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20. **What is the recommended number of units of RNase inhibitor (NEB #M0314S) to add for a 25 uL reaction?**

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22. **Are protease inhibitors included in PURExpress? If not, can they be added to the reaction?**

23. **Why does the PURExpress manual state that materials added to the reaction (template DNA, supplements, etc.) should be free of the cations (Mg^{2+} and K^{+})? What specific process in the kit do these components inhibit?**

24. **What are possible reasons for low yields?**

25. **I would like to add rat brain extracts (from newborn and adult animals) to the PURE mixture and would like to be able to differentiate afterwards between newly synthesized protein and protein from the tissue. Therefore, I'd like to label, but would prefer not to use radioactivity. Can I just add a tag to my protein of interest? Can PURExpress be used with modified amino acids?**

ANALYSIS

26. **Do you have any suggestions for performing the DHFR control reaction?**

27. **I observed some additional bands in the DHFR control reaction around 30 kDa, 15 kDa and 12 kDa.**

28. **What is the average efficiency of radioactive ^{35}S-methionine labeling of protein synthesis using PURExpress (ratio of TCA precipitated count to total counts)?**

29. **I am interested in treating my completed PURExpress reaction mix with a DNase to destroy the DNA template. Will the salt concentration in the reaction mix allow such a treatment? Which nuclease would you recommend?**

Downstream Applications

30. **Our protein is ~75kDa, which is, according to the manual, risky for losing the protein on the Amicon Ultra 100k columns. Are there alternative approaches to get rid of the ribosomes? Can we go jut straight into the Ni-NTA purification if we don't get rid of them (recognizing that the ribosomes will continue to be present if we don't get rid of them)?**

31. **I am trying to reverse-purify ribosomal complexes (for ribosome display). I am doing a 25 uL translation, diluting 1:5, using a Amicon Ultracel 0.5 ml-100 k column for ultrafiltration (basically a buffer exchange), and trying to bind up the His-tagged factors with Ni-NTA beads from Qiagen. When I run a protein gel after the ultrafiltration and after incubation with Ni-NTA beads, I still see a series of bands low on the gel, about 30 kDa or less. Are these proteins able to bind Ni-NTA? I am trying to eliminate these proteins from my preparation.**

32. **Is a completed PURExpress reaction with a DNA polymerase as the target compatible with PCR after dilution?**

FAQs for PURExpress®

GENERAL

1. **What are the MW limits of proteins that can be produced by PURExpress?**
   PURExpress reactions have the ability to produce high MW targets due to stabilization of the mRNA template in a low RNase environment. While the upper MW limits of PURExpress have not been clearly defined, a good estimate is that it can produce any protein that *E.coli* can also produce, as the translation apparatus in PURExpress is from *E.coli.* We have produced a protein of 123 kDa in our lab, while we have heard of others (outside NEB) having success with a 150 kDa and 170 kDa targets. These outside claims have not been have verified by NEB.

2. **Are there any chaperones such as GroES, GroEL, and DnaK…..etc added into the solution A or solution B in the kit?**
   At this time, there are no chaperones added in the mix, nor does NEB have any purified chaperones to offer as a supplement. Users can add chaperones (purified by the user or purchased from another vendor) to their reaction but their efficacy will be on a case by case situation. Not all proteins will benefit from chaperone addition, and there are no established rules for determining which substrates need chaperones. This is an area of an active research at NEB.

3. **Is the system capable of dealing with disulfide bonds? If not, can you recommend something else to use post synthesis?**
   The PURExpress Disulfide Bond Enhancer (NEB #E6820S) is available to specifically address this issue.

4. **Has the PURExpress system been tested for membrane protein expression?**
   Membrane proteins are a challenging target for any translation system. PURExpress is based on *E.coli* translation, a system not particularly well suited to expressing membrane proteins. As such NEB has not yet tried to produce membrane proteins with PURExpress. That being said, there are some reports of labs using modified PURE technology to produce membrane proteins. This usually entails the supplementation with liposomes, microsomes, etc. Two such publications are: Murtas et al. (2007) *BBRC.*363(1): 12-17 and Kuruma, Y. et al. (2005) *Biotechnol. Prog.* 21: 1243-1251.

5. **Did Kuruma et al. modify this system to be able to produce membrane proteins?**
   The original formulation (PURESYSTEM, sold by PGI) was used in the Kuruma et al. publication. In this article, the buffer conditions and non-recombinant factors were modified (see methods section of the paper) and the reactions supplemented with

urea-washed inverted vesicles, secretory factors and chaperones. We have not used the PURExpress formulation directly in this type of experiment, but it may be amenable. More recently, Kuruma et al. have published another paper (*Biochimica et Biophysica Acta* 1788 (2009), 567-574) in which they encapsulate PURESYSTEM from PGI and the DNA template in liposomes formed by bath sonication and vortexing in the presence of lipid films. Our understanding is that this publication uses unmodified PURESYSTEM which is nearly identical to PURExpress and thus should be reproducible with PURExpress from NEB.

6. **Which solution (A or B) includes the ribosomes?**
   Solution B.

7. **What is the concentration of ribosomes in PURExpress? Is it possible to add purified ribosomes to the system? Does NEB sell purified ribosomes?**
   The concentration of ribosomes in a standard reaction is approximately 2 uM ±20%. A quantity of 1 mg of ribosomes would be found in 167.7 ul of a PURExpress reaction. The concentration and amount of ribosomes in the PURExpress kit has been optimized for maximum yield of protein translation.
   For protocols where one is interested in using ribosomes from a different source or an amount that varies from the standard protocol, we offer the PURExpress Δ Ribosome kit (NEB #E3313S). We strongly recommend that when using your own ribosomes, you titer the amount needed for optimal protein translation in the PURExpress reaction. For researchers using our ribosomes for ribosome structure and function studies, as a target for drug screening or as starting material for isolation of native ribosomal RNAs (5S, 16S, 23S), we offer E. coli ribosomes (NEB #P0763S*) in a quantity of 1 mg (33.3 mg/ml solution).
   *Note: Although this ribosome preparation is highly active in NEB's PURExpress Protein Synthesis Kit (NEB #E6800) it is intended as a source of ribosomes only. Each pack of P0763S has enough ribosome for approximately 6 E3313S standard PURExpress reactions. For cases where in vitro transcription and translation will be performed exclusively using PURExpress components, the E6800S kit is more cost effective and easier to use. Please refer to the E3313S and E6800S manuals for a complete description of the kit and related product information.

8. **What is the concentration of leucine in the kit buffers? Do you have any experience with 3H Leucine labeling of proteins?**
   The concentration of leucine (and all the other AA's) is approximately 0.3mM (cold) in the final reaction. Most proteins contain more leucine than methionine, so ^{3}H-leu should be incorporated as well. Tritium is a weak isotope but it can be easily detected by TCA precipitation followed by counting the incorporated ^{3}H-leu.

9. **We would like to use the PURExpress system with synthetic tRNAs being aminoacylated by the native synthetases. This means our modified tRNAs will be competing with the tRNAs in the mix to be aminoacylated. Do solutions A and B contain tRNAs that are already aminoacylated? If so, should we first incubate with a DNA that would use up the native aminoacylated tRNAs and then add the message we want to be read with the custom tRNAs?**
   PURExpress uses tRNA from *E.coli* strain MRE 600. In our opinion, aminoacylated tRNA

won't survive the isolation procedure and would not be a stable complex in the kit. We do not think charged tRNA persists in any meaningful amount. Once you mix solution A and B, acylation will start. Pre-incubation with DNA will not help. Synthetic tRNAs will have a hard time competing with the native tRNA pool.

10. Is it possible that there might be UAG suppressor tRNAs present in PURExpress?

PURExpress uses commercial tRNA from *E.coli* MRE 600. We, expect the content of UAG suppressor tRNA to be very low, if present.

11. How many cycles of translation initiation does each ribosome undergo? How much reduction in synthesis should be expected when translating messages that lack a termination codon?

For the control template DHFR, we estimate the ribosome recycled 5 times successfully. We would expect an 80% reduction in synthesis when translating a message without a termination codon. However, yield varies widely with different messages.

12. Which eukaryotic proteins have been successfully produced using PURExpress?

With PURExpress, we have made some eukaryotic proteins including: GFP, Firefly luciferase, GSK, SUV39HI, *Gaussia* luciferase,*Cypridina* luciferase, HAT 2 methylase, and MuLV-RT. There are many more successful reports in the literature.

13. Do you have examples of proteins generated with PURExpress that have shown to be "functional"?

Functional *E.coli* enzymes such as beta-lactamase, beta-galactosidase, and alkaline phosphatase have been made with PURExpress. Additionally, a few eukaryotic enzymes were made by PGI and shown to be functional. At NEB, we have been using PURExpress to express active GFP, firefly luciferase, and when supplemented with isomerases and/or chaperones to produce functional eukaryotic enzymes with complex disulfide bond patterns.

The PURExpress® Disulfide Bond Enhancer (PDBE) (#E6820S) is a proprietary blend of proteins and buffer components designed to correctly fold target proteins with multiple disulfide bonds produced in PURExpress reactions. Added at the beginning of a reaction, the components promote a proper disulfide bond pattern by assisting with the oxidation of cysteine thiols and correcting mis-oxidized substrates. These enhancements can increase the yield of soluble and functionally active protein.

TEMPLATE DESIGN

14. Why do you recommend including a T7 terminator on plasmid templates and also on PCR templates?

For plasmids, the terminator sequence recommendation is to prevent excessive synthesis of mRNA. In a plasmid without a terminator, the T7 RNA polymerase will continue transcribing after the target gene ORF and into other parts of the plasmid backbone.

For a plasmid template, the terminator sequence prevents excessive synthesis of

mRNA. In a plasmid without a terminator, the T7 RNA polymerase will continue transcribing after the target gene ORF and into other parts of the plasmid backbone. A T7 terminator will also stabilize mRNA and is helpful for PCR input templates especially. We strongly recommend including either the T7 terminator (without a stop codon for example). We also advise having the stem loop be very close to the 3' end for maximum mRNA stability as shown.

15. Can cDNA be used as a template in a PURExpress reaction to check for an intact open reading frame?

cDNAs typically do not have the required transcription and translation elements to be successful templates for PURExpress. A T7 promoter, *E.coli* ribosome binding site, and ATG start codon are required.

16. Do expression plasmid templates need to be linearized to work in the PURExpress system?

No, the use of plasmids as templates for PURExpress reactions does not require them to be in a linear form, but they do require a T7 promoter and a ribosome binding site as described in the manual.

17. Is the 25-1000 ng template per reaction recommendation based on the length of DNA to be transcribed rather than the size of the whole plasmid?

This recommendation is based on the size of the whole plasmid. This broad range of template DNA recommendation was made because the optimal amount of DNA varies widely among different targets that have been examined by NEB. There is some correlation to the length of the gene but it is not a linear relationship. In fact, in some cases, it is the reverse. We recommend starting with three different concentrations of DNA, for example, 125, 250, 750 ng, and see which works best. If you need to pick just one amount we recommend starting around 250 ng. If your DNA was prepared using a kit that includes RNaseA, we strongly recommend adding a RNase inhibitor, as described in the manual, or doing a phenol/chloroform extraction to remove the contaminating nuclease that often co-purifies with plasmid in commercial plasmid DNA purification kits.

18. I obtained the expected product in addition to a smaller, secondary product. Also, I believe there is an inhibitor (e.g. EtBr) in my DNA template. While I'm aware that gel purification is not recommended, I'd like to find a way to eliminate the unwanted small PCR product. Are there any alternative staining methods (methylene blue?) that do not effect transcription and translation or ways to remove the EtBr?

EtBr is a known inhibitor of transcription/translation reactions. Gel purification is still possible by running a small fraction of the 2nd rd PCR reaction on the gel as a marker and the majority left unstained. This can be accomplished by not staining the gel with EtBr, but rather by adding a small amount of a dilute EtBr stock directly to the "marker" aliquot, and leaving the remaining sample and gel unstained. After viewing the gel under UV light, the corresponding position in the unstained reaction lane is then excised and purified before setting up the PURExpress reaction.

We have not directly tested methylene blue, so we have no data on its effect on PURExpress reactions.

Another approach would be to optimize the PCR conditions to eliminate the smaller contaminant. Sometimes, higher annealing temperatures, different primer:template ratios, or the use of a different polymerase can be helpful. There is no *a priori* way to know as each template will be amplified differently. Accordingly, NEB chose not to provide more details on PCR, as each lab has their own favorite polymerase/protocol. Yet another strategy may be to just purify the PCR without resolving the smaller contaminant. The recommendation to have a template free of unwanted PCR products is a recommendation given to decrease the likelihood of including DNA sequences that may reduce desired product yield by titrating out important transcription or translation factors. It is possible that in some cases, the smaller product has no effect on product yield.

REACTION CONDITIONS

19. Would small amounts of additional glycerol be tolerated in this system? I am trying to use this system in conjunction with purified chaperones, and the storage buffer for these contains 5-20% glycerol.

You can introduce up to an additional 6% glycerol (final concentration) in the reaction without a noticeable drop-off in performance.

20. What is the recommended number of units of RNase inhibitor (NEB #M0314S) to add for a 25 uL reaction?

We recommend RNase inhibitor be used at a concentration around 1U per ul reaction. This is true for many other RNA related reactions including RT, transcription, RNA labeling etc. RNase inhibitor is supplied at 40U/ul. Adding 0.5 ul RNase inhibitor to each 25ul PURExpress reaction works well in most cases.

21. We will be using PURExpress™ with aminoacylated yeast tRNAphe to form a crosslink with mRNA. We will need to use enough yeast tRNAphe to out compete the native tRNAphe in the kit. Have you any recommendations for the amount of aminoacylated yeast tRNAphe we should begin with? Should we run a poly-Phe message beforehand to use up the tRNAphe that is occupying the elongation factors?

We use total *E.coli* tRNA in PURExpress. Although we are unsure of the exact concentration of each individual tRNA, we estimate it is in the range of 1-5uM. In a typical translation reaction, tRNAs are reused many times, a few dozen times or more depending on translation efficiency. Thus running a poly-Phe reaction beforehand wont "use up" *E.coli* tRNA-phe. So, sensitivity may be the most important issue in your case. You will need to empirically determine how much yeast tRNA-phe is necessary for your experiment.

22. Are protease inhibitors included in PURExpress? If not, can they be added to the reaction?

Protease inhibitors are not part of the PURExpress reaction. RNase/DNase activities are much more damaging than protease for a successful PURExpress reaction. The PURExpress reaction has a very high protein concentration and contaminated DNA or H_2O added by the user is unlikely to have levels of protease that will make a difference in the PURExpress reaction. However, if protease inhibitors are going to be

added, you need to be sure the inhibitors will not inhibit transcription/translation/energy regeneration/amino acylation reactions. The inhibitor(s) should also be DNase/RNase free. Additionally, some inhibitor cocktails have EDTA, which can adversely affect the reaction by chelating the essential magnesium.

23. Why does the PURExpress manual state that materials added to the reaction (template DNA, supplements, etc.) should be free of the cations (Mg^{2+} and K^{+})? What specific process in the kit do these components inhibit?

Mg^{2+} and K^{+} are very important for transcription and translation, particularly Mg^{2+}, and it is optimized in the system. PURExpress can tolerate some additional K^{+} but raising the Mg^{2+} concentration, even a few mM, will compromise the performance. Therefore, we advise users not to introduce extra ions into the system.

24. What are possible reasons for low yields?

A likely cause of low yield is RNase contamination in the template DNA. Different preparations or batches of template often vary in performance due to differences in contamination levels. PURExpress reactions are very sensitive to RNase, so this possibility needs to be checked. If you can't add RNase inhibitor, you must be sure not to add RNase. Phenol extraction is a good method to remove contaminating RNases. For PCR templates, not having a T7 terminator at the 3' end will reduce protein yield (see the first FAQ under Template Design).

A less likely but still possible source of trouble is the method of incubation used for the reaction. They seem to work better when placed in an air incubator for 2 hr at 37°C. This kind of incubator, the same as one would use to incubate media plates used for growing bacterial colonies, produces the best results in our tests. A water bath or thermomixer, is not as consistent, and sometimes lower. Please check this variable as well.

Template concentration is another factor to consider. Template concentration can affect reaction yield. Evaporation of H_2O can effectively concentrate the sample, which can reduce yield.

25. I would like to add rat brain extracts (from newborn and adult animals) to the PURE mixture and would like to be able to differentiate afterwards between newly synthesized protein and protein from the tissue. Therefore, I'd like to label, but would prefer not to use radioactivity. Can I just add a tag to my protein of interest? Can PURExpress be used with modified amino acids?

Addition of tissue extracts to a PURExpress reaction may be difficult. Mammalian tissues have high levels of RNases and direct addition of extracts may degrade target gene transcripts, even in the presence of RNase inhibitor. It would be best to do a small reaction with and without the extract using the control protein, or another gene that is known to work well, to determine whether or not the extract will cause a problem.

An antibody that recognizes a tag could be used for labeling protein (His, FLAG, etc). Modified amino acids will probably not be charged by the cognate tRNA synthetase, and thus not incorporated into newly synthesized protein. Radioactive amino acids will be fine.

ANALYSIS

26. Do you have any suggestions for performing the DHFR control reaction?

One common issue our customers have had is the use of the wrong % acrylamide gel for resolving the reactions. The DHFR protein is quite small (around 20 kD) and will run near the dye front during SDS-PAGE. If the customer chooses a low % gel, they will not be able to resolve the DHFR band from another resident protein. This also happens when people try to run gels too quickly, etc. It is critical to run the gel long enough to resolve this MW region.

27. I observed some additional bands in the DHFR control reaction around 30 kDa, 15 kDa and 12 kDa.

The DHFR plasmid contains the selectable marker beta-lactamase that encodes a product in the 28-30 kDa range. All plasmids contain a marker gene, so any PURExpress reaction with a plasmid template will have the marker ORF as well as the target ORF. The difference is the marker is not regulated by a T7 promoter. Only the target ORF should have a T7 promoter. As T7 RNAP is the only polymerase activity in the reaction, only the target gene should be transcribed. Regarding the additional bands in reactions with the DHFR control template, the explanation depends on how one is analyzing the reaction. On a Coomassie stained gel, the only additional band observed (compared to a reaction with no template DNA) is the desired 20 kDa DHFR target. On autoradiographs of ^{35}S-methionine labeled reactions resolved by SDS-PAGE, we do see additional bands at 12 and 15 kDa. See figure 2 in the product manual. We do not know the origin of these bands. They could be truncated products of the DHFR gene or degradation products from the full-length molecule. The 30 kDa band you mentioned is the same size as beta-lactamase, but it is clearly not present in our reactions with the DHFR control plasmid. For comparison, we do have a modified form of beta-lactamase that is also on our gels in the manual (mss-Blac). It runs a few kDa higher than the beta-lactamase on the plasmid?. Unfortunately, the marker positions on the autoradiograph in figure 2 in the manual are shifted a few kDa higher than their true position. They should be identical to the Coomassie marker, so you can use DHFR (which migrates at 20kDa) to position things.

28. What is the average efficiency of radioactive ^{35}S-methionine labeling of protein synthesis using PURExpress (ratio of TCA precipitated count to total counts)?

The efficiency of incorporation is dependent on templates. For DHFR we see approximately 17% incorporation, others are lower. If you have trouble reading the total counts in your scintillation counter, it might be because the counts are too high and exceed the counter limit (>10 mil counts). This can happen when using 2ul of very fresh ^{35}S-methionine per 25ul reaction and counting 5ul for the total. In our experience, 1ul ^{35}S-met per reaction is sufficient for most proteins or just count 2.5ul for the total counts.

29. I am interested in treating my completed PURExpress reaction mix with a DNase to destroy the DNA template. Will the salt concentration in the reaction mix allow such a treatment? Which nuclease would you recommend?
Commercial wild type DNase I is quite sensitive to salt. It will perform better if you can dilute the reaction with a no salt buffer. Be sure the DNase you use is protease-free. Micrococcal nuclease, will degrade both DNA and RNA and it needs 2mM Ca^{2+} to work.

Downstream Applications

30. Our protein is ~75kDa, which is, according to the manual, risky for losing the protein on the Amicon Ultra 100k columns. Are there alternative approaches to get rid of the ribosomes? Can we go jut straight into the Ni-NTA purification if we don't get rid of them (recognizing that the ribosomes will continue to be present if we don't get rid of them)?
Your 75 kDa protein may or may not be retained by the Amicon Ultra 100K columns. We have not tried to purify proteins produced by PURExpress without also trying to remove the ribosomes. During the product development phase, samples of reactions that had only been incubated with nickel resin were analyzed as part of the purification fractions. Those samples showed an abundance of lower MW proteins. These ribosomal proteins were present in nearly quantitative amounts so we do not think they will be retained at all by the nickel resin and its subsequent removal. Additionally, many proteins, especially all these translation factors, may interact with ribosomes and be co-purified. The easiest way to know for sure is to try it. It's possible to use smaller scale samples that are diluted (to improve flow through the columns) prior to incubation with the nickel resin as long as your downstream application (enzyme assay, western blot, etc) can tolerate the lower concentration of the target protein.

31. I am trying to reverse-purify ribosomal complexes (for ribosome display). I am doing a 25 uL translation, diluting 1:5, using a Amicon Ultracel 0.5 ml-100 k column for ultrafiltration (basically a buffer exchange), and trying to bind up the His-tagged factors with Ni-NTA beads from Qiagen. When I run a protein gel after the ultrafiltration and after incubation with Ni-NTA beads, I still see a series of bands low on the gel, about 30 kDa or less. Are these proteins able to bind Ni-NTA? I am trying to eliminate these proteins from my preparation.
The lower MW bands you refer to may be ribosomal proteins indicating the ribosomes were not removed by ultrafiltration. These proteins are not his-tagged so they cannot be removed by affinity chromatography to nickel-resin. They require the 100kD membrane in the ultrafiltration step to separate them from the target protein. That being said, the reverse purification does not always work. Target proteins near the MWCO are difficult to purify as they often do not pass through the membrane. Additionally, poorly expressed targets are often hard to enrich and ribosomal complexes with nascent proteins may complicate matters. Using salt to disrupt any association of the target with ribosomes can improve the resolution of this technique. In our hands, the method works well when performed as described in the manual. Trying to do the his tag removal first, or other changes to the protocol never

improved the results and often made them worse.

32. Is a completed PURExpress reaction with a DNA polymerase as the target compatible with PCR after dilution?

PURExpress reactions contain high concentrations of many proteins, enzymes, salts, nucleotides, RNAs, etc. Even at 1:100 dilution, these components still exist at a concentration high enough to potentially interfere with a sensitive PCR reaction. PURExpress is optimized for coupled transcription and translation. DNA polymerases may not be compatible with the PURExpress reaction conditions. If this is the case, It may be necessary to clean up the reaction before doing PCR.