

PROTEIN EXPRESSION & ANALYSIS

PURExpress™
In Vitro Protein Synthesis Kit

Instruction Manual



NEB #E6800S
Store at -80°C



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Kit Components:

All kit components should be stored at **-80°C**.

Each kit contains sufficient reagents for 10 x 25 µl reactions.

Solution A (Yellow) ● 2 x 62.5 µl

Thaw on ice just before use.

Avoid multiple freeze-thaw cycles, if possible. Aliquot as necessary.

Solution B (Red) ● 2 x 25 µl

Thaw on ice just before use.

Avoid multiple freeze-thaw cycles, if possible. Aliquot as necessary.

Control (DHFR) Template 1 x 5 µl

Plasmid DNA (0.2 µg/µl) encoding *E.coli* dihydrofolate reductase for use as a positive control.

Universal Primer 1 x 25 µl

A forward primer (2 µM) used to add regulatory sequences to linear templates generated by overlapping extension PCR.

Materials Not Included:

General:	37°C incubator
Labeling:	³⁵ S-Methionine (>1000Ci/mmol recommended, <i>in vitro</i> translation grade)
TCA Precipitation:	TCA solutions (25%, 10%), 1M NaOH, casamino acids, ethanol, glass fiber filters, vacuum filtration manifold
SDS-PAGE:	gels and running buffer, gel apparatus, power supply, gel dryer
Western Blotting:	transfer apparatus, membrane, antibodies and detection reagent
Purification:	Ni-NTA Agarose, Microcon YM-100 spin concentrators

Introduction:

Overview

PURExpress is a novel coupled cell-free transcription/translation system reconstituted from purified components necessary for *E. coli* translation. Recombinant histidine-tagged aminoacyl-tRNA synthetases (20), initiation factors (3), elongation factors (3), release factors (3), ribosome recycling factor, methionyl-tRNA transformylase, T7 RNA polymerase, creatine kinase, myokinase, nucleoside-di-phosphate kinase, and pyrophosphatase provide the activities required for coupled transcription and translation, as well as energy regeneration. Purified 70S ribosomes, amino acids, rNTP's, and tRNA's complete the system.

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 the Post Genome Institute (PGI) (Toyko, Japan). PURExpress improves upon the **PURESYSTEM**[®] Classic II kit by optimizing the components to increase the yield of protein synthesis. PURExpress is an easy-to-use one-step reaction that requires the mixing of only two tubes. Protein synthesis is initiated by the addition of template DNA and is largely complete within one hour. Products of translation can be analyzed by SDS-PAGE (Coomassie stained, autoradiograph of ³⁵S-labeled proteins, or western blot) or in direct activity assays. Purification of the target protein can often be accomplished by ultrafiltration to remove the high MW ribosomes followed by IMAC (immobilized metal affinity chromatography) to remove the His-tagged components.

Due to its reconstitution of recombinant components, PURExpress is essentially free of contaminating exonucleases, RNases, and proteases. Template DNA is not exposed to digestion and target proteins are free of post-translational modifications (glycosylation, phosphorylation, and proteolysis).

Considerations for Template Preparation and Detection Methods:

PCR products, linear, or circular plasmid DNA can be used as the template DNA with PURExpress. While higher yields are often obtained with circular plasmid DNA as the template, PCR products can generate acceptable yields and can provide many timesaving advantages. The use of PCR to prepare template lends itself to projects where throughput is important, as transformation and plasmid purification steps are bypassed. PCR also affords the user the ability to modify coding or regulatory sequences (deletions, point mutations, addition of tags or other sequence elements, etc.) and prepare multiple templates at once.

Template purity is very important for successful *in vitro* transcription/translation. For best results, template DNA should be free of nucleases (DNases and RNases). Plasmid DNA prepared from many commercial kits (e.g. Qiagen)

often contains inhibitory amounts of RNase A. Excluding RNase A from the lysis buffer during plasmid preparation will dramatically reduce the amount of RNase in the sample. For samples already containing RNase, phenol:chloroform extraction and ethanol precipitation will remove the unwanted activity. For samples where RNase can't be removed, inclusion of RNase Inhibitors (e.g. NEB #M0314S) to the reaction will generally provide good results.

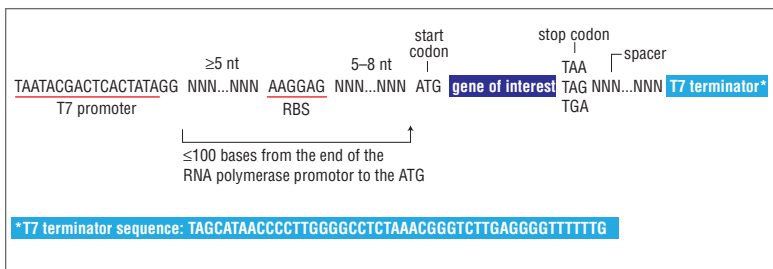
PCR templates should be **free of non-specific amplification products** that can interfere with transcription and/or translation.

In general, we recommend a starting concentration of 250 ng template DNA per 25 μ l reaction. In our experience, the optimal amount will fall in a range of 25–250 ng template per 25 μ l reaction. Template amounts for larger or smaller reactions should be scaled up or down accordingly. Templates are usually suspended in 10 mM Tris, pH 8.0. Samples should be free of potential inhibitors, including NaCl (> 50 mM), glycerol (> 1%), EDTA (> 1 mM) and Mg²⁺ or potassium salts (> 1-2 mM).

In addition to an in-frame coding sequence for the target protein, the template DNA must contain the following:

- start codon (ATG)
- stop codon (TAA, TAG, or TGA)
- T7 promoter upstream (approximately 20-100 nucleotides) of the coding sequence
- ribosome binding site (RBS, aka Shine-Dalgarno sequence) upstream (approx. 6-8 nucleotides) of the start of translation
- spacer region \geq 6 bp downstream from the stop codon (PCR products)
- T7 terminator downstream from the stop codon (recommended for plasmid DNA)

Figure 1:

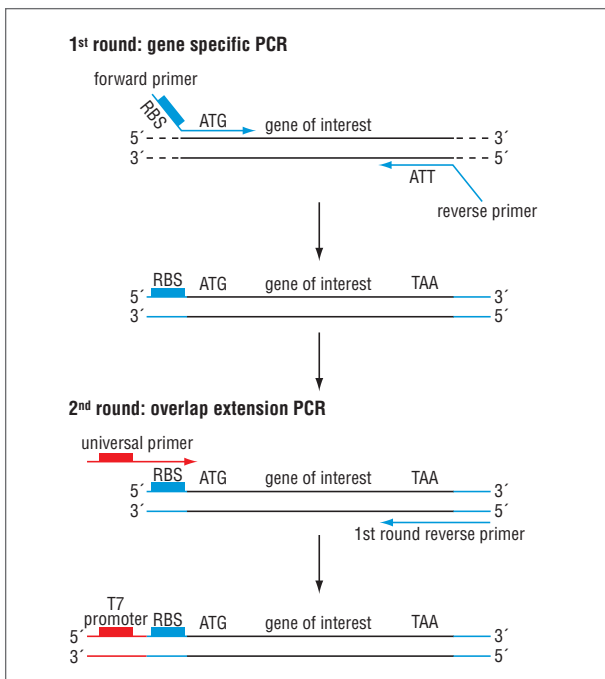


Required elements for template DNA

Generation of Template DNA by Overlap Extension PCR:

Overlap extension PCR can be used to generate template DNA for use with PURExpress. In the first round of PCR, gene specific primers are used to add adapter sequences (homologous to part of the regulatory region DNA) to the 5' and 3' ends of the gene of interest. In the second round of PCR, flanking primers bind the homologous regions of the first round product and extend through the coding region during the first cycle. In the next cycle of the second round, the first cycle products are self primed and extended to form full length linear expression constructs which are then exponentially amplified by the flanking primers in the subsequent cycles to amplify the template DNA containing the gene of interest with complete regulatory sequences and tags.

Figure 2:



Overlap extension PCR to generate linear template DNA

Add Solution B to Solution A, do not dilute Solution B unbuffered. We recommend a starting concentration of 250 ng template DNA per 25 μ l reaction. The optimal amount of input DNA can be determined by setting up multiple reactions and titrating the amount of template DNA added to the reaction. Typically, the optimal amount will fall in a range of 25–250 ng template per 25 μ l reaction.

3. Mix gently and pulse-spin in microfuge to collect mixture at the bottom of the tube.
4. Incubate at 37°C for 1 hour.
We recommend using an incubator rather than a water bath, to prevent evaporation. Some reactions can benefit from an additional hour of incubation to achieve maximum yield. Some proteins are also more soluble at reduced temperatures; however, incubating reactions below 37°C will likely reduce yield.
5. Stop the reaction by placing the tube(s) on ice.
6. Use samples for analysis or purification or freeze at –20°C for use at a later time.

The PURExpress components are highly purified and present in known quantities. This reconstituted nature of the product makes it amenable to modifications. As such, it is easy to perform *in vitro* labeling reactions with ³⁵S-methionine to allow visualization of the product. It is also straightforward to supplement the reactions with a component under investigation that is believed to have an effect on transcription or translation. *In vitro* labeling with ³⁵S-methionine can be performed by setting up a standard reaction with the addition of 2 μ l of ³⁵S-methionine. Unlabeled methionine is present at 0.3 mM in PURExpress. When supplemented with 1.2 μ M ³⁵S- L-methionine, we observe levels of incorporation compatible with autoradiographic detection of the synthesized protein. Reactions (1-5 μ l) can then be directly resolved by SDS-PAGE (no need for acetone precipitation), the gels are then briefly fixed in an methanol /acetic acid solution (45%/10%) for 5 minutes at 25°C and dried down onto filter paper (2 hrs at 80°C). The dried gel is then exposed to autoradiographic film (overnight at –20°C) or detected with a phosphorimager.

We encourage safe handling of radioisotopes and suggest consulting with your institution's radiation safety officer for guidelines and advice on the practical aspects of performing labeling reactions in your workplace.

Analysis of Synthesized Protein:

After *in vitro* transcription/translation, the reaction can be analyzed by SDS-PAGE followed by staining with Coomassie (Figure 3), silver or other dye, western blotting or autoradiography (for labeled proteins, Figure 4). PURExpress reactions are amenable to direct analysis; there is no need to precipitate the proteins by acetone, TCA, or ethanol prior to SDS-PAGE. Alternatively, if the target protein has enzymatic activity, the reaction can be used directly in the enzymatic assay provided the reaction mixture components do not interfere with the assay.

The yield of the target protein will vary. On average, we observe between 10–200 µg/ml, which translates to 250–5000 ng/25 µl reaction volume. It is useful to run a portion of the reaction on a protein gel and compare the banding pattern to a control reaction with no template DNA. The target protein is usually observed as a unique band, not present in the control reaction. Sometimes, the target has the same apparent MW as one of the endogenous proteins. In these cases, the target protein will enhance or “darken” the co-migrating band. Use 2.5 µl of each reaction and directly load them on a 10-20% Tris-glycine mini-gel and stain the gel with fresh Coomassie Blue R. Alternatively, excision of a band of interest from a gel with labeled proteins followed by scintillation counting provides a means to determine the yield of full-length protein.

If the reactions will be visualized by autoradiography (for ³⁵S-met labeled proteins) or by western blotting (for target proteins recognized by an available antibody) the amounts of reaction needed will vary and usually be less than the 2.5 µl used for Coomassie stained gels. Aliquots between 0.5–2.5 µl should be sufficient depending on the efficiency of the labeling, age of the label, or quality of the antibody. Again, we note that *in vitro* protein synthesis reactions produced by PURExpress can be directly loaded onto SDS-PAGE gels with no need for acetone precipitation and clean-up.

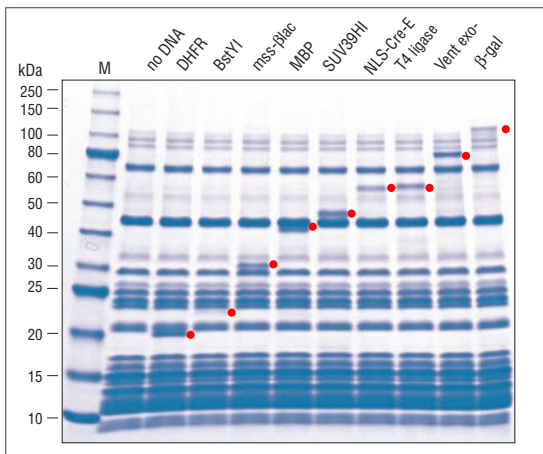
The choice of method for visualization is dependent on the needs of the investigator. One thing to note is target protein with low methionine content (below 3%, unpublished observation) may not label as well as those with a higher methionine composition. In these cases, ¹⁴C-leucine can be used as an alternative label. Similarly, for targets to be visualized by Coomassie staining, lysine content is important for good staining, and again, lysine content (below 3%, unpublished observation) may not stain well.

Measurement of ³⁵S-Methionine Incorporation by TCA Precipitation and Yield Determination

Using TCA to precipitate labeled protein after synthesis in the presence of ³⁵S-methionine allows the measurement of radiolabel incorporation and provides a means to estimate the amount of protein synthesized in a reaction. When compared to a reaction without template DNA (negative control reaction), the overall efficiency of the protein synthesis reaction is revealed.

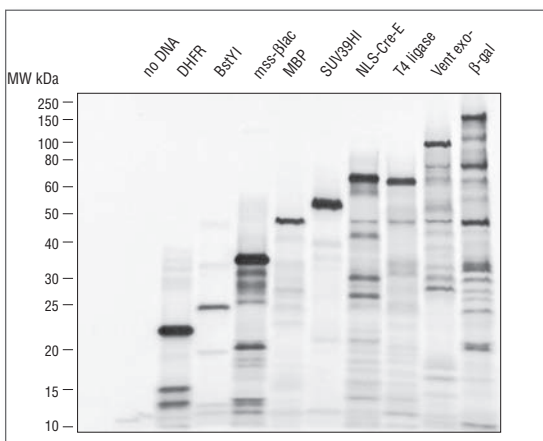
1. Following incubation, mix 5 µl of the labeled PURExpress reaction with 250 µl of 1M NaOH in a glass test tube and incubate at RT for 10 min. NaOH will deacylate all charged tRNA's, including ³⁵S-Met-tRNA, to ensure that all TCA precipitable counts originate from labeled protein.
2. Add 2 ml cold TCA/CAA mix (25% trichloroacetic acid/ 2% casamino acids) to sample and vortex briefly. Incubate on ice for 5 min. Acidifying the solution with TCA will precipitate all the protein.

Figure 3: Protein expression using the PURExpress™ *In Vitro* Protein Synthesis Kit.



25 μ l reactions containing 250 ng template DNA and 20 units RNase Inhibitor were incubated at 37°C for 2 hours. 2.5 μ l of each reaction was analyzed by SDS-PAGE using a 10–20% Tris-glycine gel. The red dot indicates the protein of interest. Marker M is the Protein Ladder (NEB #P7703).

Figure 4: Incorporation of 35 S-methionine enables visualization of protein by autoradiography.



25 μ l reactions containing 250 ng template DNA, 20 units RNase Inhibitor and 2 μ l 35 S-met were incubated at 37°C for 2 hours. 2.5 μ l of each reaction was analyzed by SDS-PAGE, the gel was fixed for 10 minutes, dried for 2 hours at 80°C and exposed to x-ray film for 5 hours at -80°C.

3. Use vacuum filtration to collect the precipitated protein. Pre-wet glass fiber filters with 10% TCA and transfer sample to the filter with vacuum. Rinse the tubes 3X with cold 10% TCA and transfer to the vacuum filter. Wash once with 95% ethanol to dry the filters and prevent quenching.
 - 3a) Alternatively, soak 2.5 cm glass or paper filters in 10% TCA and allow to dry. Spot 20 μ l of the base-treated reaction (step 1) on the filter and transfer to a beaker containing 100 ml ice-cold TCA and incubate w/ swirling for 15 minutes on ice. Repeat wash three times (total), then wash with ethanol and dry.
4. Place dry filters into scintillation vials with 2 ml scintillation fluid.
5. Prepare a control filter to measure the total counts in a labeling reaction. Directly pipet 5 μ l of a reaction onto a dry glass fiber filter and place the filter into scintillation fluid.
6. Measure samples in a scintillation counter. Multiply all values by 5 to determine the counts in a 25 μ l reaction. The TCA precipitated counts is a measure of the efficiency of the labeling and can be represented as a percentage of the total counts by dividing the TCA sample value by the total counts control filter value and multiplying by 100.

Determination of Yield:

Using the equations below, one can calculate the yield of protein synthesized in the reaction. The calculations do not differentiate full-length protein from truncated products and as such, all translation products contribute to the calculation of yield. Prior to using the equations, it is necessary to have determined the number of picomoles of both labeled and unlabeled methionine in the reaction, the number of counts produced by no template (background), target protein (TCA-precipitable) and the total counts in a reaction.

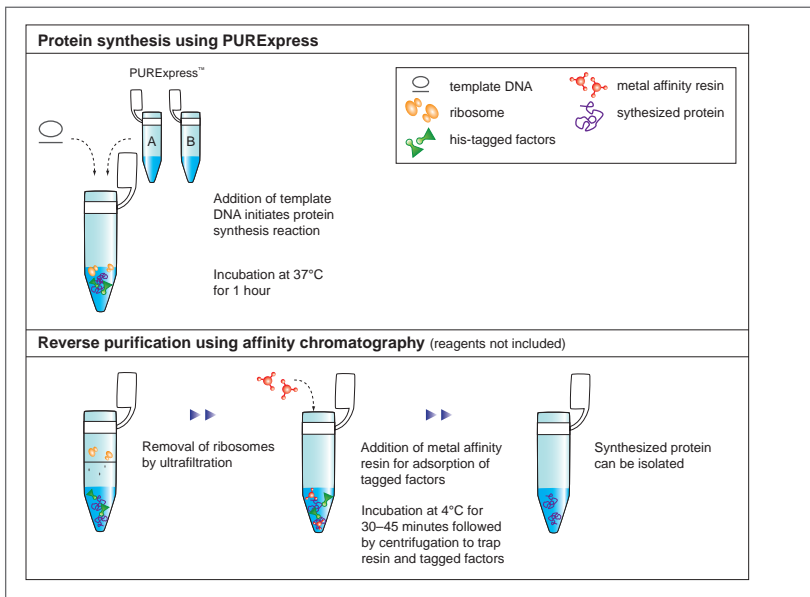
picomoles of Met:

unlabeled	= 0.3 mM in reaction = 300 pmol/ μ l x 25 μ l rxn = 7,500 pmol
labeled	= 2 μ l of ^{35}S -Met (15 mCi/ml, 1,000 Ci/mmol) per rxn = 2 μ l x 15 mCi/ml = 30 μ Ci x μ mol/1 x 10^6 μ Ci = 3×10^{-5} μ mol = 30 pmol
total	= 7,500 pmol unlabeled Met + 30 pmol labeled Met = 7,530 pmol Met
Total counts	= total cpm per 5 μ l control x (reaction volume/5)
Specific Activity	= $\frac{\text{Total counts}}{\text{pmoles methionine (labeled and unlabeled)}}$
Met incorporation (pmoles)	= $\frac{[(\text{TCA ppt cpm} - \text{background cpm}) \times \text{total reaction volume}/5]}{\text{Specific Activity}}$
pmoles of protein	= $\frac{\text{pmoles of incorporated Met}}{\# \text{ of Met residues in target protein}}$
Yield of protein (μ g)	= $\frac{\text{pmoles of protein} \times \text{MW of protein}}{10^6}$

Example of Calculation:

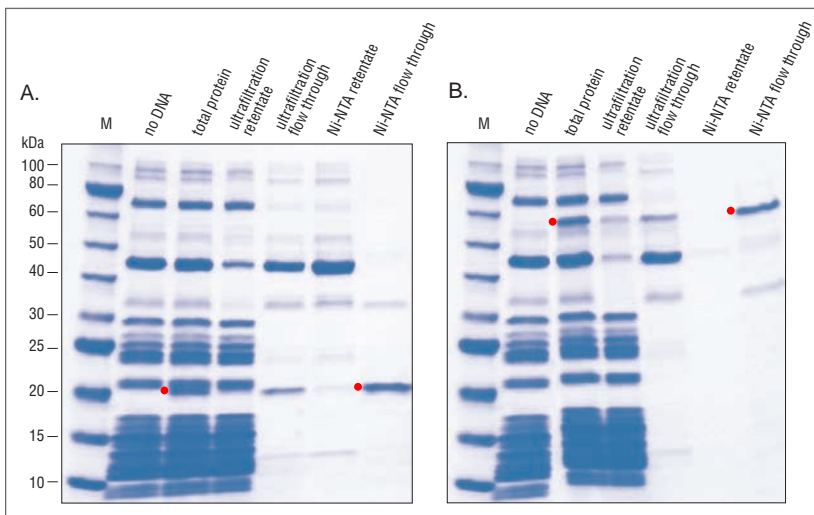
DHFR:	17,998 Daltons, 5 methionine residues
CPM's Measured:	1.12 x 10^7 total, 5 μ l aliquot 1.8 x 10^4 background 1.95 x 10^6 TCA ppt
Total Counts	= 1.12 x 10^7 cpm x 5 = 5.6 x 10^7 cpm/25 μ l rxn
Specific Activity	= $\frac{5.6 \times 10^7 \text{ cpm}}{7530 \text{ pmol}} = 7437 \text{ cpm/pmol}$
Methionine Incorporation	= $\frac{(1.95 \times 10^6 \text{ cpm} - 1.8 \times 10^4 \text{ cpm}) \times 5}{7437}$ = 1297 pmol Met
pmoles DHFR	= $\frac{1297 \text{ pmol Met}}{5 \text{ Met/DHFR}} = 259.4 \text{ pmol DHFR}$
Yield (μ g)	= $\frac{259.4 \times 17,998}{10^6} = 4.67 \mu\text{g}/25 \mu\text{l rxn} \times 40/\text{ml}$ = 187 μ g/ml

Figure 5:



Schematic diagram of protein synthesis and purification by PURExpress.

Figure 6: Expression and reverse purification of DHFR (A) and T4 DNA Ligase (B) using PURExpress.



125 μ l reactions were carried out according to recommendations in accompanying manual. Samples were analyzed on a 10–20% Tris-glycine gel and stained with Coomassie Blue. Note that in both cases, the desired protein can be visualized in the total protein fraction. The red dot indicates the protein of interest. Marker M is the Protein Ladder (NEB #P7703).

Purification of Synthesized Protein using Reverse His-tag Purification

The following protocol is designed to rapidly purify analytical amounts of translated protein from a PURExpress reaction (Figure 5). It requires the target protein be less than 100kDa in molecular weight and not capable of binding to IMAC resin. In practice, proteins less than 60 kDa are more readily purified using this procedure than proteins near the MW cut-off of the spin-column membrane. Additional equipment is necessary and includes: Ni-NTA Agarose (Qiagen), Microcon YM-100 spin concentrators (Millipore). An example is illustrated in Figure 6.

1. Add an equal volume of H₂O to the reaction to increase the volume and make handling of the sample easier.

We recommend a minimum volume of 100 µl after dilution to minimize losses during purification. If the sample cannot be diluted, we suggest a larger reaction volume be used. Use of concentrated NaCl (e.g. 0.8 M) to dilute reaction may help dissociate complexes between the target protein and translation factors. However, the NaCl will remain after the final elution and downstream applications may require microdialysis.

2. Apply the diluted reaction mixture to a Microcon YM-100 spin concentrator (0.5 ml maximum load volume) and centrifuge for 30–60 min at 1,500 x g at 4°C.
3. Transfer the permeate/flow-thru to a new tube, preferably a 2-ml round-bottom microfuge tube with a leak-proof cap.
4. Add 0.25 volumes Ni-NTA Agarose and mix thoroughly for 30–45 min at 4°C to allow His-tagged components to bind the resin.
5. Apply the reaction mixture slurry to an empty Bio-Rad micro-spin column and centrifuge for 2 min at 1500 xg at 4°C.
6. Collect eluate containing purified protein and proceed with experimental analysis.

Troubleshooting:

1. Control protein is not synthesized.

1.1 Kit component(s) inactivated

Storage of all materials at –80°C is required and number of freeze-thaw cycles should be minimized.

1.2 Nuclease contamination

To avoid nuclease contamination, wear gloves and use nuclease-free tips and tubes. We also recommend adding RNase Inhibitor to reactions.

2. Control protein is synthesized, target sample is not present or present in low yield.

2.1 RNase contamination

Commercial mini-prep kits are a useful tool for the preparation of template DNA but are often the source of introducing RNase A to the *in vitro* protein synthesis reaction. See our guidelines for template DNA preparation (Page 3). The inclusion of RNase Inhibitor (NEB #M0314S, 20 units/25 μ l reaction) often overcomes this problem.

2.2 Template DNA design is compromised

Ensure that the sequence of the template DNA is correct. The coding region as well as the regulatory sequences need to be correct and in-frame to ensure that translation is initiated properly and that a full-length product is made. Non-optimal regulatory sequences and/or spacing may adversely affect translational efficiency.

Translation initiation is a key step for successful protein synthesis. Secondary structure or rare codons at the beginning of the mRNA may compromise the initiation process and adversely affect protein synthesis. The addition of a good initiation region (e.g. first ten codons of maltose binding protein) may help, assuming that adding residues to the target sequence can be tolerated. Alternatively, using PCR to modify the 5' end of the target gene can be a successful strategy to eliminate secondary structural elements or rare codons.

2.3 Template DNA is contaminated

Inhibitors of transcription or translation may be present in the DNA. A simple mixing experiment (control DNA + target DNA, compared to control DNA alone) will reveal whether inhibitors are present. Inhibitors in the target DNA will reduce the yield of the control protein. Do not use DNA purified from agarose gels as they often contain inhibitors of translation (e.g. ethidium bromide). Residual SDS from plasmid preparation protocols is another common contaminant and can be removed by phenol:chloroform extraction and ethanol precipitation. When performing ethanol precipitation we recommend the use of sodium acetate rather than ammonium acetate, a known inhibitor of translation. Be careful to remove all traces of ethanol.

Templates produced by PCR need to be free of non-specific amplification products. These contaminants may contain transcription signals and thus compete for and titrate out transcription and/or translation components. As a result, yields may suffer and unwanted truncated products may be produced.

2.4 Template DNA concentration is not optimal.

The concentration of template DNA is important as *in vitro* protein synthesis is a balance of transcription and translation. Too little template reduces the

amount of actively translated mRNA while too much template results in the overproduction of mRNA and the overwhelming of the translational apparatus. We recommend 250 ng of template DNA for a 25 μ l reaction. Optimization with different amounts of template DNA (e.g. 25–250 ng) may improve yield of a particular target protein.

If UV absorbance was used to calculate the concentration of the template DNA, be aware that RNA or chromosomal DNA will also absorb UV light. If your sample has significant amounts of RNA or chromosomal DNA, the actual amount of template DNA may be lower than the calculated amount. The 260 nm/280 nm ratio should be 1.8. Running some of the template DNA on an agarose gel may reveal the presence of other nucleic acids as well as any degradation or incorrect size of the template DNA.

3. Target protein synthesized but full-length product is not major species

3.1 Translation initiation and/or termination not correct

The production of full-length protein requires proper initiation and termination. Internal ribosome entry sites and/or premature termination can produce unwanted truncated proteins. Initiation at non-authentic AUG codons and premature termination are difficult to control. If many rare codons are present or the target has an unusually high percentage of a particular amino acid, supplementation of the “missing” tRNA may help.

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About PURE:

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Ordering Information

PRODUCT	CATALOG #	SIZE
PURExpress™ <i>In Vitro</i> Protein Synthesis Kit	E6800S	10 reactions
COMPANION PRODUCTS		
Murine RNase Inhibitor	M0314S/L	3,000/15,000 units

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e-mail: info@ca.neb.com

China, People's Republic

New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
Fax: 010-82378262
e-mail: info@neb-china.com

Germany

New England Biolabs GmbH
Telephone: +49/(0)69/305 23140
Free Call: 0800/246 5227 (Germany)
Fax +49/(0)69/305 23149
Free Fax: 0800/246 5229 (Germany)
e-mail: info@de.neb.com

Japan

New England Biolabs Japan, Ltd.
Telephone: +81 (0)3 5669 6192
e-mail: info@uk.neb.com

United Kingdom

New England Biolabs (UK), Ltd.
Telephone: (01462) 420616
Call Free: 0800 318486
Fax: (01462) 421057
Fax Free: 08-- 435682
e-mail: info@uk.neb.com

