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Breaking through the Limitations of Golden Gate Assembly– The Co-Evolution of Test Systems, Engineered Enzymes and Understanding Ligase Fidelity

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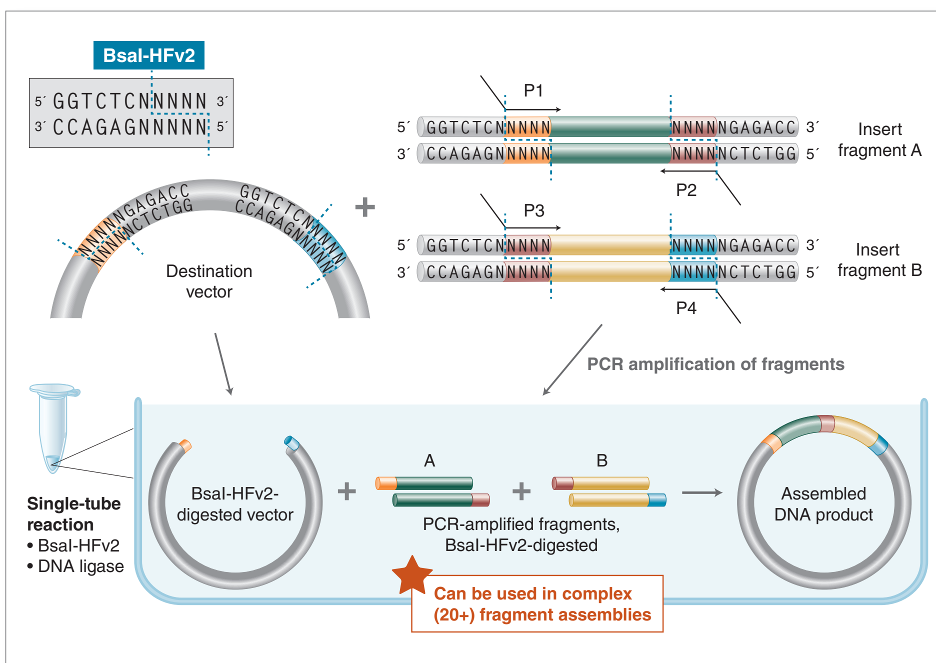
INTRODUCTION

Golden Gate Assembly is a molecular assembly technique that utilizes simultaneous digestion with Type IIS restriction enzymes and ligation by a DNA ligase to enable the scarless, ordered assembly of multiple fragments (1). Embraced by the synthetic biology community, as well as the general molecular biology community, it is commonly used to assemble 2–10 inserts, or modules, in a single "one-pot" reaction to form complex, multi-insert modular assemblies that enable biosynthetic pathway engineering and optimization. However, Golden Gate is also useful for both single-insert cloning, and inserts from diverse populations that enable library creation.

Current best practices for assemblies of more than 10 modules often rely on two-step hierarchical approaches using different Type IIS restriction enzyme specificities at each step. In the past, factors such as enzyme efficiency, stability and buffer compatibility have placed practical limits on single- or two-step assemblies, and large-scale downstream screening is required to find correct assemblies – a highly desirable outcome. The constraints of this assembly approach called for further development. As a result, NEB has been able to reduce those limitations through ongoing engineering efforts of Type IIS restriction enzymes and careful choice of junction sequences guided by experimentally-derived DNA ligase fidelity data. Our work demonstrates that it is now possible to achieve 20+ fragment assemblies with both robust efficiency and accuracy.

FIGURE 1:
Golden Gate Assembly workflow for complex assemblies



DEVELOPMENT OF GOLDEN GATE ASSEMBLY TEST SYSTEMS

New England Biolabs has been committed to further developing Golden Gate protocols and enzymes for a number of years, a commitment that has enabled the development of a variety of test systems with increasing complexities. These more difficult assemblies have allowed us to identify and implement improvements in Golden Gate assembly that could not be detected with simpler 1–10 insert test systems. Our research focused on three different levels of assembly:

- high efficiency and accurate single-insert assembly
- intermediate 5- or 12-fragment assembly, mirroring the commonly perceived "upper limit" for assembly
- more complex 24-fragment assembly

Table 1 (page 2) illustrates the breadth of systems used by NEB to address this range of usage for Golden Gate as assembly approaches have evolved.

Before assembly optimization, each test system was evaluated in a variety of ways. Single insert cloning based on the acquisition of a selectable antibiotic marker allowed fast throughput testing of efficiencies. This cloning was also compared to a similar-sized lambda amplicon to indicate any possible bias towards suppression of background by antibiotic selection. Screening of transformants by colony PCR confirmed the insertion of the lambda insert at the same high frequencies. The 5-, 12- and 24-assembly systems are based on the ability to correctly

TABLE 1:
Assembly test systems of increasing complexities designed at New England Biolabs

All inserts self-assemble into the pGGA destination construct. Reactions were incubated at 37°C for 5-min. or 60-min. for single inserts, and 30 cycles of (5 min. 37°C → 5 min. 16°C) for the 5, 12 and 24 fragment assemblies. In both cases, a terminal soak of 5 min. at 55°C was used to complete the assemblies at a temperature that favors cutting of any uncut/re-formed pGGA to reduce background. Single-insert assemblies used purified amplicon inserts at a 2:1 insert:vector ratio, while the *lacI/lacZ* cassettes used precloned inserts at equimolar levels to pGGA. Full details for complex assemblies using BsaI-HFv2 and T4 DNA Ligase are included at the end of this paper.

NUMBER OF INSERTS	GOLDEN GATE ASSEMBLY TEST SYSTEM	SIZE OF INDIVIDUAL INSERTS	SIZE OF ASSEMBLED INSERTS	INDICATION OF CORRECT ASSEMBLIES INTO pGGA (Cam^R) DESTINATION PLASMID
1	Amp^R, Kn^R, or Lambda amplicon	~1 kb	~1 kb	Growth on Cam/Amp, Cam/Kn or Cam/colony PCR
5	*lacI/lacZ* Cassette	~1 kb	~5 kb	Blue colony on Cam/X-gal/IPTG plates
12	*lacI/lacZ* Cassette	~300–600 bp	~5 kb	Blue colony on Cam/X-gal/IPTG plates
24	*lacI/lacZ* Cassette	~100–300 bp	~5 kb	Blue colony on Cam/X-gal/IPTG plates

assemble a *lacI/lacZ* cassette (designed by NEB for use in optimization of assembly systems) to produce a blue color phenotype upon growth on LB/Cam/X-gal/IPTG agar plates, indicating successful reconstruction of the coding sequence for beta-galactosidase in the *lacI/lacZ* cassette. Additional confirmation of accurate assembly was achieved by the sequencing of plasmids isolated from blue or white colonies. Sequencing of blue colonies showed the expected complete sequence for the *lacI/lacZ* genes (2), while sequences of white colonies revealed a mixture of mis-assemblies and very occasional uncut or cut/re-ligated pGGA destination plasmids. A final validation of the 5-, 12- or 24-fragment test systems was performed by setting up assembly reactions in which a single component was purposefully omitted. Since any assembly is dependent on the presence of every module, destination construct and functioning Type IIS restriction enzyme and DNA ligase, any single omission should completely block the formation of a complete assembly that would result in a blue phenotype. Indeed, this was seen in all *lacI/lacZ* assembly test systems; no blue colonies were obtained if any single component was omitted.

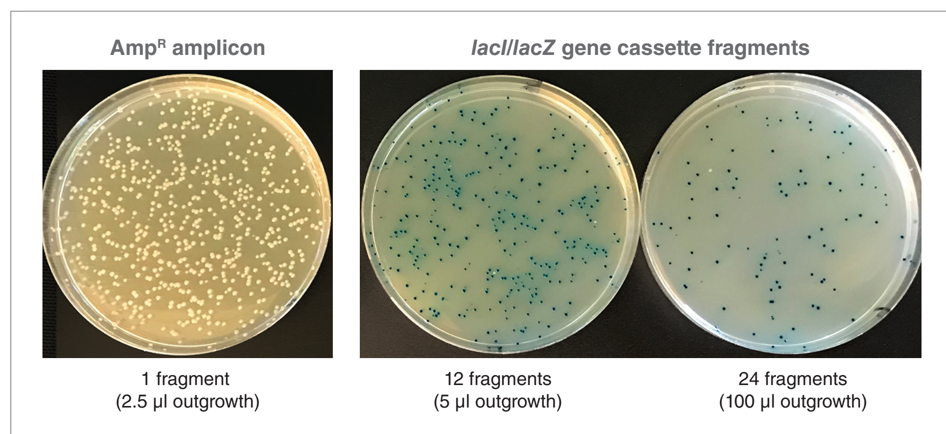
All Golden Gate assemblies feature an inverse proportionality between the complexity of the assembly (number of inserts or modules) and the resulting efficiency of assembly (number of transformants); the greater the number of inserts, the lower the number of transformants. This is often compensated for by plating greater volumes of the outgrowth on the selection plate to achieve enough transformants for downstream screening. Figure 2 shows representative transformation plates obtained from 1-, 12- and 24-fragment assemblies of the *lacI/lacZ* cassette, and illustrates how the volume of the 1 ml outgrowth spread on each transformation plate can be manipulated to result in appropriate levels of colony plating densities.

BREAKING THROUGH THE LIMITS OF GOLDEN GATE ASSEMBLY

Five fragment *lacI/lacZ* cassette assembly was easily achievable with high levels of transformants and low backgrounds – so much so that there was little range for detectable improvements in the methodology. The decision was made to re-design the test system for 12 and 24 fragments. This was guided by both advances in the re-engineering of the original BsaI-HF® Type IIS restriction enzyme and the completion of DNA ligase fidelity studies conducted by Potapov, et al. at NEB (1,2). While T4 DNA Ligase, the mainstay of most biotechnological cloning efforts for over 50 years, prefers ligation of Watson-Crick base pair substrates, it can show significant activity on some mismatch-containing pairings. During Golden Gate Assembly, ligation of mismatched pairs of overhangs can lead to incorrect assemblies, so care must be taken to minimize this possibility. Recently, NEB researchers profiled the comprehensive fidelity of cohesive end ligation by this enzyme for all three- and four-base overhang sequences under standard reaction conditions. This data set allows quantitation of sequence-dependent ligation efficiency and identification of mismatch-prone pairings. Using these 4-base pair overhang observations, accurate "high-fidelity" junction

FIGURE 2:
Representative Transformation Plates of Golden Gate Assemblies Featuring Increasing Complexities

Assembly reactions were transformed into competent *E. coli* strains NEB 10-beta (#C3019) (1 fragment) and T7 Express (#C2566) (12 and 24 fragments) and incubated for 16 hours at 37°C. While many strains support assembly protocols, and 10-beta is routinely recommended due to its ability to stably maintain large construct plasmid sizes, the non-alpha complementing T7 Express cell strain was used for the *lacI/lacZ* cassette testing to avoid any possibility of alpha-fragment LacZ complementation.



sets for both the 12- and 24-fragment versions of the *lacI/lacZ* cassette were designed and synthesized. In conjunction with BsaI-HFv2 (NEB #R3733), re-engineered to provide improved Golden Gate performance, a series of optimization experiments for these more complex assemblies were performed. It was found that high efficiencies and accurate assembly levels were indeed possible, with correct, in-frame assembly proceeding in 99% of 12-fragment assemblies and over 90% for 24-fragment assemblies (Figure 3, Table 2). The fidelity data can be applied to derive similar high-fidelity overhangs for any Golden Gate assembly design. Additionally, the stability of the enzymes allowed use of a greater number of cycles, pushing the efficiency levels even higher than afforded by the standard 30 cycles for those wishing maximal transformation levels. Extended reaction cycling is only successful when using highly stable enzymes that maintain activity beyond the standard 5 hours required for 30 cycles, utilizing 5-minute stages at temperatures favoring digestion (37°C) and ligation (16°C).

FIGURE 3:
Golden Gate Assembly of 24 fragments can be achieved with high efficiency and accuracy

Twenty-four fragment assemblies of the *lacI/lacZ* cassette were performed using the protocol included in this article. While 30 cycles is sufficient to achieve 24 fragment assemblies, the stability of the BsaI-HFv2 and T4 DNA Ligase allows continued assembly through 45 and 60 cycles with a low background. (a) Efficiency of assembly and (b) accuracy of assembly versus cycle number. This continued functionality past the traditional 30 cycles of assembly indicates a high level of enzyme stability.

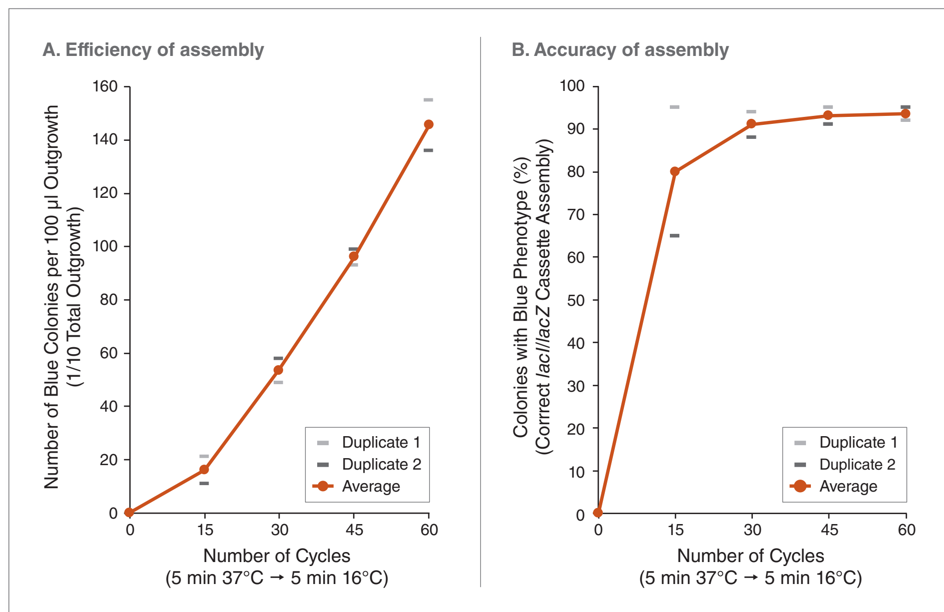


TABLE 2:
Yields and fidelities for Golden Gate Assemblies with BsaI-HFv2 and T4 DNA Ligase

Efficiency of assemblies per plate using outgrowth volumes described in Figure 3, with calculated yields from entire outgrowth built from 2 µl of the assembly reaction, and from the entire assembly reaction. All assembly protocols had a 5 min, 55°C terminal soak before transformation.

NUMBER OF FRAGMENTS ASSEMBLED	GOLDEN GATE ASSEMBLY PROTOCOL* (VOLUME OF 1 ml OUTGROWTH PLATED)	CORRECT ASSEMBLIES PER PLATE	FIDELITY OF ASSEMBLY (PERCENT CORRECT)	CALCULATED COLONY TOTALS	
				PER 2 µl ASSEMBLY REACTION	PER FULL ASSEMBLY REACTION**
1	5 min., 37°C (2.5 µl)	687	100%	274,200	2,742,000
1	60 min., 37°C (2.5 µl)	1,623	100%	649,200	6,492,000
12	(5 min., 37°C → 5 min. 16°C) x 30 (5 µl)	245	99.5%	48,900	489,000
24	(5 min., 37°C → 5 min. 16°C) x 30 (100 µl)	78	90.7%	783	9,792

* All assembly reactions had a 5 minute terminal soak after either 37°C incubations or cycling.

** Assembly reaction volumes were 20 µl (1, 12 fragments) or 25 µl (24 fragments).

LOOKING TO THE FUTURE

DNA assembly methods are important tools for many areas of science, and researchers continue to test the limits of DNA assembly approaches with increasingly complex experimental conditions. The ability to construct more complex, multi-fragment assemblies, as shown in this work, will fuel additional efforts to push the technique forward. Our research and development efforts continue to focus on providing optimized reaction components for a wider number of Type IIS specificities and substantial improvements to the methodology, with the goal of enabling routine, efficient and accurate assembly of 50 fragments in a single tube in the not-so-distant future.

References:
1. Potapov, V., et al. (2018) *Nucleic Acids Research*, gky303; doi: https://doi.org/10.1093/nar/gky303
2. Potapov, V., et al. (2018) *bioRxiv*, 322297; doi: https://doi.org/10.1101/322297

Golden Gate (24 Fragment) Assembly Protocol

Note: For complex (>10 fragment) assemblies, high efficiencies are achievable with increased ligase and BsaI-HFv2 levels (1000 units T4 DNA Ligase, 30 units BsaI-HFv2), as listed in this protocol. For assemblies involving 10 fragments and less, the standard amounts (500 units T4 DNA Ligase, 15 units BsaI-HFv2) are sufficient. Note the reaction volume of 25 µl is used to allow sufficient volume for precloned insert additions, if needed.

Materials

- T4 DNA Ligase (NEB #M0202)
- BsaI-HFv2 (NEB #R3733)
- pGGA Destination Plasmid*
- NEB 10-beta Competent *E. coli* (NEB #C3019)
- NEB 10-beta/Stable Outgrowth Medium (NEB #B9035)
- LB Agar plates with chloramphenicol

* Included in the NEB Golden Gate Assembly Mix (NEB #E1600)

Assembly Reactions

1. Set up 25 µl assembly reactions as follows:

REAGENTS	ASSEMBLY REACTION	NEGATIVE CONTROL (IF DESIRED)
pGGA Destination Plasmid*, 75 ng/µl	1 µl (75 ng)	1 µl (75 ng)
24 precloned inserts cloned into pMiniT 2.0, 100 ng/ul each plasmid	0.75 µl (75 ng) each, (18 µl total)	-
T4 DNA Ligase Buffer (10X)	2.5 µl	2.5 µl
T4 DNA Ligase (NEB #M0202), 2000 U/µl	0.5 µl (1000 units)	0.5 µl (1000 units)
BsaI-HFv2 (NEB #R3733), 20 U/µl	1.5 µl (30 units)	1.5 µl (30 units)
Nuclease-free H_2O	1.5 µl	19.5 µl

*or user provided

2. Mix gently by pipetting up and down 4 times.
3. Briefly microcentrifuge (1 sec.) to bring material to the bottom of tube.
4. Transfer to thermocycler and program as follows: (5 min 37°C → 5 min 16°C) x 30 cycles followed by 5 min 60°C. If reactions are done overnight, add a 4°C terminal hold to the protocol, but repeat the final 5 min 60°C step the next day before the transformations.

Transformation

1. For each assembly, thaw a 50 µl tube of NEB 10-beta competent *E. coli* cells on ice for 5–10 min.
2. Add 2 µl of the assembly reaction; gently mix by flicking the tube 4-5 times.
3. Incubate on ice for 30 min.
4. Heat shock at 42°C for 30 sec.
5. Place back on ice for 5 min.
6. Add 950 µl of room temperature NEB 10-beta/Stable Outgrowth Medium (NEB #B9035). Incubate at 37°C for 60 min., shaking vigorously (250 rpm) or using a rotation device.

Plating

1. Warm LB agar plates containing chloramphenicol (for pGGA) at 37°C for 15 min.
2. Mix the cells thoroughly by flicking the tube and inverting, then spread 100 µl outgrowth onto each plate.
3. Incubate the plates overnight at 37°C, or 24 hrs at 30°C, or 48 hrs at 25°C.

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