun A. (1998) Curr. Opin. Biotechnol. 9, 534-548.
3. Shimizu Y., et al. (2006) FEBS J. 273, 4133-4140.
4. Endo Y. and Sawasaki T. (2006) Curr. Opin. Biotechnol. 17,373-380.
5. Zárate X. et al. (2010) Proteome Sci. 8, 32.
6. Klammt C. et al. (2007) Methods Mol. Biol. 375, 57-78.
7. Murtas G. et al. (2007) Biochem. Biophys. Res. Commun. 363, 12-17.
8. Endo S. et al. (2006) Mol. Biotechnol. 33, 199-209.
9. Kim T.W., et al. (2006) J. Biotechnol. 126, 554-561.
10. Zawada J.F., et al. (2011) Biotechnol. Bioeng. doi: 10.1002/bit.23103. [Epub ahead ofprint]

General Protocol

1. ***in vitro* Expression:**
   Thaw PURExpress tubes A, B, and Disulfide Bond Enhancer (PDBE 1 and 2) on ice. Adjust template DNA to 100 ng/µl.
   Assemble reactions on ice in nuclease-free, 0.5 ml microcentrifuge tubes as indicated*:

TUBE	1	2	3	4	5
H_2O	7.5 µl	6.5 µl	4.5 µl	1.5 µl	3.5 µl
Solution A	12.5 µl	12.5 µl	12.5 µl	12.5 µl	12.5 µl
Solution B	5.0 µl	5.0 µl	5.0 µl	5.0 µl	5.0 µl
PDBE 1**	0.0 µl	0.0 µl	1.0 µl	1.0 µl	2.0 µl
PDBE 2**	0.0 µl	0.0 µl	1.0 µl	4.0 µl	1.0 µl
Template DNA	0.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl

   * *Refer to current PURExpress (http://www.neb.com/nebecomm/products/productE6800.asp) and PDBE (http://www.neb.com/nebecomm/products/productE6820.asp) manuals for latest protocols, as recommended volumes and incubation times have been updated.*

   ** *Typical PDBE starting amounts are 1 µl. Titration may be necessary.*

   Incubate at 37°C for 1 hour to express and fold the protein. Cool and store at 4°C to stop reaction.

2. ***in vivo* Expression:**
   To produce larger quantities of purified enzyme, clone the *E3* gene into a suitable expression plasmid and transform into SHuffle using the protocol found on the NEB website (http://www.neb.com/nebecomm/products/protocol390.asp). Plate onto antibiotic selection plates and incubate for 24 hours at 37°C. Resuspend a single colony in 3 ml LB containing antibiotic and grow the starter culture overnight at 37°C. Inoculate 50 ml MagicMedia™ (Life Technologies) with 50 µl of the starter culture and grow in 250 ml baffled flasks at 37°C until reaching 1 OD, upon which transfer to growth at 25°C for a total of 24 hours.
   Harvest cells by centrifugation at 3,000 rpm for 30 minutes at 4°C. Resuspend cell pellets in BugBuster Plus solution containing protease inhibitors and lysozyme at 4 ml/g wet cell mass. Lyse for 30 minutes according to the manufacturers protocol. Affinity purify EG3 from the lysate (e.g. immobilized metal affinity purification), quantitate by using absorbance at 280 nm, and assess for purity by SDS-PAGE.

3. **Assay:**
   For each *in vitro* expression reaction, prepare triplicate CMC digestions in 200 µl PCR tubes as follows: Combine 45 µl 1.1% w/v CMC solution with 2.5 µl of the *in vitro* expression reaction and 2.5 µl H_2O (1% CMC final). Similarly, combine 0.25 µg purified *in vivo*-expressed enzyme with 45 µl 1.1% w/v CMC solution and water to a final 50 µl volume. Place the tubes in a thermal cycler with a heated lid and incubate at 50°C for 1 hour. Cool and hold at 4°C.
   Prepare DNS solution. Transfer 120 µl DNS solution to each CMC reaction and mix gently by pipetting. Cap the PCR tubes and heat to 95°C for 5 minutes with the lid set to 105°C. Cool to room temperature. Transfer 100 µl to a half-area spectrophotometer plate and read the absorbance at 540 nm. Similarly processed glucose standards may be used to calculate liberated glucose-equivalents in the CMC digestion reactions. Dispose of residual DNS solution and used pipette tips in appropriate chemical waste.

SHuffle™ Competent *E. coli* Strains

Exclusively from NEB, SHuffle™ competent *E.coli* strains are engineered to enable the folding of recombinantly expressed proteins *in vivo*. The expression of disulfide bond isomerase (DsbC) within the SHuffle cytoplasm enhances the correct formation of disulfide bonds in proteins that require them for biological activity. SHuffle can aid in the expression of many proteins, including antibodies, hormones, proteases, toxins and extra cytoplasmic signal proteins.

Ordering Information

PRODUCT	NEB #	SIZE
E. coli K12 STRAINS		
SHuffle™ Competent *E. coli*	C3025H	6 x 0.05 ml
SHuffle™ T7 Competent *E. coli*	C3026H	6 x 0.05 ml
SHuffle™ T7 *lysY* Competent *E. coli*	C3027H	6 x 0.05 ml
E. coli B STRAINS		
SHuffle™ Express Competent *E. coli*	C3028H	6 x 0.05 ml
SHuffle™ T7 Express Competent *E. coli*	C3029H	6 x 0.05 ml
SHuffle™ T7 Express *lysY* Competent *E. coli*	C3030H	6 x 0.05 ml

Advantages

- Oxidizing cytoplasmic environment enables disulfide bond formation
- DsbC directs correct disulfide bond formation
- Enhanced biological activity of correctly folded proteins
- Cytoplasmic expression increases yield
- DsbC also acts as a general chaperone for protein folding (1)
- Resistant to either Spectinomycin or Chloramphenicol only (in *lysY* strains), allowing a wide range of antibiotics to be used for selection purposes
- Transformation efficiency: 1 x 10^6 cfu/µg pUC19 DNA

1. Chen, J., et al. (1999) *J. Biol. Chem.* 274, 19601–19605.

PURExpress® *In Vitro* Protein Synthesis Kit

A rapid method for gene expression analysis, PURExpress® is a novel cell-free transcription/translation system reconstituted from the purified components necessary for *E. coli* translation. The nuclease-free and protease-free nature of the PURExpress platform preserves the integrity of DNA and RNA templates/complexes and results in proteins that are free of modification and degradation. Transcription and translation are carried out in a one-step reaction, and require the mixing of only two tubes. With results available in a few hours, PURExpress saves valuable laboratory time and is ideal for high throughput technologies. NEB offers the full PURExpress kits, along with various delta kits in which components are provided in separate tubes for specific applications.

PURExpress is based on the PURE system technology originally developed by Dr. Takuya Ueda at the University of Tokyo and commercialized as the **PURESYSTEM**® by Biocomber (Tokyo, Japan).

Ordering Information

PRODUCT	NEB #	SIZE
PURExpress® *In Vitro* Protein Synthesis Kit	E6800S	10 rxns
PURExpress® Δ Ribosome Kit	E3313S	10 rxns
PURExpress® Δ (aa, tRNA) Kit	E6840S	10 rxns
PURExpress® Δ RF123 Kit	E6850S	10 rxns
COMPANION PRODUCTS		
PURExpress® Disulfide Bond Enhancer	E6820S	50 rxns
E. coli Ribosome	P0763S	1 mg

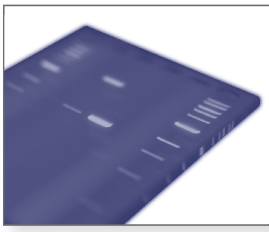
Advantages

- Lack of endogenous proteases or nucleases eliminates sample degradation
- Formulation amenable to modification
- Synthesized protein can often be visualized on a Coomassie stained gel
- Requires the mixing of two tubes followed by the addition of template DNA, either circular or linear template
- Reaction is complete in approximately two hours
- Purified components enable more control of translational and folding machinery
- Resistant to multiple freeze/thaw cycles

Protein Expression & Purification: Selection Chart

There are many factors to consider when choosing the optimal system for protein expression and purification. Each NEB expression technology offers different advantages that enable you to choose the strategy best suited to your particular needs. Many of the expression vectors share a compatible polylinker, enabling the gene of interest to be easily shuffled between systems. Various applications along with the recommended NEB kit are highlighted below.

APPLICATION	KIT	ADVANTAGES
High Yield Expression	pMAL™ Protein Fusion and Purification System	Substantial yields (up to 100 mg/L); uses the strong P_{tac} promoter
	K. lactis Protein Expression Kit	Uses the strong LAC4 promoter; multiple integrations of plasmid results in higher yield
	IMPACT™ Kit	Uses the T7 promoter for high level regulated expression
Cell-free Expression	PURExpress® In Vitro Protein Synthesis Kits	Quickly generates analytical amounts of protein
Co-expression of Multiple Proteins	*K. lactis* Protein Expression Kit	Easily co-express 2–4 proteins
	PURExpress® In Vitro Protein Synthesis Kits	Bicistronic constructs or multiple plasmids with appropriate transcription and translation regulatory elements can be used
Enhanced Solubility	pMAL™ Protein Fusion and Purification System	Fusion to MBP enhances solubility of proteins in *E.coli*
	K. lactis Protein Expression Kit	Utilizes *K. lactis* eukaryotic folding pathway
Affinity Tag Chromatography	IMPACT™ Kit	Utilizes an intein-chitin binding domain (CBD) tag on either the N- or C- terminus, offering single-step purification
	pMAL™ Protein Fusion and Purification System	Fusion to MBP or CBD allows for purification on amylose resin
	K. lactis Protein Expression Kit	Vectors are sold separately that generate fusions to MBP allowing for purification on amylose resin
Post-translational Modification	*K. lactis* Protein Expression Kit	Secretion of both N- and O- glycosylated proteins
Periplasmic Expression	pMAL™ Protein Fusion and Purification System	Periplasmic expression enhances folding of proteins with disulfide bonds
Secreted Expression	*K. lactis* Protein Expression Kit	Eliminates cell lysis step, simplifying purification
Toxic Proteins	*K. lactis* Protein Expression Kit	Secretion of protein from the cell
	IMPACT™ Kit	Can express the toxic gene in two pieces and ligate proteins together
	pMAL™ Protein Fusion and Purification System	Can export toxic proteins into periplasmic space
	PURExpress® In Vitro Protein Synthesis Kits	Cell-free environment not affected by "toxicity" of expressed protein
Protein Labeling or Ligation	IMPACT™ Kit	Generates proteins with reactive ends (N-terminal cysteine and/or C-terminal thioester)
	PURExpress® In Vitro Protein Synthesis Kits	Allows introduction of modified, unnatural, or labeled amino acids
No Additional Amino Acid Residues	IMPACT™ Kit	Start of native protein is fused adjacent to site of intein cleavage
	pMAL™ Protein Fusion and Purification System	Start of protein is fused adjacent to protease site
Reduced Proteolysis	*K. lactis* Protein Expression Kit	Protease deletion strains improve quality of secreted proteins



For protein analysis, NEB also offers a selection of highly pure protein markers and ladders. For more information, visit **www.neb.com/proteinladder**

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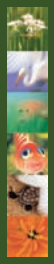

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