Accelerating DNA Construction to Protein Expression: A Rapid 1-Day Workflow Using NEBridge® Golden Gate Assembly

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INTRODUCTION

Molecular biology tools enable the custom generation of proteins with complete control of sequence, purification tags, secretion signals, and other performance characteristics. While the breadth of tools allows researchers to create their desired protein, this process often involves a low-throughput and time-consuming, multi-day workflow using live cells. To overcome these limitations, we have demonstrated a completely *in vitro* workflow that combines Golden Gate DNA Assembly, rolling circle amplification (RCA), and cell-free protein expression (CFPE) to rapidly screen the impact of multiple protein designs simultaneously (Figure 1, page 2). This workflow enables researchers to generate an array of protein variants in as little as a single day using a basic set of custom DNA vectors or insertion fragments. It also provides a means to assess the engineering constraints that are unique to each protein of interest and allows for the rapid identification of soluble protein.

Golden Gate Assembly utilizes Type IIS restriction enzymes to cleave DNA in a manner that permits scarless assembly in a modular fashion, thereby facilitating the rapid creation of protein variant libraries. Efficient assemblies from low complexity (2 fragments) to high complexity assemblies (7–50+ fragments) are supported by NEB's optimized NEBridge reagents and accompanying protocols (1, 2). While Golden Gate Assembly has been widely used for over a decade, recent computational advances (3) by NEB have made this DNA assembly approach more robust and user-friendly, thereby enabling the creation of simplified workflows with a high probability of generating the desired DNA construct after a single round of assembly. As such, the workflow demonstrated here can be leveraged to generate many variants of any desired protein with minimal customization of DNA sequences.

While Golden Gate Assembly provides a standardized and efficient approach for gene assembly, RCA provides an ideal method to rapidly amplify assembled genes. RCA is a robust and highly sensitive isothermal amplification approach that continuously amplifies circular DNA, resulting in a high yield without specific PCR primers. Two-hour amplification times are achieved by employing a new phi29 polymerase engineered by NEB called phi29-XT. This polymerase generates more DNA product in a shorter amount of time versus wild-type phi29 polymerase, while sharing key qualities that are ideal for RCA applications, including high processivity, strong strand displacement activity, and high fidelity. phi29-XT polymerase is also more thermostable, with an optimal reaction temperature of 42°C, and has improved sensitivity, supporting amplification down to 1 fg of DNA input (4).

This speed advantage over PCR allows faster workflows while generating ample amounts of template that are amenable to cell-free protein expression reactions such as the NEBExpress Cell-free *E. coli* Protein Synthesis System. The NEBExpress Cell-free *E. coli* Protein Synthesis System is an extract-based, transcription/translation (TXTL) system derived from *E. coli* cells engineered for high *in vitro* synthesis performance. It is designed to synthesize proteins ranging in size from 17–230 kDa in just 2–4 hours under the control of T7 RNA Polymerase (5).

MATERIALS

- NEBridge Ligase Master Mix (NEB #M1100)
- BsaI-HF®v2 Type IIS restriction enzyme (NEB #R3733)
- Q5® Hot Start High-Fidelity 2X Master Mix (NEB #M0494)
- phi29-XT RCA Kit (NEB #E1603)
- NEBExpress® Cell-free *E. coli* Protein Synthesis System (NEB #E5360)
- NEBExpress Ni-NTA Magnetic Beads (NEB #S1423)
- SNAP-Capture Magnetic Beads (NEB #S9145)
- Monarch® DNA Gel Purification Kit (5 µg) (NEB #T1020)

Rapid expression of proteins using NEBridge® Golden Gate Assembly and NEBExpress Cell-free *E. coli* Protein Synthesis System

NEBridge® Golden Gate Assembly Kit | phi29-XT RCA Kit | NEBExpress® Cell-free *E. coli* Protein Synthesis System

This system removes the need for costly and time-consuming steps of live-cell transformation and screening (6). Together, the use of Golden Gate Assembly, RCA, and cell-free protein synthesis provides a valuable tool to maximize laboratory productivity, especially when combined with automation, as high-throughput screening has become increasingly prevalent in research labs.

METHODS

Gene coding sequences were split into three fragments using NEBridge SplitSet® tool, and fragments were ordered as dsDNA fragments (gBlocks®, Integrated DNA Technologies) with Golden Gate sites appended to the 5´ and 3´ ends. PCR and clean-up were carried out to ensure the integrity and purity of the DNA fragments. dsDNA fragments were resuspended to 10 ng/µl, and 1 ng was used as a template in a 50 µl PCR reaction using Q5 Hot Start High-Fidelity 2X Master Mix (NEB #M0494). Amplification specificity was verified by agarose gel electrophoresis. The PCR amplicons were then purified using Monarch DNA Gel Extraction kit (NEB #T1020) and eluted into 25 µl. For higher purity protein, users should consider using clonal sequence-verified templates and switching the RCA reaction with PCR amplification by Q5 High-Fidelity DNA Polymerase, followed by PCR clean-up.

PCR products (25 fmol each) and vector (25 fmol) were used in a 10 µl reaction using BsaI-HFv2 (NEB #R3733) with NEBridge Ligase Master Mix (NEB #M1100) according to the online protocol. The reaction was allowed to proceed with 30 cycles of 1 minute at 37°C followed by 1 minute at 16°C and a final 5 minutes hold at 60°C.

Golden Gate Assembly reaction mix (1 µl) was directly used in a 10 µl phi29-XT reaction with 0.5X LAMP fluorescent dye (NEB #B1700). The reaction was allowed to proceed at 42°C with fluorescence monitoring on a Bio-Rad® C1000 qPCR instrument every minute on the FAM channel. After 2 hours, the reaction was heated to 65°C for 10 minutes to heat inactivate the polymerase. It is important to set up a control phi29-XT amplification without template as RCA materials can easily become contaminated. The use of filter tips is an absolute requirement when setting up RCA reactions. Caution must be exercised when handling phi29-XT reagents and products to minimize cross-contamination of reagents and pipettes.

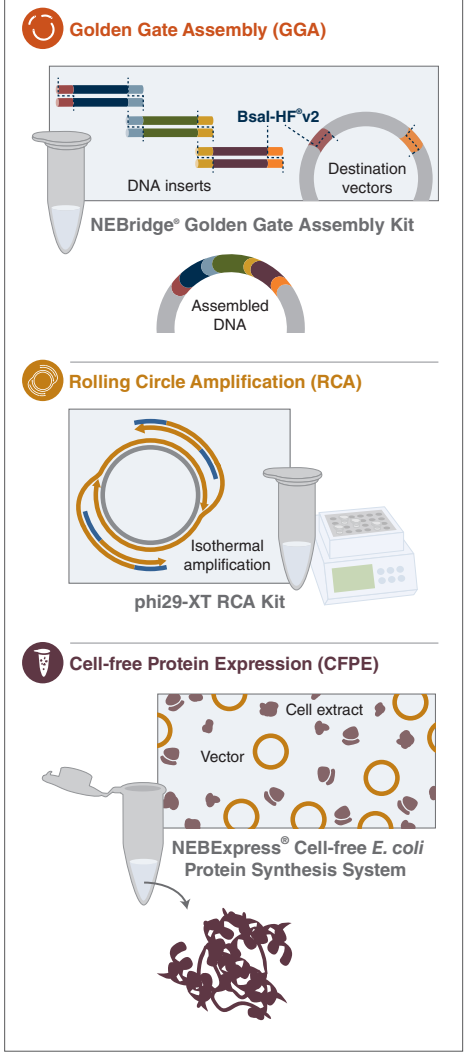
In a separate reaction, 1 µl of Golden Gate Assembly reaction mix was used in a 10 µl phi29-XT reaction without dye in a 1.5 ml tube and incubated on an Eppendorf® Thermomixer® at 42°C, 0 rpm, for 2 hours. The temperature was then raised to 65°C for 10 minutes.

Cell-free expression was carried out using the entire phi29-XT-based amplification product in a reaction volume of 200 µl with the NEBExpress Cell-free *E. coli* Protein Synthesis System (NEB #E5360) in the same 1.5 ml tube. The reaction was carried out at 25°C with shaking on an Eppendorf Thermomixer at 2000 rpm overnight (14 hours). Protein expression can be visualized by loading 2 µl of the reaction with 10 µl water and 6 µl 3X Blue Protein Loading Dye (NEB #B7703S) onto an SDS-PAGE gel. In the event your protein is not expressed, consider digesting a portion of the RCA product to visualize amplification quality.

Next, 50 µl of NEBExpress Ni-NTA Magnetic Beads (NEB #S1423) were washed twice with 200 µl 1X IMAC buffer, and the washes removed. Cell-free expression reactions (100 µl) corresponding to the N- and C-terminally His_6-tagged constructs were diluted with 100 µl 2X IMAC buffer and added to the washed beads. The resulting mixture was resuspended repeatedly for 30 minutes allowing the target proteins to bind. The mixture was applied to an external magnet and the supernatant removed. The beads were washed twice using resuspension with 500 µl 1X IMAC buffer supplemented with 20 mM imidazole. The beads were then eluted in 50 µl 1X IMAC buffer supplemented with 500 mM Imidazole.

To affix the protein to a bead, 50 µl of SNAP-Capture Magnetic Beads (NEB #S9145) were spun down and washed with 100 µl 1X IMAC buffer. The supernatant was removed and 50 µl of the SNAP-tagged constructs were added to the beads with 50 µl of 2X IMAC buffer. DTT was added to 12.5 mM. The mixture was mixed at room temperature for 30 minutes on a thermomixer before sitting overnight on the benchtop. The following day the beads were spun down and washed 3 times with 100 µl 1X IMAC buffer with vortexing between washes. The beads were then resuspended in 100 µl 1X IMAC buffer.

FIGURE 1: **Overview of Workflow**



DNA parts are assembled using NEBridge Golden Gate Assembly before acting as template for a phi29-XT RCA reaction. The amplification product can then be used in a cell-free protein synthesis reaction from which protein can be purified. The entire workflow is completed *in vitro*.

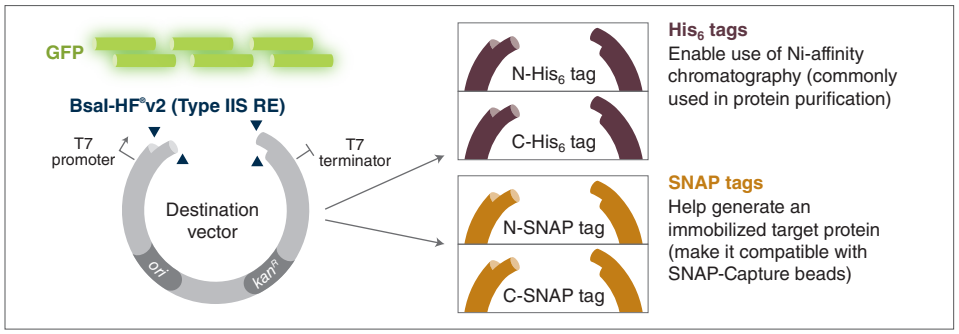
RESULTS

DNA constructs were rapidly generated and expressed in a 24-hour, *in vitro* workflow. Assemblies were constructed using 3 insert fragments to further demonstrate the utility and efficiency of Golden Gate Assembly with modular systems. The three-fragment assemblies were constructed into expression vectors that were subjected immediately to RCA to generate templates for cell-free expression. After assembly construction, the entire workflow was conducted on a Thermomixer providing support that such workflows could be scaled and automated. By using Golden Gate Assembly with standardized vectors, multiple assemblies could be performed in parallel using the same DNA inputs with different accepting vectors. This plug-and-play methodology enables rapid screening of various expression constructs without the need to order custom DNA for each expression. By cloning multiple constructs at once, researchers investigating a single target or multiple targets in a molecular foundry can rapidly construct, express, and assay their target proteins. Here we show a rapid workflow expressing tagged constructs with minimal perturbance to native sequences from amplification through protein purification.

NEBridge Ligase Master Mix performs high-efficiency and high-fidelity Golden Gate Assembly with a broad assortment of NEB Type IIS restriction enzymes. NEB has optimized the conditions that allow for convenient, efficient, and accurate Golden Gate Assembly; users need only choose their preferred NEB Type IIS restriction enzyme and add DNA substrates to be assembled. By using NEBridge Ligase Master Mix, minimal ligation sequences of 4 bp can be added to 5′ and 3′ ends of inserts, thereby facilitating the use of common overhangs between destination vectors adding different functionality. If homologous sequence cloning methods were used, adding approximately 6 amino acids to each N- and C-terminus would be required, which could greatly disrupt the protein sequence to be investigated.

Four destination vectors were designed: N-terminal His_6, C-terminal His_6, N-terminal SNAP, and C-terminal SNAP (Figure 2). The His_6 tags enable the use of Ni-affinity chromatography commonly used in protein purification. The SNAP tags functionalize the target protein with a fusion that is compatible with NEB SNAP-Capture beads to generate an immobilized protein through a covalent linkage in the presence of a reducing agent. Using these two systems, we aimed to show that this construction-to-expression workflow is compatible with both soluble protein purification as well as immobilized products.

FIGURE 2: **Vector and construct design using Golden Gate Assembly**

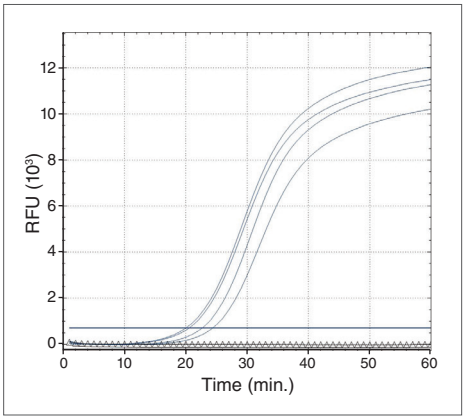


NEBridge Golden Gate Assembly allows for plug and play assembly of multiple vector constructs. The GFP protein is seamlessly cloned and assembled from 3 fragments into the vector containing either N- or C-terminal His tags, or N- or C-terminal SNAP tags, including the necessary T7 promoter, RBS, T7 RNAP terminator sequences, origin of replication and antibiotic resistance marker.

Destination vectors containing an origin and an antibiotic marker were designed to utilize the same overhangs when treated with the Type IIS restriction enzyme, BsaI-HFv2 (Figure 2). The multiple cloning sites were all flanked with a T7 promoter sequence upstream and a T7 terminator sequence downstream. The 5′ overhang was designed as "ACCA" where "A" represents the start codon of the ORF being investigated. This tag adds an additional threonine between any N-terminal tag and the investigated ORF. When using destination vectors not encoding an N-terminal tag, the "ACC" in the overhang is part of the spacer sequence between the ribosomal binding site and the coding sequence (CDS), generating a seamless CDS without an N-terminal addition. The 3′ overhang is "ATTC" wherein the stop codon is not included. The C-terminus has an isoleucine followed by a leucine ("CTG"). For vectors generating a C-terminal tag, the tag sequence immediately follows this isoleucine-leucine (IL) linker. If the vector does not encode a C-terminal tag, a stop codon (*) follows the IL linker sequence adding a total C-terminal linker of: IL*.

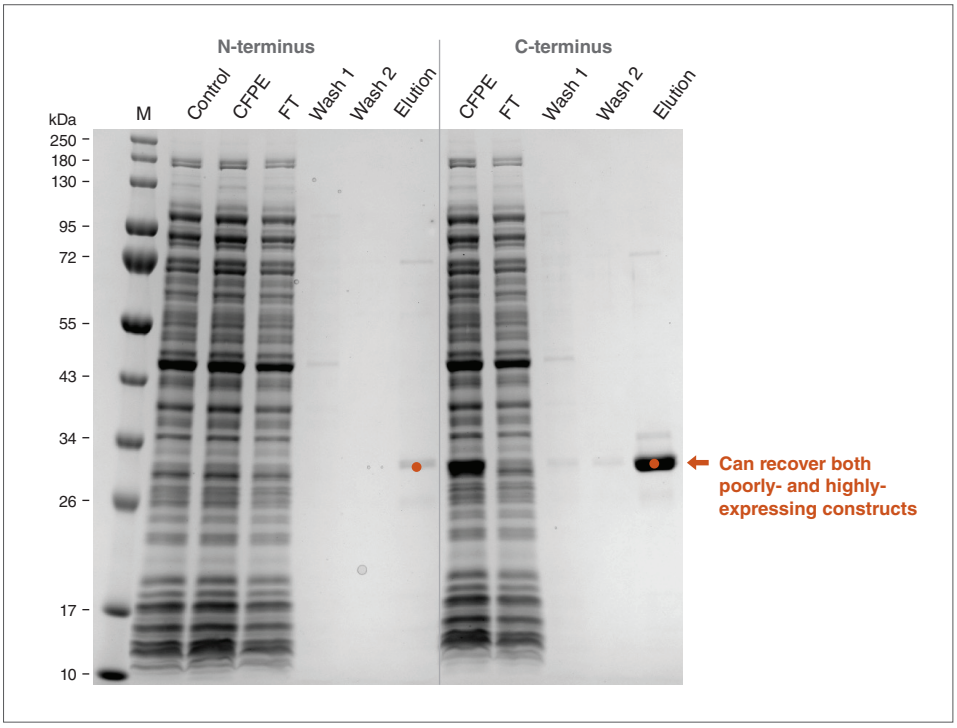
Inserts were designed using NEBridge SplitSet® Tool to divide the desired sequence into 3 fragments. The overhangs used were then verified with NEBridge Ligase Fidelity Viewer® with the added overhangs: "ACCA and ATTC" to ensure the robustness of the assembly. Fragments were next amplified using Q5 Hot Start High-Fidelity 2X Master Mix from dsDNA fragments. The amplified fragments were purified using the Monarch DNA Gel Extraction kit and quantified by absorbance. Stoichiometric amounts of each piece and backbone were then used in a Golden Gate Assembly reaction with BsaI-HFv2 and NEBridge Ligase Master Mix.

FIGURE 3: **Rolling Circle Amplification quickly produces high yield of DNA from NEBridge Golden Gate Assembly reactions**



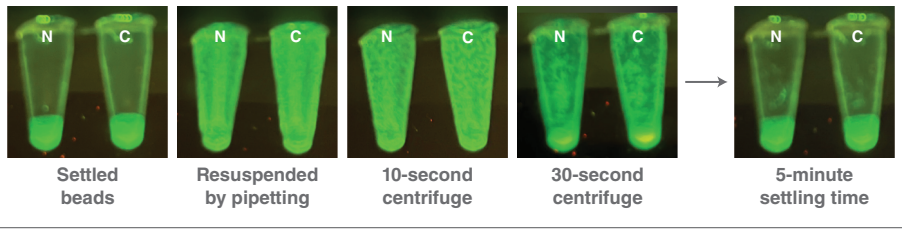
Fluorescence monitoring using phi29-XT DNA polymerase demonstrates fast and efficient amplification of DNA template within 2 hours. Image of real-time fluorescence results shows saturation at 60 minutes or less. Black triangles are non-templated amplification.

FIGURE 4: **GFP is highly pure following cell-free protein expression and purification**



SDS-PAGE showing the different His_6-tagged GFP proteins that were expressed by NEBExpress Cell-free *E. coli* protein synthesis system and purified using NEBExpress Ni-NTA Magnetic Beads. While the N-terminally tagged construct expressed poorly, protein was still obtained after purification. The C-terminally tagged construct expressed well resulting in high yield of purified protein.

FIGURE 5: **GFP is immobilized and functionalized on magnetic beads**



SNAP-functionalized proteins were captured using SNAP-Capture Magnetic Beads. As shown, SNAP-tagged GFP is detected on the beads when they are settled. After resuspension and pelleting, GFP fluorescence is only detected on the beads showing a strong association between the protein of interest and bead. N = N-terminus, C = C-terminus.

Following the assembly of 3 parts into the destination vectors, the assembly mixes were diluted into a phi29-XT amplification reaction performed on a temperature block without shaking, and a high yield of DNA was quickly obtained (Figure 3, page 3). After amplification for 2 hours and heat inactivation at 65°C for 10 minutes, the reaction was cooled to room temperature, and a master mix of NEBExpress Cell-free *E. coli* Protein Synthesis was directly added to the tube. The mixture was incubated overnight with vigorous shaking. The NEBExpress Cell-free *E. coli* Protein Synthesis System was developed using several strategies to enhance performance, ease of use, and ensure robustness. These include using an *E. coli* strain genetically engineered to maximize the stability of template DNA and RNA and the protein products, a highly optimized reaction buffer, and a stringent biomanufacturing process.

The following morning, after 14 hours of incubation, protein synthesis was verified by SDS-PAGE. In one workflow, 100 µl of the His_6-tagged reactions were purified using 50 µl NEBExpress Ni-NTA Magnetic Beads (Figure 4). A stark contrast in yield was observed between the N- and C-terminally His_6-tagged proteins. Despite the low yield of the N-terminally tagged construct, the purified protein was isolated and observed. The difference in yield demonstrates the need for screening multiple constructs in parallel. In a separate workflow, the SNAP-tagged expressed proteins were affixed to 50 µl SNAP-Capture Magnetic beads in the presence of DTT (Figure 5). These two workflows demonstrated the compatibility of the material from NEBExpress Cell-free *E. coli* Protein Synthesis with our protein purification products.

CONCLUSION

The workflow described herein represents a seismic shift in the time it takes to go from cloning reaction to purified protein relative to typical cloning and subsequent protein expression protocols. NEBridge Golden Gate Assembly reactions were successfully used as templates for amplification by phi29-XT. The amplified material was suitable as an input for cell-free protein synthesis as shown by the demonstration of purifying His_6-tagged constructs as well as SNAP-Capture. Due to the precision of assemblies using NEBridge Golden Gate Assembly, the rapid amplification of the phi29-XT RCA kit, and the high yield of the NEBExpress Cell-free *E. coli* Protein Synthesis system, genes can be cloned into expression constructs and expressed within a day.

Learn more about NEBridge Golden Gate Assembly at www.neb.com/goldengate. Try our NEBridge Ligase Fidelity Tools for Golden Gate Assembly at ligasefidelity.neb.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

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