Scaling down to scale up – Miniaturizing cell-free protein synthesis reactions with the *Echo 525 Acoustic Liquid Handler*

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INTRODUCTION

The introduction of recombinant protein expression revolutionized the field of biochemistry by offering researchers simplified workflows for protein production (1). Advances in automation offer the potential for a similar paradigm shift; however, alternatives to traditional cell-based expression methods are needed as current methods suffer from limitations in speed, throughput, and automation complexity. These shortcomings can be overcome by synthesizing proteins using cell-free protein synthesis (CFPS) (2,3). CFPS provides a rapid and reproducible alternative to traditional expression methods by circumventing the need to construct plasmids, transform and grow cells, and express and purify proteins. Users simply apply their genetic material directly to standardized synthesis mixtures and go from genes to proteins in a matter of hours instead of days. Furthermore, by divorcing the researcher's interests from cellular fitness requirements, CFPS systems provide new opportunities for toxic protein expression.

In addition to simplifying and accelerating workflows, CFPS components are readily dispensable by automated liquid handling devices, thus enabling high-throughput workflows. To maximize the accessible throughput of two CFPS products from New England Biolabs® (NEB®), here we present an Application Note highlighting the ease and reproducibility in which two CFPS reactions can be miniaturized on the Echo 525 Acoustic Liquid Handler. Using this approach, we show that miniaturization reliably increases the throughput of NEBExpress® and PURExpress® reactions by 50–100X with no discernable loss in activity.

Cell-free Protein Synthesis at NEB

NEBExpress Cell-free *E. coli* Protein Synthesis System (NEB #E5360, Table 1) is a lysate-based product derived from *E. coli* cells engineered for high *in vitro* expression (4,5). It is designed to synthesize analytical amounts of proteins ranging in sizes from 17–230 kDa in 2–4 hours. In addition to the highly active cellular extract, this system includes an optimized T7 RNA polymerase (T7 RNAP), an RNase inhibitor, and a reaction buffer containing all additional cofactors required for protein synthesis (e.g., nucleotides, amino acids). The user-provided genetic material must contain the gene of interest downstream of a T7 RNAP promoter, an appropriately spaced ribosome binding site (RBS), an in-frame start/stop codon, and a downstream T7 RNAP terminator (Figure 1, page 2) (6). The genetic material can

MATERIALS

- NEBExpress Cell-free *E. coli* Protein Synthesis System (NEB #E5360)
- PURExpress *In Vitro* Protein Synthesis Kit (NEB #E6800)
- PURExpress Δ Ribosome Kit (NEB #E3313)
- PURExpress Δ (aa, tRNA) Kit (NEB #E6840)
- PURExpress Δ RF123 Kit (NEB #E6850)
- NEBExpress GamS Nuclease Inhibitor (NEB #P0774)

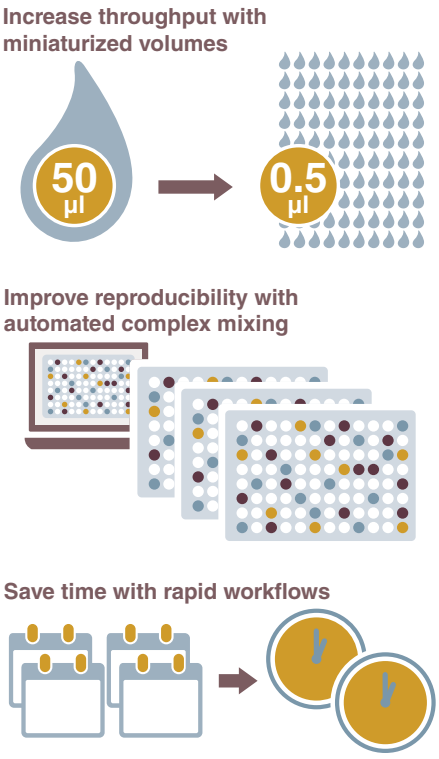
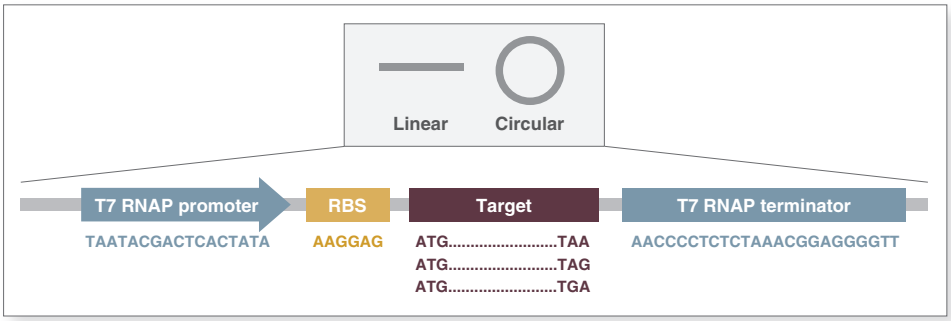


TABLE 1: **Features of NEBExpress and PURExpress**

	NEBEXPRESS (NEB #E5360)	PUREXPRESS (NEB #E6800)
Composition	Cell-extract – components supplied separately: *E. coli* lysate, reaction buffer, T7 RNA polymerase and RNase Inhibitor	Reconstitution – all components purified from *E. coli* and mixed in a multicomponent solution
RNA Polymerase	T7 RNA polymerase system	T7 RNA polymerase system
Components	Not His-tagged	His-tagged
Customization	Not customizable	Customizable
Preferred Substrate	Circular DNA	Linear DNA
Standard Volume	50 µl	25 µl

FIGURE 1: **CFPS template structure**

Linear or circular DNA molecule containing a T7 RNAP promoter, a ribosome-binding site (RBS), an open-reading frame (target) with in-frame start (ATG) and stop codons (TAA, TAG, TGA), and a T7 terminator.



be supplied as circular DNA, linear DNA (e.g., PCR product) or an RNA molecule. Notably, amplification products can serve as templates, thus removing the need for upstream cloning. If linear DNA molecules are provided as templates, it is important to supplement the reaction with the nuclease inhibitor, GamS (NEB #P0774).

PURExpress *In Vitro* Protein Synthesis Kit (NEB #E6800, Table 1, page 1) is a reconstituted system comprising over 26 individually purified *E. coli* proteins (7,8). This system permits *in vitro* expression of proteins without unwanted activities present in lysate products (e.g., nucleases and proteases). It supports reverse purification of the target, as the purified elements supplied in the reaction solution are His-tagged. As with NEBExpress, PURExpress accepts both DNA (linear or circular) and RNA substrates as template for protein synthesis. Additional formulations of PURExpress are available that provide ribosomes (NEB #E3313), tRNAs (NEB #E6840) or releaser factors (NEB #E6850) separately, thus enabling a variety of translational studies or the incorporation of non-natural amino acids.

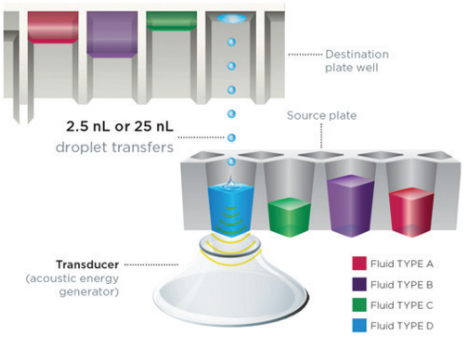
Using the Echo 525 Acoustic Liquid Handler in combination with NEBExpress and PURExpress

High-throughput CFPS studies can be cost prohibitive. One way to reduce cost is to reduce reaction volumes. However, miniaturization is difficult using traditional tip-based liquid handlers, as these devices display poor accuracy and precision when transferring volumes below 0.5 µl. For this reason, we employed the Echo 525 Acoustic Liquid Handler (Figure 2) as its tip-less dispensing mechanism overcomes these difficulties and enables the accurate, precise, and robust transfer of small volumes. The Echo 525 uses a transducer to acoustically transfer reagents from a source plate to an inverted destination plate in 25 nl increments. This tip-less technology allows for complex (i.e., scripted) and rapid transfers from any well between source and destination plates, thus minimizing potential cross-contamination and consumable waste. In this application note, we will demonstrate the utility of NEB's CFPS products – NEBExpress and PURExpress – coupled with the Echo 525.

METHODS

Protein synthesis was measured via fluorescence using a Biotek Synergy Neo2 with YFP settings (Abs 500/20 nm, Emm 541/20 nm). The Bio-Rad HSP3805 was used as the destination plate. Plates were sealed with MicroAmp Optical Adhesive Films, and end-point measurements were taken after incubation at 25°C for ≥ 18 hr. For the NEBExpress reactions, all components except the template and back-fill were mixed and dispensed as a master mix. For PURExpress reactions, Solution A and Solution B were dispensed separately, which enabled retrieval and reuse, effectively minimizing the dead volume of the instrument. Apart from water, all components were transferred from a 384-Well PP 2.0 Microplates (source plate) using the 384PP_Plus_AQ_GPSB fluid class. Water was transferred from an Echo Qualified Reservoir source plate using the

FIGURE 2: **Echo 525 Acoustic Liquid Handler**



Acoustic droplet ejection - The transducer is positioned below the source microplate well and emits focused sound energy repeatably to the meniscus of the fluid to be transferred. A stream of 2.5 or 25 nL droplets (model dependent) is reliably ejected into a well of an inverted destination microplate

TABLE 2: **Miniature CFPS reaction volumes**

	50 µl REACTION	THROUGHPUT VOLUME		
		20X	50X	100X
NEBEXPRESS MASTER MIX				
S30 Synthesis Extract	12 µl	2 µl of Master Mix	0.8 µl of Master Mix	0.4 µl of Master Mix
Protein Synthesis Buffer	25 µl			
T7 RNA Polymerase	1 µl			
RNase Inhibitor, Murine	1 µl			
Nuclease-free Water**	1 µl			
DNA Input (Circular)**	**10 µl**	**0.5 µl**	**0.2 µl**	**0.1 µl**
Total Reaction Volume	**50 µl**	**2.5 µl**	**1.0 µl**	**0.5 µl**

** Substitute GamS for water when using linear DNA input

	25 µl REACTION	THROUGHPUT VOLUME		
		10X	25X	50X
PURExpress MASTER MIX				
Solution A	10 µl	1.00 µl	0.4 µl	0.20 µl
Solution B	7.5 µl	0.75 µl	0.3 µl	0.15 µl
DNA Input (Linear or Circular)	**7.5 µl**	**0.75 µl**	**0.3 µl**	**0.15 µl**
Total Reaction Volume	**25 µl**	**2.5 µl**	**1.0 µl**	**0.5 µl**

6RES_AQ_BP2 fluid class. Notably, all endpoint reactions were diluted to 25 µl with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) prior to fluorescence detection.

The linear or circular plasmid template encoded a fast-maturing, yellow fluorescent protein (vGFP, Venus) optimized for expression in *E. coli* under the T7 promoter/terminator with a pMB1 ori. Plasmid was purified from NEB 5-alpha Competent *E. coli* (NEB #C2988) using Monarch® Plasmid Miniprep Kit (NEB #T1010) and diluted in nuclease-free water prior to dispensing. The linear input was a PCR amplification product from the above plasmid using Q5® Hot Start High-fidelity DNA Polymerase (NEB #M0494) with primers specific to the T7 promoter or T7 terminator. The resulting amplification reaction was purified with the Monarch PCR & DNA Cleanup Kit (NEB #T1030) and diluted in nuclease-free water. When working with small volumes, exceptional care must be taken to maintain appropriate temperature and humidity levels to limit evaporation. For these experiments, the NEBExpress GamS Nuclease Inhibitor (NEB #P0774) was not added but may be added to inhibit Exonuclease V and stabilize linear DNA templates.

To learn more, visit www.neb.com/E5360

RESULTS

Successful CFPS reactions require a correct ratio of components and not a specific volume. Consequently, CFPS reactions can be scaled down, but until now, the reproducibility of small volume transfer has limited our miniaturization attempts. The NEBExpress and PURExpress reactions afford the user-provided DNA input to constitute 20–30% of the total reaction volume. In our hands, the limit for miniaturization of CFPS reactions assembled by hand (i.e., pipette) is 2.5 µl, as the 0.50–0.75 µl allotted for DNA input (Table 2) approaches the lower limit for reproducible, multi-channel transfer. Therefore, we used 2.5 µl reactions to compare the reproducibility and yield of CFPS reactions assembled by hand versus those assembled by the Echo 525. We manually pipetted or acoustically transferred 10 nM of linear DNA template encoding a fast-maturing yellow fluorescent protein and determined end-point fluorescence values for 10 replicates after incubation at 25°C for ≥ 18 hr (Figure 3). The Echo 525 successfully assembled both NEBExpress and PURExpress reactions and produced synthesis levels that were similar to or better than manual pipetting both in terms of total yield and reproducibility (coefficient of variation ≤ 5%).

Having shown the Echo 525 capable of successfully assembling both CFPS mixtures, we next took advantage of the Echo's scripted mixing capabilities to titrate DNA input. We used a linear or circular DNA template (Figure 4, page 4) in 2.5 µl reactions with end-point fluorescence measurements determined after ≥ 18 hr at 25°C. Both the lysate-based NEBExpress and the reconstituted PURExpress systems displayed strong fluorescence values independent of input type, indicative of high synthesis levels. The yield of the PURExpress synthesis reactions displayed a positive response relative to DNA input, with yield saturating at ~2 nM for both linear and circular DNA. The yield of the

FIGURE 3: **Echo-mediated CFPS reactions are reproducible**

End-point fluorescence values for CFPS yields assembled by pipette or the Echo 525 Acoustic Liquid Handler after >18 hrs at 25°C. CV values were ~5% for all conditions tested.

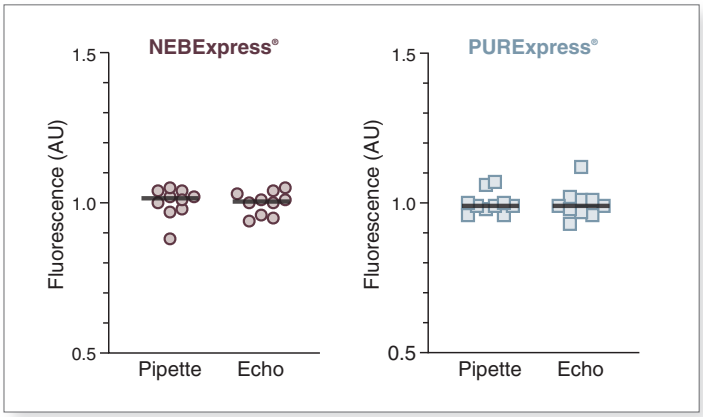


FIGURE 4: **Echo enables rapid and reliable input titration**

Endpoint fluorescence values illustrate influence of input type (linear or circular) and concentration on CFPS yield.

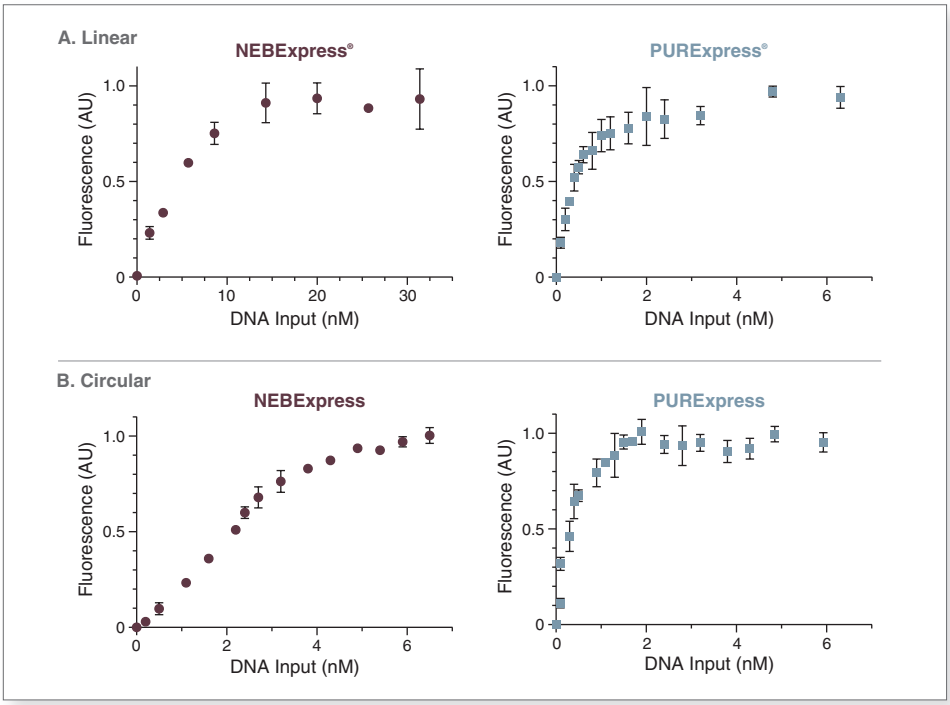
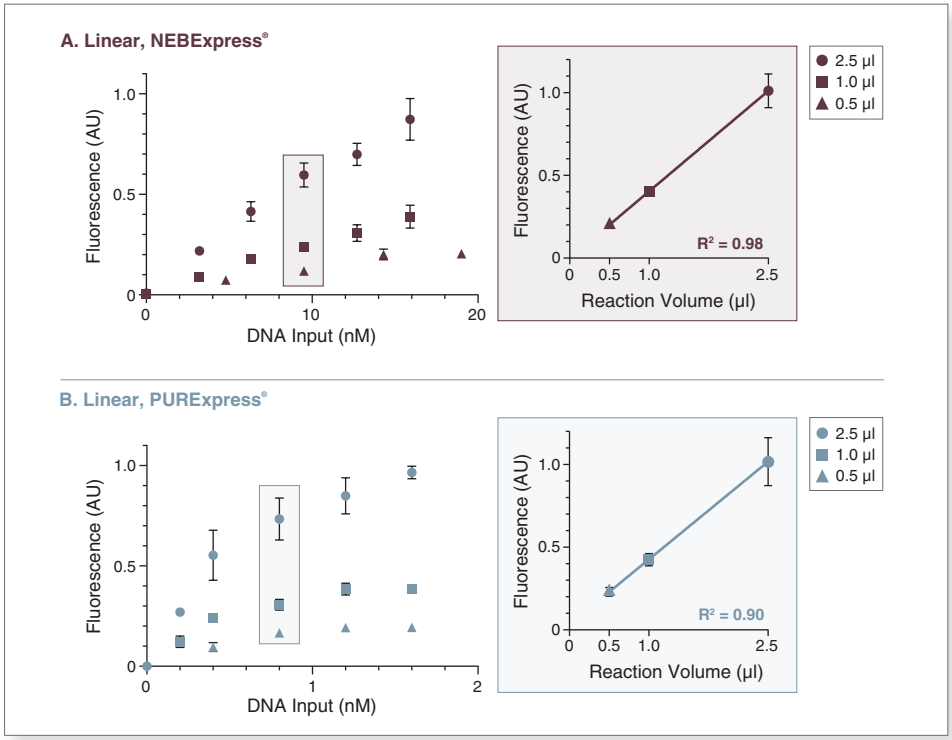


FIGURE 5: **Miniature CFPS reactions with linear input are linearly scalable**

Echo-mediated titration of linear template in three reaction volumes (0.5, 1.0 and 2.5 µl) for NEBExpress (A) and PURExpress (B). Replot of highlighted data (inset) illustrate the linear scalability of yield by volume.

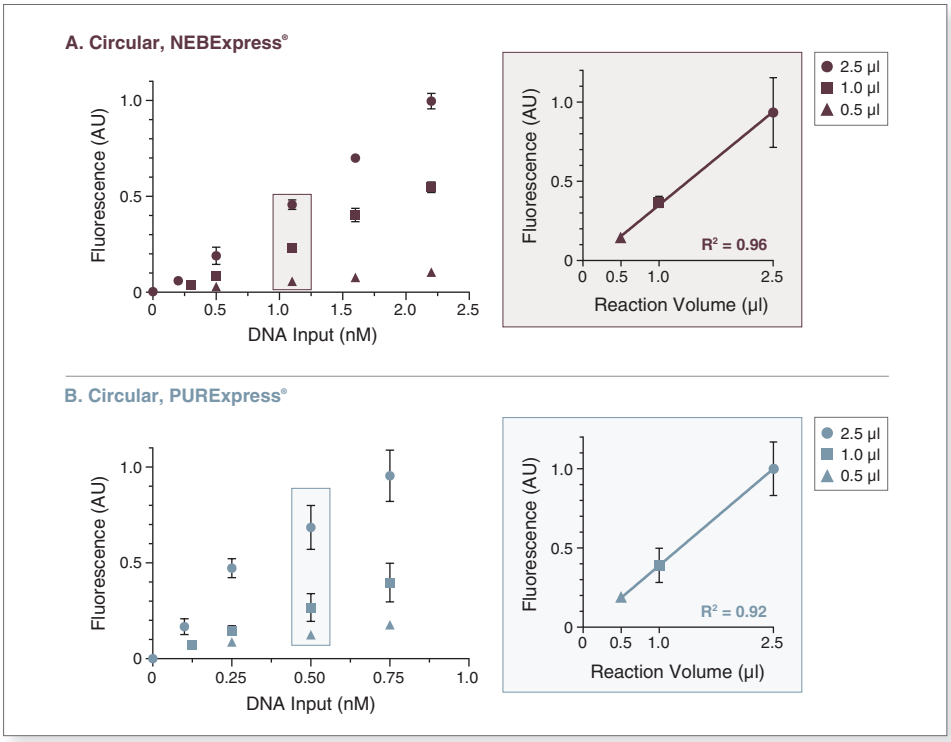


NEBExpress synthesis reactions also displayed a positive response with respect to DNA input, but saturation levels varied between input types, with circular and linear DNA producing maximum yield at ~5 nM input and ~15 nM, respectively. The differences in input response for NEBExpress are consistent with previous results and highlight the reconstituted nature of PURExpress.

We further characterized the miniaturization of the CFPS reactions to determine if reduced reaction volume affected yield. We titrated linear (Figure 5) and plasmid DNA templates (Figure 6) within the linear input ranges determined previously for both NEBExpress and PURExpress in three reaction volumes – 2.5, 1.0 and 0.5 µl. We determined that synthesis levels were positively correlated with reaction volume, with 2.5 µl reactions displaying the highest yield. To better illustrate the relationship between yield and reaction volume, we plotted the highlighted data against the reaction volume. We observed a strong linear relationship for both DNA input types in both CFPS systems (R_2 = 0.90–0.98). Our data are consistent with the notion that a 0.5 µl CFPS reaction produces half as much protein as a 1.0 µl reaction. This finding suggests that yield is linearly scalable with reaction volume, and this attribute is independent of input type. Strikingly, these results also demonstrate the ability to reduce reaction volumes by 50- to 100-fold compared to standard reaction volumes for PURExpress and NEBExpress, respectively, with little to no decrease in effective yield. In addition to successful miniaturization, we maintained a high degree of reproducibility across all volumes, indicating that reduced reaction volume has no significant effect on reproducibility.

FIGURE 6: **Miniature CFPS with circular input are linearly scalable**

(Left) Echo-mediated titration of circular template in three reaction volumes (0.5, 1.0 and 2.5 µl) for NEBExpress (A) and PURExpress (B). (Right) Replot of highlighted data (inset) illustrate the linear scalability of yield by volume.



CONCLUSION

PURExpress and NEBExpress allow researchers to generate active proteins *in vitro* in a rapid and reproducible manner. The performance of these products is robust in small scale reactions that are easily dispensed by Echo Acoustic liquid handlers. This facilitates high-throughput biological analyses in 96- or 384-well, generating more data at a lower cost than manual approaches.

Compare PURExpress to NEBExpress at www.neb.com/PURExpressVsNEBExpress

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