A Streamlined Cell-Free Workflow for On-Demand Protein Expression Using NEBuilder® HiFi DNA Assembly

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INTRODUCTION

NEBuilder® HiFi DNA Assembly is a versatile tool used for seamless cloning and high-fidelity assembly of multiple DNA fragments into a desired construct. It supports applications such as construction of plasmids for recombinant protein expression, sgRNAs, cassettes as linear donors for yeast integration, and site-directed mutagenesis. Due to its high efficiency and accuracy, NEBuilder HiFi can be scaled to assemble many constructs in parallel over a wide range of volumes[1]. Often, these assemblies are used to prototype variants for protein engineering or screening applications.

Traditionally, these assembled expression vectors are introduced into hosts through transformation, verified by sequencing, and subsequently expressed *in vivo*. While reliable, this method can be cumbersome and time-consuming, and many sequences pose difficulties for *in vivo* expression due to toxicity.

NEBExpress Cell-free *E. coli* Protein Synthesis System expresses proteins from DNA sequences completely *in vitro* by providing both transcription and translational components in a single reaction. This system is compatible with various forms of DNA, including plasmid and linear templates. It can be miniaturized to submicroliter volumes for screening[2], or scaled up for automated purification using magnetic particle processors[3].

While convenient, most cell-free protein synthesis (CFPS) systems require significant amounts of DNA, which are not typically available directly from DNA assembly reactions. To overcome this challenge, rolling circle amplification (RCA) can be employed using universal primers to yield high concentrations of DNA (> 1 µg/µl) in just a few hours, providing ample template for CFPS reactions. RCA preferentially amplifies circular DNA, thus enriching only successful ligation products circumventing the need for additional cleanup steps. In this study, we show that NEBuilder HiFi assemblies can be used as templates for phi29-XT RCA, just as they can be used as templates for subsequent PCR amplification.

Here we demonstrate that RCA-amplified NEBuilder HiFi assemblies from unpurified PCR products can be used with the NEBExpress Cell-free *E. coli* Protein Synthesis System to provide a rapid and streamlined workflow for assembling and expressing different target proteins (Figure 1, page 2).

MATERIALS

- Q5® Hot Start High-Fidelity 2X Master Mix (NEB #M0494)
- NEBuilder® HiFi DNA Assembly Master Mix (NEB #E2621)
- phi29-XT RCA Kit (NEB #E1603)
- NEBExpress® Cell-free *E. coli* Protein Synthesis System (NEB #E5360)
- NEBExpress Ni-NTA Magnetic Beads (NEB #S1423)
- 2X IMAC Buffer (NEB #B1076SVIAL, part of NEB #S1423)
- 2M Imidazole (NEB #B1077SVIAL, part of NEB #S1423)
- Blue Protein Loading Dye (NEB #B7703)
- Unstained Protein Standard, Broad Range (10-200 kDa) (NEB #P7717)
- Quick-Load® 1 kb DNA Ladder (NEB #N0468)
- NEBNext® Magnetic Separation Rack (NEB #S1515)
- Thermocycler
- Eppendorf Thermomixer C

Streamlined workflow for rapid protein expression using NEBuilder HiFi DNA Assembly and NEBExpress Cell-free *E. coli* Protein Synthesis System

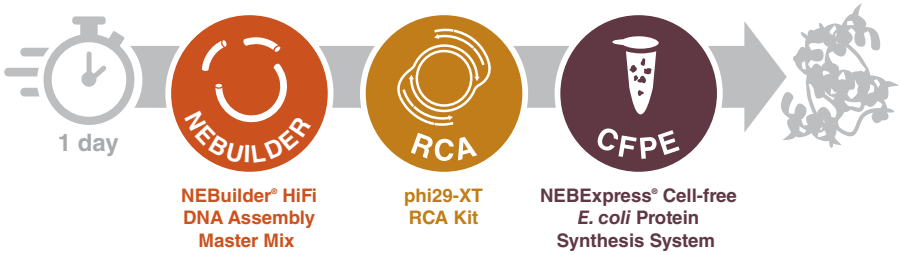
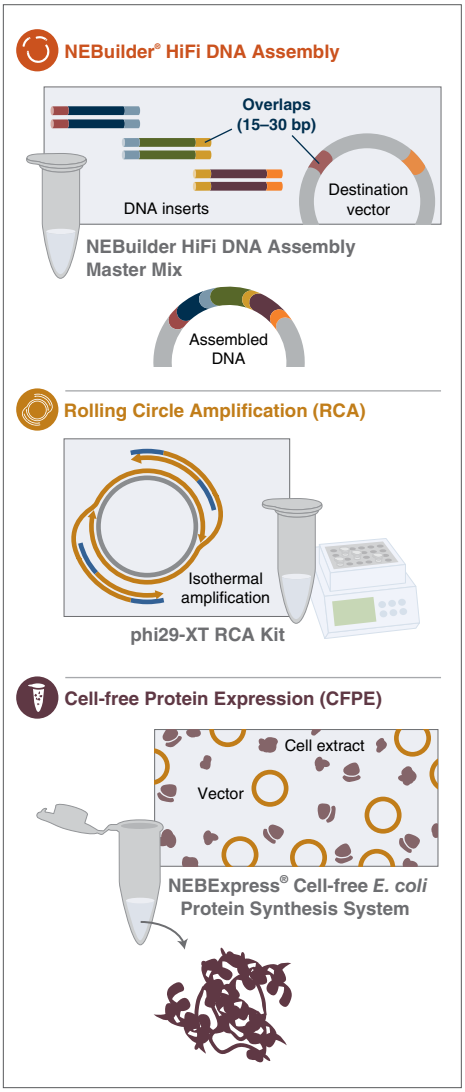


FIGURE 1: **Overview of cell-free cloning and expression workflow**



DNA fragments required for assembly are amplified using a universal PCR cycling protocol with Q5 Hot Start High-Fidelity 2x Master Mix. After PCR amplification of fragments, unpurified DNA is directly assembled into circular vectors using NEBuilder HiFi DNA Assembly. Following assembly, an isothermal rolling-circle amplification reaction using phi29-XT is performed to generate a template used directly in the NEBExpress Cell-free *E. coli* Protein Synthesis System.

METHODS

PCR of DNA fragments using Q5® Hot-Start Master Mix

Fragments for NEBuilder HiFi were generated by PCR using Q5 Hot Start High-Fidelity Master Mix. Primers were designed to create 21 bp overlaps between adjacent fragments so that when assembled, they would yield full expression vectors. Annealing regions of primers were designed to be at least 15 nucleotides long, with a melting temperature of at least 59°C. All annealing regions were predicted to have annealing temperatures of less than 64°C. Each 50 µl reaction contained 0.2 µl of the corresponding sequence-verified template with 0.5 µM of each primer. All PCRs were carried out on the same thermocycler during the same run. Cycling conditions were as follows: an initial denaturation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98°C for 5 seconds, annealing at 59°C for 15 seconds, and extension at 72°C for 2 minutes, followed by a final extension at 72°C for 2 minutes. 5 µl of PCR product was then mixed with 2 µl 6X Gel Loading Dye before analysis by gel electrophoresis.

NEBuilder HiFi DNA Assembly reactions

Following confirmation of robust and specific amplification of the inserts and vector, 5 µl of CutSmart® Buffer and 2 µl of DpnI were added to the remaining 45 µl of PCR. The mixtures were then incubated at 37°C for 30 minutes, followed by heat inactivation at 80°C for 20 minutes. 2-fragment (1 insert + 1 vector), 20 µl NEBuilder HiFi DNA Assembly reactions were set up by mixing 0.5 µl of each unpurified insert with 0.5 µl of amplified vector backbone and adjusting the volume to 10 µl with water (total of 1 µl in 20 µl reaction, 5% v/v). 10 µl of NEBuilder HiFi DNA Assembly Master Mix was then added, mixed, and the reaction was incubated at 50°C for 30 minutes. Note that designs incorporating additional insert fragments can be efficiently assembled using NEBuilder HiFi, with the optimal number of total DNA fragments ranging from 2 to 6.

phi29-XT rolling circle amplification of assemblies

After assembly, 2 µl of NEBuilder HiFi reaction was mixed with 7 µl water, 2 µl 10 mM dNTPs, 4 µl of phi29-XT 5X Reaction Buffer, 2 µl of 500 µM Exonuclease-Resistant Random Primers, and 2 µl phi29-XT DNA Polymerase. The reaction was incubated for 3 hours at 42°C before heat inactivation at 65°C for 10 minutes. To verify assembly fidelity, the RCA reactions were vortexed, and 1 µl was combined with 7 µl water, 1 µl NEBuffer™ r2.1, and 1 µl BsrDI. The mixture was incubated for 30 minutes at 37°C, followed by heat inactivation at 80°C. The mixture was then combined with 2 µl Gel Loading Dye and analyzed by agarose gel electrophoresis.

NEBExpress Cell-free *E. coli* Protein Synthesis using RCA products

Small test expressions using 5% v/v of RCA product in CFPS were conducted by mixing 1 µl 2-fold diluted RCA product with 0.2 µl T7 RNAP, 0.2 µl RNase Inhibitor, 5 µl Protein Synthesis buffer, 2.4 µl NEBExpress S30 Synthesis Extract, and water to a final volume of 10 µl. We have found that adding more than 10% RCA products (v/v) to a CFPS reaction can become inhibitory. Reactions were incubated for 16 hours at 30°C in capped PCR tubes on a thermocycler with the lid set to 30°C. Large reactions were directly scaled to 100 µl and conducted in 1.5 ml Eppendorf® DNA LoBind tubes in an Eppendorf Thermomixer with a heated lid at 30°C and shaking at 1200 rpm for 16 hours. To assess expression, 2 µl of the reaction was mixed with 10 µl water and 6 µl Blue Protein Loading Dye prior to boiling for 5 minutes, followed by analysis by SDS-PAGE.

Protein purification using NEBExpress Ni-NTA Magnetic beads

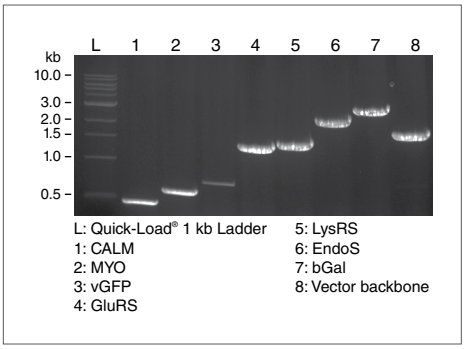
50 µl of NEBExpress Ni-NTA magnetic beads were washed twice and resuspended in 100 µl 2X IMAC buffer (20 mM sodium phosphate, 300 mM NaCl, pH 7.4) containing 20 mM imidazole in a PCR tube. 100 µl of NEBExpress Cell-free reaction was transferred to the beads and vortexed every 2 minutes for 10 minutes to allow binding. The tubes were pulse spun before being placed on a NEBNext Magnetic Separation Rack. The flow-through was collected, and the beads were washed twice, resuspending them in 150 µl IMAC buffer supplemented with 10 mM imidazole. The beads were then eluted with 50 µl IMAC buffer containing 500 mM imidazole and analyzed by SDS-PAGE. For higher throughput, this type of magnetic bead purification strategy is amenable to automation via magnetic particle processors or decks with magnetic modules.

RESULTS

Universal PCR cycling conditions can be used with Q5 Hot-Start 2x Master Mix to amplify a variety of sequences

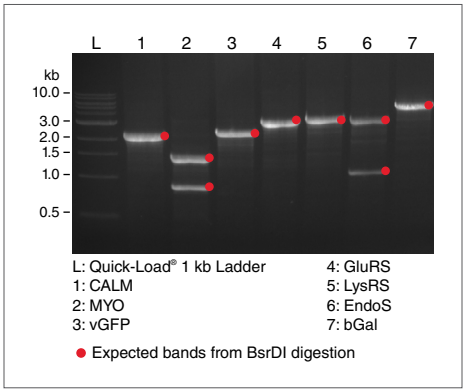
When constructing a large variety of DNA sequences, it can be challenging to accommodate the various annealing temperatures and extension times required for each amplicon. Fortuitously, when working with sequences destined for expression in cell-free protein synthesis, these are usually codon optimized and have balanced GC content. By designing primers with annealing ranges within 5°C, a common annealing temperature can be used that matches the lowest temperature of the set. To accommodate a variety of amplicon lengths, the extension temperature can be set to meet the expected time required for the longest amplicon. In Figure 2, we show that various amplicons can be obtained using a single PCR protocol due to the robustness of Q5 and its ability to handle a wide range of GC content and disparate melting temperatures. In addition to the robustness provided by Q5, the Hot-Start technology allows users to take their time setting up reactions at room temperature without the risk of off-target product formation.

FIGURE 2: **Robust amplification of diverse templates under universal PCR conditions.**



Q5 Hot Start High-Fidelity Master Mix and a single thermocycling protocol (59°C annealing, 2 min extension) were used to amplify the vector and a variety of inserts from different sources across a range of lengths from 486 to 3126 bp with annealing temperatures ranging from 59 to 64°C.

FIGURE 3: **phi29-XT RCA reactions yield high amounts of specific products from NEBuilder HiFi reactions**



After NEBuilder HiFi assembly using unpurified DNA inputs (total of 1 µl in 20 µl reaction, 5% v/v), 20 µl RCA reactions with phi29-XT are templated with 2 µl of assembly reaction (10% v/v). Following amplification, 1 µl of the RCA reaction was digested with BsrDI and visualized via gel electrophoresis to verify that the RCA reactions yielded the expected products.

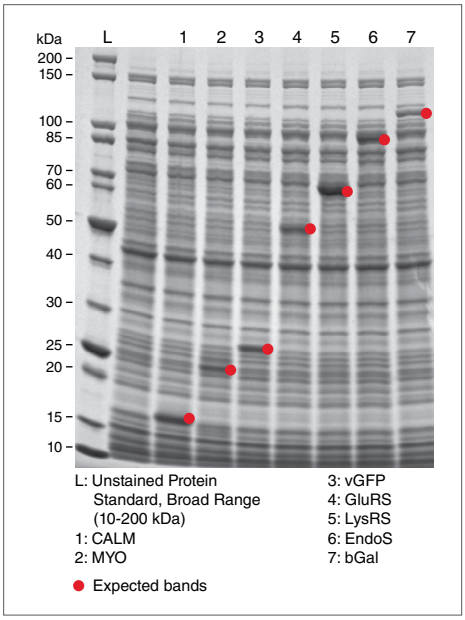
RCA can efficiently replicate NEBuilder HiFi DNA Assembly reactions

NEBuilder HiFi achieves assembly through *in vitro* recombination using a 5′ exonuclease activity to expose homologous regions. Cross-annealing of these regions, followed by extension and nick ligation produces assembled DNA. While quantification and molar normalization of DNA inputs may increase assembly efficiency, it is not always necessary. NEBuilder HiFi reactions can use unpurified DpnI-treated Q5 PCR products as inputs as long as they make up less than 20% of the total reaction volume[4], which streamlines assembly workflows. Note that larger volumes of unpurified PCR products can significantly inhibit both assembly and transformation. In such cases, it is recommended to purify the PCR products using Monarch® DNA purification kits. Following a 30-minute assembly, this reaction can be used directly as a template with the phi29-XT RCA kit, as only successfully ligated, closed-circle DNA molecules serve as a as only successfully ligated, closed-circle DNA molecules serve as efficient RCA templates. The reaction is amplified in 3 hours to increase template concentration by more than 300-fold. When a portion of the RCA product was analyzed by restriction digest, only bands corresponding to the desired product were observed (Figure 3), showing that both the NEBuilder HiFi assembly and RCA amplification produced high-quality assembled and amplified DNA.

RCA products can be used directly in the NEBExpress Cell-free *E. coli* Protein Synthesis system

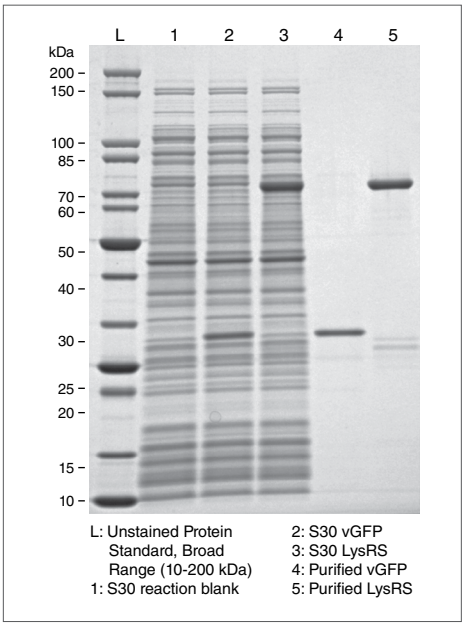
NEBExpress Cell-free *E. coli* Protein Synthesis system is compatible with both plasmid and linear DNA inputs, as well as RCA products. Following amplification, the RCA products can be used directly without digestion or purification in NEBExpress Cell-free *E. coli* protein synthesis reactions for protein expression. Figure 4 illustrates a wide range of protein sizes that are expressed using this simple and convenient workflow. Following expression, proteins can be purified with several affinity metal chromatography products available from NEB, including Ni resin, spin columns, and magnetic beads. Purification can also be automated as shown in our other application notes[3]. Purification of these products shows high fidelity construction and robust expression of these constructs at the expected molecular weights (Figure 5, page 3). The approach described here is also compatible with other affinity tags, expanding the utility of this workflow for a range of protein designs.

FIGURE 4: **RCA reactions successfully template NEBExpress CFPS of diverse targets**



Small test reactions of 10 µl total volume were supplemented with 0.5 µl of unpurified RCA products (5% v/v) to serve as templates, and expression proceeded overnight at 30°C. The expressed proteins were observed at the expected molecular weights: CALM (16.7 kDa), Myokinase (25.5 kDa), vGFP (26.9 kDa), GluRS (54.9 kDa), LysRS (75.0 kDa), EndoS (89.0 kDa), and β-gal (100.0 kDa).

FIGURE 5: **Rapid Ni-NTA purification following cell-free cloning and expression yields high purity and concentration**



Purification demonstrates high-fidelity construction, robust amplification and efficient expression. The purified proteins were observed at the expected molecular weights: vGFP (26.9 kDa) and LysRS (75.0 kDa).

CONCLUSIONS

Here we present a quick workflow spanning DNA assembly to protein purification using Q5 Hot-Start 2x Master Mix, NEBuilder HiFi DNA Assembly, phi29-XT RCA kit, and NEBExpress Cell-free *E. coli* Protein Synthesis System. When speed and convenience are the main priorities, this workflow can replace traditional, low-efficiency processes that require several days and microbial cultivation. We demonstrate that leveraging New England Biolabs' high-efficiency cloning tools, such as NEBuilder HiFi DNA Assembly and NEBridge Golden Gate Assembly, DNA can be directly amplified and subsequently used for expression in cell-free protein synthesis systems[5].

These workflows allow for rapid and scalable screening suitable for applications in synthetic biology and protein engineering, as outlined in our application note: *Scaling down to scale up – Miniaturizing cell-free protein synthesis reactions with the Echo 525 Acoustic Liquid Handler*[2]. Additionally, these protocols can also be coupled with automated purification as shown in *Automated Cell-free Protein Expression and Purification for High-Throughput Screening using NEBExpress® Cell-free E. coli Protein Synthesis System and NEBExpress Ni-NTA Magnetic Beads*[3]. For examples of a similar workflow using NEBridge Golden Gate, see our application note titled *Accelerating DNA Construction to Protein Expression: A Rapid 1-Day Workflow Using NEBridge® Golden Gate Assembly.*

Learn more about NEBuilder HiFi DNA Assembly at www.NEBuilderHiFi.com

For more information about the phi29-XT RCA Kit, please visit www.neb.com/E1603

Learn more about protein expression at NEB at www.neb.com/proteinexpression

References

1. Bailey, J.B. et al. (2018) Application Note: *Nanoliter Scale DNA Assembly Utilizing the NEBuilder HiFi Cloning Kit with the Labcyte® Echo® 525 Liquid Handler.* Labcyte Inc.
2. Buss, J.A. et al. (2023) Application Note: Scaling down to scale up – *Miniaturizing cell-free protein synthesis reactions with the Echo 525 Acoustic Liquid Handler.* New England Biolabs, Inc.
3. Buss, J.A. et al. (2024) Application Note: *Automated Cell-free Protein Expression and Purification for High-Throughput Screening using NEBExpress® Cell-free E. coli Protein Synthesis System and NEBExpress Ni-NTA Magnetic Beads.* New England Biolabs, Inc.
4. *NEBuilder® HiFi DNA Assembly Master Mix/ NEBuilder HiFi DNA Assembly Cloning Kit.* Instruction Manual. New England Biolabs, Inc.
5. Lund, S. et al. (2023) Application Note: *Accelerating DNA construction to protein expression: A rapid 1-Day workflow using NEBridge Golden Gate Assembly.* New England Biolabs, Inc.