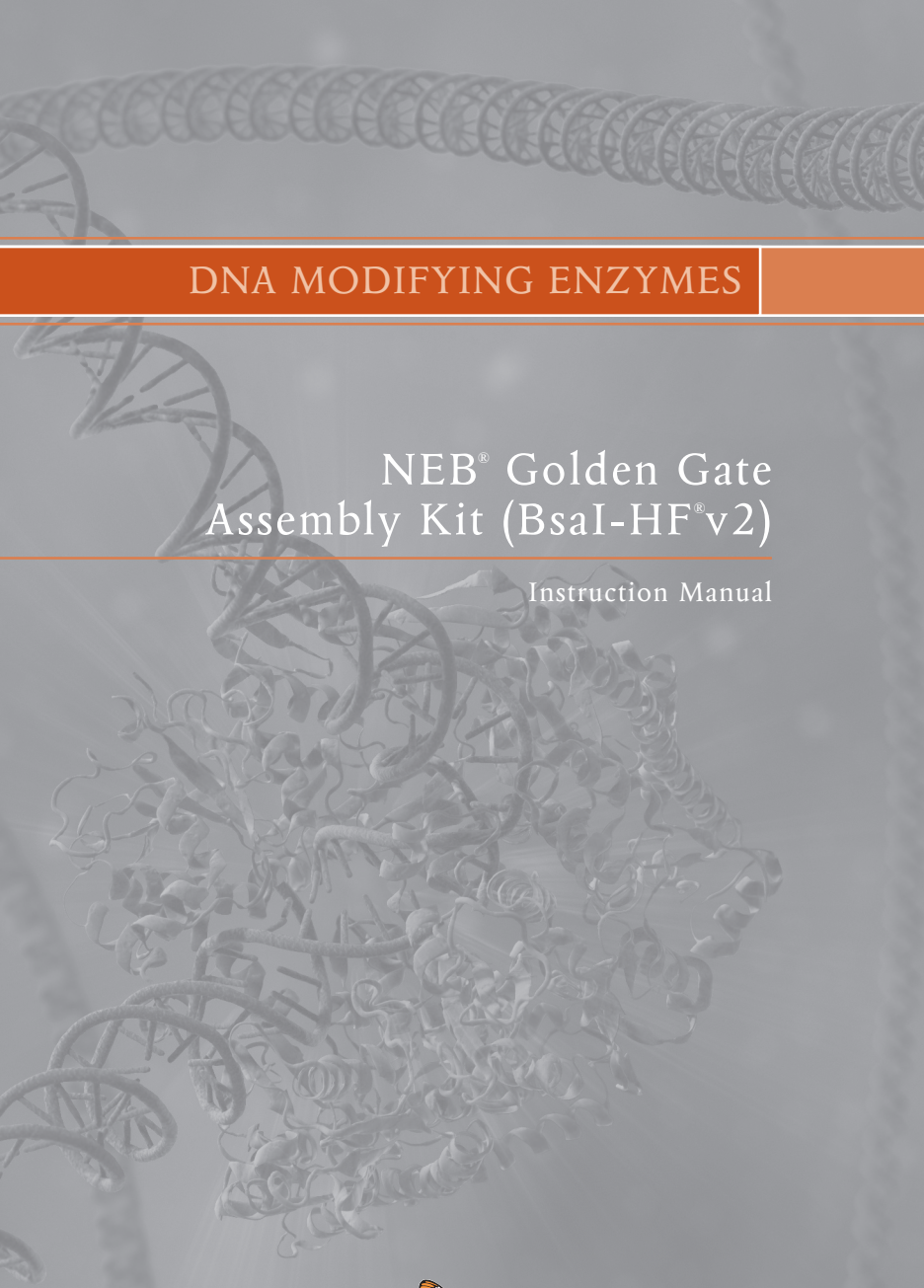
DNA MODIFYING ENZYMES

NEB® Golden Gate Assembly Kit (BsaI-HF®v2)

Instruction Manual



NEB #E1601S/L
20/100 reactions
Version 1.0 9/18

NEW ENGLAND BioLabs® Inc.

be INSPIRED
drive DISCOVERY
stay GENUINE

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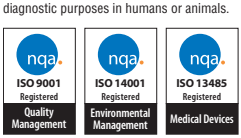


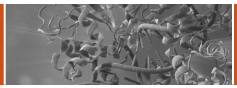


Table of Contents:

Components:

Important Note: Upon arrival, store the kit components at –20°C.

NEB Golden Gate Enzyme Mix (BsaI-HFv2)
20 µl (NEB #E1601S), 100 µl (NEB #E1601L)
Contains an optimized mix of BsaI-HFv2 and T4 DNA Ligase.

pGGA Destination Plasmid
100 µl
Provides the vector backbone for assemblies.

T4 DNA Ligase Buffer (10X)
1.5 ml
Contains buffer components optimal for BsaI-HFv2 digestion and ligation of DNA.

Required Materials Not Included:

User-defined inserts
Competent cells
Other materials for transformation

Introduction:

The NEB Golden Gate Enzyme Mix contains an optimized mix of BsaI-HFv2 and T4 DNA Ligase. Together these enzymes, along with an optimal buffer, can direct the assembly of multiple inserts/modules using the Golden Gate approach. Also provided is the pGGA destination plasmid, which provides a backbone for your assembly, features convenient restriction enzyme sites for subcloning, and has T7/SP6 promoter sequences to enable *in vitro* transcription.

The efficient and seamless assembly of DNA fragments, commonly referred to as Golden Gate Assembly (1,2), had its origins in 1996, when for the first time it was shown that multiple inserts could be assembled into a vector backbone using only the sequential (3) or simultaneous (4) activities of a single Type IIS restriction enzyme and T4 DNA Ligase.

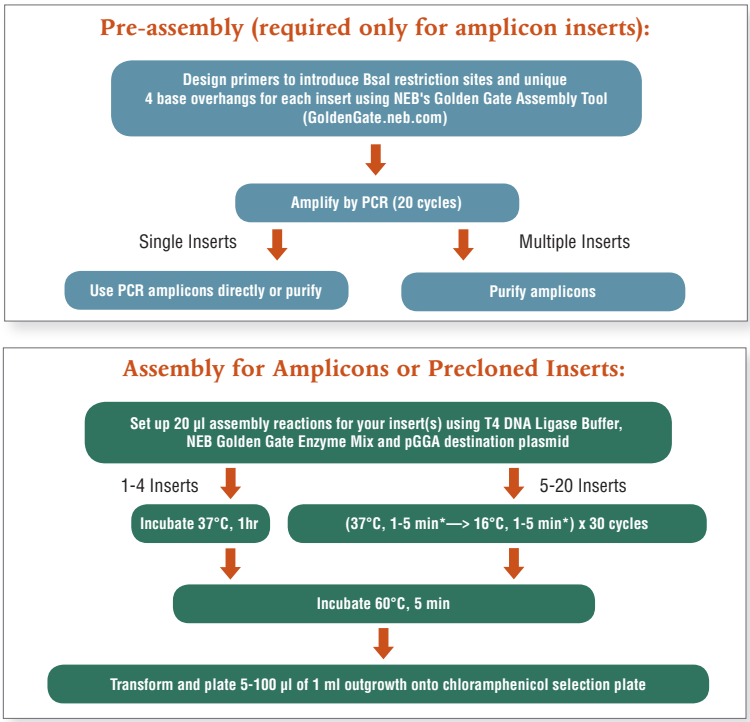
Type IIS restriction enzymes bind to their recognition sites but cut the DNA downstream from that site at a positional, not sequence-specific, cut site. Thus, a single Type IIS restriction enzyme can be used to generate DNA fragments with unique overhangs. As an example, BsaI has a recognition site of GGTCTC(N1/N5), where the GGTCTC represents the recognition/binding site, and the N1/N5 indicates the cut site is one base downstream on the top strand, and five bases downstream on the bottom strand. Assembly of digested fragments proceeds through annealing of complementary four base overhangs on adjacent fragments. The digested fragments and the final assembly no longer contain Type IIS restriction enzyme recognition sites, so no further cutting is possible. The assembly product accumulates with time.

While particularly useful for multi-fragment assemblies such as Transcription Activator Like Effectors (TALEs)(5) and TALEs fused to a FokI nuclease catalytic domain (TALENs)(6), the Golden Gate method can also be used for cloning of single inserts and inserts from diverse populations that enable library creation.

A technical note describing Golden Gate Assembly initiatives at NEB and typical results for assemblies differing in complexity is available on our website www.neb.com/GoldenGate.

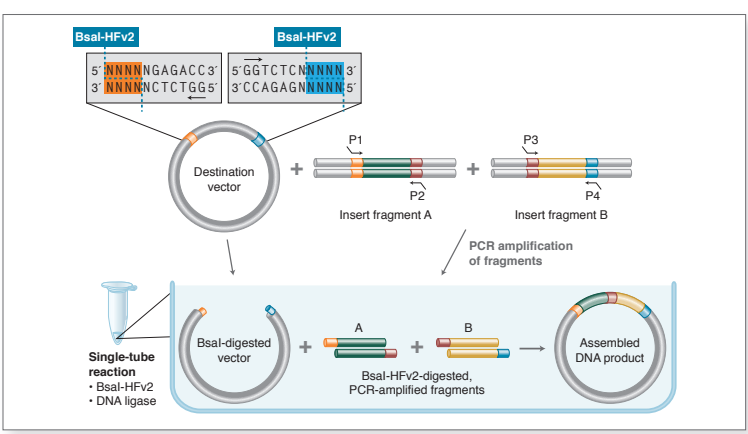
Please note that while general descriptions regarding Golden Gate Assembly use the BsaI nomenclature, this kit and protocols feature the specific engineered form optimized for Golden Gate Assembly, BsaI-HFv2.

Figure 1: Overview: Assembly Protocol of Golden Gate Assembly



* Refer to detailed protocols for specific time recommendations

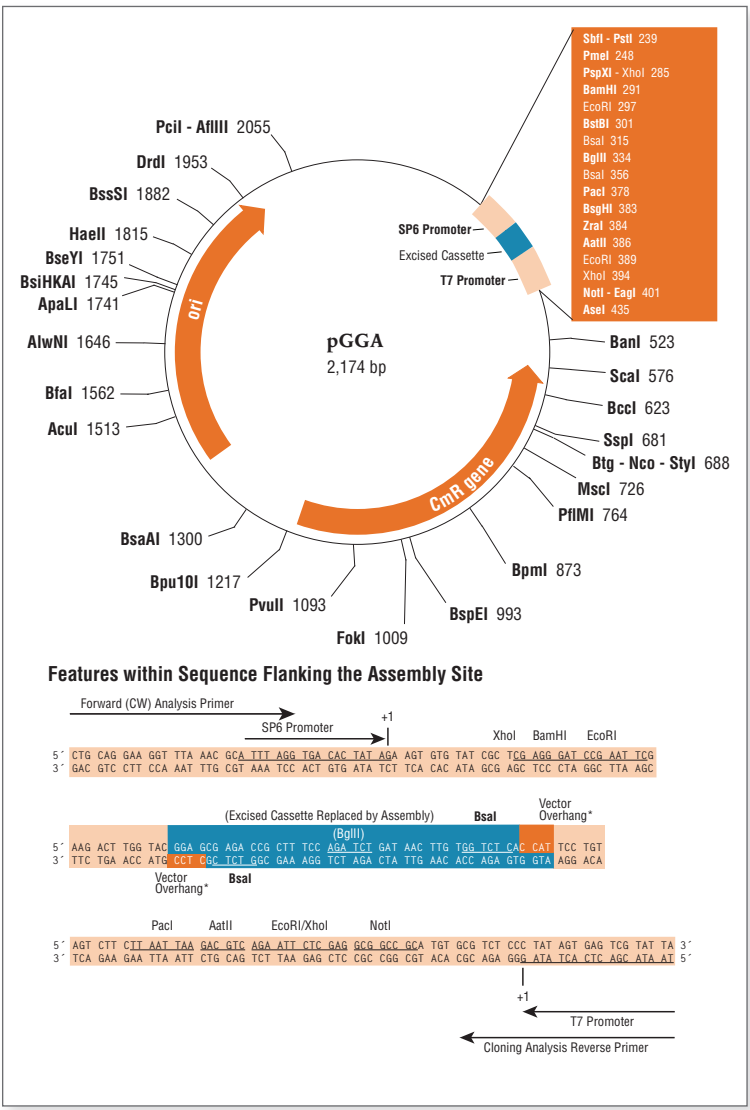
Figure 2: Golden Gate Workflow



In its simplest form, Golden Gate Assembly requires a Type IIS recognition site, in this case, BsaI-HFv2 (GGTCTC), added to both ends of a dsDNA fragment. After digestion, these sites are left behind, with each fragment bearing the designed 4-base overhangs that direct the assembly.

pGGA Destination Plasmid:

pGGA is a 2,174 bp cloning vector useful for Golden Gate Assembly. The plasmid contains two BsaI restriction sites; digestion with BsaI releases a 41 bp fragment and a 2,133 bp vector backbone fragment to receive your insert or assembly.



Features within Sequence Flanking the Assembly Site

Insert Considerations:

Historically, Golden Gate inserts were precloned into plasmid constructs having flanking BsaI restriction sites to generate the appropriate 4 base overhang sequences that guide the assembly. However, the use of amplicon inserts without precloning also supports efficient assembly levels and saves time. See below for specific recommendations for precloned inserts, and amplicon inserts for single insert cloning and multiple insert assembly:

A. Precloned Inserts: Precloning is always an option, and is recommended for inserts/modules < 250 bp or > 3 kb, or those containing repetitive elements that might accumulate errors during PCR amplification to produce amplicon inserts. The pMiniT 2.0 vector backbone used in the NEB PCR Cloning Kit (NEB #E1202/#E1203) is an excellent cloning option as the pMiniT 2.0 Vector backbone has no BsaI sites. Note that all sequences that will be part of the assembly must be flanked by correctly oriented BsaI restriction sites, facing towards the insert on the top and bottom strands.

B. Amplicon Inserts: The 5´ flanking bases and BsaI restriction enzyme recognition site are introduced through PCR primer design upstream and downstream of sequences to be assembled. In all cases, the 2:1 insert:vector (pGGA, 2,174 bp) ratio is suggested to achieve assembly efficiencies similar to that with precloned inserts. For molar calculations we recommend using the NEBioCalculator® (nebiocalculator.neb.com).

(a) Single Insert Cloning/Assembly Primer Design:

Upstream (CW) primer: 5´...GGCTAC **GGTCTC** C GGAG NNNN→

Downstream (CCW) primer: 5´...GGCTAC **GGTCTC** C ATGG NNNN→

flanking bases — **BsaI** — N1 4BO* — insert sequence

- 4 base overhang to allow annealing/ligating into pGGA vector backbone in CW orientation; for CCW assembly orientation, switch the 4 base overhang sequences in the 2 primers.

Single insert amplicons can be used directly from PCR without purification **if a specific amplicon is evident upon analysis of part of the reaction by gel electrophoresis.** Use the amounts approximating a 2:1 insert:vector ratio and never more than 1 µl to minimize carryover of PCR components that can interfere with assembly. If specificity is not evident, or the concentration is too low, purify amplicon inserts with a spin column. [Monarch® DNA Gel Extraction Kit (NEB #T1020) or the Monarch PCR & DNA Cleanup Kit (5 µg) (NEB #T1030)]. Use of these kits will result in purified, higher concentration DNA due to smaller elution volumes.

(b) Multiple Insert Assembly Primer Design. We recommend using the NEB Golden Gate Assembly Tool (goldengate.neb.com) for the design of PCR primers to ensure the correct 4 base overhangs will be generated and to scan

inserts for internal BsaI sites. Purify all amplicon inserts with a spin column protocol (see page 5).

Detailed Protocols:

Golden Gate Assembly Protocol:

1. Set up assembly reactions as follows:

REAGENT	NEGATIVE CONTROL	ASSEMBLY REACTION
pGGA Destination Plasmid (1), 75 ng/µl	1 µl	1 µl
Inserts (user provided): - if precloned (2) - if in amplicon form (3)	–	75 ng each plasmid 2:1 molar ratio, (insert:vector; pGGA = 2,174 bp; 75 ng = 0.056 pmol)
T4 DNA Ligase Buffer (10X)	2 µl	2 µl
NEB Golden Gate Enzyme Mix	1–2 µl (4)	1–2 µl
Nuclease-free H_2O	to 20 µl	to 20 µl

(1) or user provided.
(2) Precloned inserts must possess BsaI restriction sites at both ends of the insert sequence and in the proper orientation.
(3) Amplicon inserts must possess 5´ flanking bases and BsaI restriction sites at both ends of the amplicon and in the proper orientation.
(4) For assemblies ≤ 10 inserts, use 1 µl : for assemblies ≥ 10 inserts, use 2 µl.

Note: Negative controls are not routinely done for assembly reactions, but are described for first time users.

2. Choose the appropriate assembly protocol:

INSERT NUMBER	SUGGESTED ASSEMBLY PROTOCOL
For 1 insert	37°C, 5 min (cloning) or 37°, 1 hr (library preparation) → 60°C, 5 min
For 2-4 Inserts	37°C, 1 hr → 60°C, 5 min
For 5-10 Inserts	(37°C, 1 min → 16°C, 1 min) x 30 → 60°C, 5 min
For 11-20+ Inserts	(37°C, 5 min → 16°C, 5 min) x 30 → 60°C, 5 min

Transformation Protocol:

The following protocol is designed for NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019), as this strain is highly efficient for the stable maintenance of large plasmids. For other strains (discussed further in the FAQ section) please refer to the protocol specific to the strain. If using electrocompetent cells, such as NEB 10-beta Electrocompetent *E. coli* (NEB #C3020), follow the protocol provided with the cells, which can also be found at https://www.neb.com/protocols/1/01/01/electroporation-protocol-c3020.

For NEB 10-beta Competent *E. coli*:

1. Thaw a 50 µl tube of NEB 10-beta Competent *E. coli* cells on ice for 10 minutes.

2. Add 2 µl assembly reaction; gently mix by flicking the tube 4–5 times.
3. Incubate on ice for 30 minutes.
4. Heat shock at 42°C for 30 seconds.
5. Place on ice for 5 minutes.
6. Add 950 µl of room temperature NEB 10-beta/Stable Outgrowth Medium. Incubate at 37°C for 60 minutes, shaking vigorously (250 rpm) or using a rotation device.

Plating Protocol:

1. Warm LB agar plates containing chloramphenicol (for pGGA) or other appropriate antibiotic at 37°C.
2. Mix the cells thoroughly by flicking the tube and inverting, then spread 50 µl of a 1:10 dilution (single inserts) or 50–100 µl (multiple inserts) of the 1 ml outgrowth onto each plate.
3. Incubate the plate overnight at 37°C, or if desired, 24–36 hours at 30°C or 48 hours at 25°C.

Recommended Screening Protocols:

The following are different ways to screen your assemblies:

1. Colony PCR screening using an appropriate DNA polymerase for amplification of the insert region. While many DNA polymerases are suitable for colony PCR, One*Taq*® DNA Polymerase (NEB #M0480) or One*Taq* Hot Start DNA Polymerase (NEB #M0481) is recommended. *Taq* DNA Polymerase (NEB #M0267) can also be used. For PCR of larger assemblies, LongAmp® *Taq* DNA Polymerase (NEB #M0323) or LongAmp Hot Start *Taq* DNA Polymerase (NEB #M0534) are strongly recommended.
2. Preparing plasmid mini-preps using the Monarch Plasmid Miniprep Kit (NEB #T1010) and mapping by using appropriate restriction endonucleases to confirm the correct assembly.

Regardless of the screening protocol used, the correct assembly of insert(s) should always be confirmed by sequencing of the plasmid construct across the 4 base junctions and inserts.

Frequently Asked Questions (FAQs):

Why does the Golden Gate Assembly Mix now feature BsaI-HFv2?

Research using more challenging 12 and 24 fragment assembly test systems showed the clear superiority of the recently re-engineered BsaI-HFv2 enzyme over BsaI. This is evident in efficiencies of assembly (number of transformants), accuracies of assembly (fidelity) and continued increases in assembly formation at higher than usual cycle numbers if desired. The mix is identical to the original except for the Type IIS restriction enzyme replacement.

How does NEB Golden Gate Assembly work?

Assembly utilizes two simultaneous enzymatic activities in a single reaction; endonuclease digestion by BsaI and ligation by T4 DNA Ligase. With optimized buffer components and enzyme ratios, a single reaction containing a destination plasmid such as pGGA and inserts (PCR amplicons or precloned) will result in ligation of inserts with complementary cohesive ends and the accumulation of assembled product as a function of time. The final assembly has no BsaI recognition sites, rendering the assembly inert to further digestion by BsaI. For more information, view our online tutorial at www.neb.com/goldengate.

What affects the efficiency of Golden Gate Assembly?

Single insert cloning is significantly more efficient than multiple insert cloning. Assembly efficiency decreases as the number of fragments increases. The presence of repetitive sequences in an insert will also decrease efficiency. For inserts < 250 bp or > 3 kb, precloning will increase efficiency. Lastly, the normal restrictions on overall plasmid size to allow stable maintenance in *E. coli* apply to Golden Gate Assemblies. Efficiencies are highest with assembled product plasmid constructs < 12 kb. Larger assemblies can be made but will require larger numbers of colonies to be screened for the correct full length assembled products. For experimental examples of complexity vs. efficiency, refer to the Golden Gate Assembly Technical Note on our website www.neb.com/GoldenGate.

Why do many of the published Golden Gate Assembly articles feature precloned inserts as opposed to inserts generated by PCR?

Precloned inserts allow stable storage of inserts and make it easy to calculate the molarity of inserts, while using amplicon inserts saves time. Stable storage of amplicon inserts is important. Single insert cloning/assemblies can use unpurified amplicons, while multiple insert amplicons should be purified, for example by spin column protocols such as the NEB Monarch PCR and DNA Cleanup Kit (5 µg), NEB #T1030S. For long term storage at –20°C, store DNA in 10 mM Tris (pH 8.5), 1 mM EDTA (TE) or short-term storage in 10 mM Tris (pH 8.5), 0.1 mM EDTA (modified TE). EDTA at these levels will not significantly lower the 10 mM $MgCl_2$ present in the T4 DNA Ligase Buffer.

Using amplicons directly without precloning seems much easier, but is the assembly efficiency decreased?

No. While in general DNA is more stable in circular form than in linear form due to the absence of free ends, amplicons are a viable and easy way to build assemblies as long as they have been purified and stored in the appropriate buffer (see above). The suggested 2 fold molar ratio of amplicon inserts:pGGA vector brings the assembly efficiency to that of precloned inserts (using 75 ng of each plasmid) for most assemblies.

Can PCR amplicons be used directly in single insert (cloning) assembly reactions without purification?

Yes, although efficiencies will be decreased by 3–8 fold. BsaI digestion generates 5´-four base overhangs that can be filled-in by the carryover DNA polymerase used in PCR when using unpurified amplicons, producing blunt ends. This will lead to nonspecific assembly. For single insert cloning/assembly, the ligase successfully competes with the carryover DNA polymerase such that unpurified PCR amplicon inserts can be used. Tests done with unpurified PCR material amplified by *Taq*, Hot-Start *Taq*, Q5® and Hot-Start Q5 confirmed single insert cloning worked well if the volume of material brought into the 20 µl Golden Gate Assembly reaction was never more than 1 µl. For multiple insert Golden Gate assemblies, amplicons must be purified and if non-specific products are present, amplicons must be gel purified.

For amplicon inserts, why are the calculations suggested as using the overall pGGA destination plasmid length and the overall amplicon lengths? Shouldn't only the part of pGGA functioning as the vector backbone be used?

It can, but the length of the vector backbone in pGGA differs only by 41 bp from its full plasmid length; since this 41 bp "fall away" fragment closely approximates the lengths of the 5´ flanking bases and BsaI sites on each amplicon insert, the simpler overall plasmid:insert calculation can be used.

Why is Golden Gate also used for single insert cloning?

While Golden Gate is normally used for insert assemblies of 5–10 or more fragments, it also allows easy and highly efficient cloning of single inserts following the provided directions. Golden Gate can also be used with diverse single insert populations for library preparations.

What if there is an Internal BsaI site(s) in my inserts?

Golden Gate Assembly with single inserts can tolerate a single internal site simply because the ligase can complete successfully with the BsaI restriction enzyme, although the efficiency of assembly will decrease. For multiple insert assemblies it is best to use site-directed mutagenesis to eliminate the internal site(s), or consider another assembly approach such as NEBuilder® HiFi DNA Assembly. This alternative aproach can be used for assemblies involving up to 5 inserts.

Why is there a 60°C, 5 min heat step at the end of the assembly reaction?

The final incubation step at 60°C favors BsaI cutting, in the absence of DNA ligation. Digesting any uncut or cut/religated destination plasmid still present in the assembly reactions reduces background.

How can I minimize PCR-generated errors in my amplicon inserts?

Use a high-fidelity DNA polymerase and avoid over-amplification. We recommend Q5 High-Fidelity DNA Polymerase formulations for maximal fidelity (NEB #M0491, #M0493), which is also available in Master Mix format (NEB #M0492, #M0494). Also, use the minimum number of cycles required to generate the amount of DNA required for assembly; this is usually 20 cycles or less.

I'm doing a moderate (4–5 insert) assembly but don't have access to a thermal cycler; can I use the simpler protocol using the 1 hr 37°C incubation?

Yes, but anticipate your assembly efficiency will decrease 4-fold; plate a larger amount of outgrowth to compensate.

Can the Golden Gate assembly reactions be scaled down?

Except for very complex, multi-insert assembly reactions, the reactions can be scaled down 2–3 fold if input volumes allow. This can be done by scaling down the reaction volume and components proportionately, or keeping the 20 µl reaction volume but using 2–3 fold less of each DNA component. In this latter case plate a larger amount of outgrowth to compensate.

Can I use other competent E. coli strains than NEB 10-beta? Can I use subcloning efficiency cells?

Yes, other cell strains can be used, but large assemblies will require strains known to maintain large plasmid stability. In our experience NEB 10-beta competent *E. coli* (High Efficiency; NEB #C3019) is an excellent choice for Golden Gate Assembly. For smaller assemblies other strains such as NEB 5-alpha Competent *E. coli* (High Efficiency; NEB #C2987), NEB Stable Competent *E. coli* (High Efficiency; NEB #C3040), NEB Turbo Competent *E. coli* (High Efficiency, NEB #C2984) or NEB T7 Express Competent *E. coli* (High Efficiency; NEB #C2566) can also be used. Subcloning efficiency cells will result in lower transformation levels and should not be used for multi-component assemblies.

I would like to use colony PCR to screen my transformants and sequence my assembly. Are there any recommended sequencing or colony PCR primers?

Analysis primers are not included with the Golden Gate Assembly Mix product. We recommend the following oligos for pGGA-based assemblies be custom ordered through any oligo synthesis provider:

Forward (CW) primer 54 bp upstream from assembly point:
5´-CTGCAGGAAGGTTTAAACGCATTTAGG-3´

Reverse (CCW) primer 51 bp downstream from assembly point:
5´-TAATACGACTCACTATAGGGAGACGC-3´

Note that larger assemblies will require internal assembly-specific primers to verify assembly sequence. As an alternative, the cloning analysis forward primer featured in the NEB PCR Cloning Kit can be used, but is located further upstream of the assembly point. Also many commercially available T7 promoter oligos can be used from the reverse direction.

Golden Gate Assembly Tips

1. Use of the NEB Golden Gate Assembly Tool (GoldenGate.neb.com) is strongly recommended; this tool will check insert sequences for internal BsaI sites and design primers to amplify your inserts for Golden Gate Assembly. The primers will feature 6 bases at the 5´ end flanking the BsaI recognition site, the recognition site itself, plus the 4-base unique overhangs that determine correct annealing and ligation of the inserts. All overhangs will automatically be designed as non-palindromic (to eliminate self insert ligations), unique, and in the correct orientations to ensure correct assembly.

2. Research at NEB has led to an increased understanding of ligase fidelity, including the development of a comprehensive method for profiling end-joining ligation fidelity in order to predict which overhangs will result in improved accuracy (7). This ligase fidelity information can be used in conjunction with the NEB Golden Gate Assembly Kit (BsaI-HFv2) to achieve high efficiency and accurate complex assemblies. Please visit www.neb.com/GoldenGate for more information and examples of 24 fragment assemblies with > 90° accuracy.

3. Two basic protocol approaches exist for assembly – constant 37°C single temperature incubations, or cycling protocols alternating between 37°C (optimal temperature for endonuclease digestion within a temperature range for ligase stability), and 16°C (optimal temperature for ligation). The assembly protocol suggestions are based on the number of inserts in your assembly reaction, but there is considerable flexibility; match the protocol to your desired efficiency of assembly and scheduling needs. In general:

    a. Assembly of 1–4 inserts does not require cycling; single 37°C incubations work well.

b. Cycling assemblies using 1 min temperature steps work well for multiple inserts.

c. Cycling assemblies using 5–10 minute temperature steps work well for larger scale assemblies (> 10 inserts) and for any assembly for which maximal assembly yields and transformation levels are desired.

d. Regardless of the number of inserts, if it is more convenient, any Golden Gate Assembly can be done overnight with 30 cycles of 37°C, 5 min → 16°C, 10 minutes, followed by 60°, 5 min and 4°C, ∞. There is no downside to longer protocols being used.

4. For precloning of inserts, we recommend using the NEB PCR Cloning Kit as the kit's pMiniT 2.0 vector backbone has no BsaI sites present.

5. The standard protocol using 30 cycles (alternating between 37°C and 16°C) results in high levels of accurate assemblies with low background, (even for 24 fragment assemblies). BsaI-HFv2 and T4 DNA Ligase however are very stable and they will continue to function up to 60 cycles.

6. While BsaI and BsaI-HFv2 are blocked by overlapping dcm methylation (methylation at the C5 position of cytosine in the sequences CCAGG or CCTGG), this is usually not an issue for Golden Gate Assembly. Commonly used destination vectors are designed to avoid upstream CC(A or T) bases in front of the BsaI GGTCTC recognition site that would create an overlapping dcm methylation site.

Specifications:

A 20 µl reaction containing 2 µl T4 DNA Ligase Buffer (10X), 75 ng pGGA (Golden Gate destination plasmid, Cam^R), 75 ng each of 5 plasmids carrying fragments of a gene encoding *lacIZ* and 1 µl Golden Gate Enzyme Mix is incubated for 30 cycles of 37°C for 1 minute, 16°C for 1 minute, then at 60°C for 5 minutes to linearize any remaining plasmid.

Successfully assembled fragments result in *lacIZ* gene in pGGA vector and yield blue colonies on an IPTG/Xgal/Chloramphenicol plate.

Transformation of T7 Express Competent *E. coli* (High Efficiency) (NEB #C2566) with 2 µl of the assembly reaction yields greater than 250 colonies and > 80% blue colonies when 5% of the outgrowth is spread on an IPTG/Xgal/Chloramphenicol plate and incubated overnight at 37°C.

References:

1. Engler, C. et al. (2008) *PLoS ONE*, 3: e3647.
2. Engler, C. et al. (2009) *PLoS ONE*, 4: e5553.
3. Lee, J.H. et al. (1996) *Genetic Analysis: Biomolecular Engineering*, 13; 139–145.
4. Padgett, K.A. and Sorge, J.A. (1996) *Gene*, 168, 31–35.
5. Weber, E. et al. (2011) *PLoS ONE*, 6; e19722.
6. Christian, M. et al. (2010) *Genetics*, 186, 757–761.
7. Potapov, V. et al. (2018) bioRxiv, 32297; doi: https://doi.org/10.1101/322297.

Ordering Information

PRODUCT	NEB #	SIZE
NEB Golden Gate Assembly Kit (BsaI-HFv2)	E1601S/L	20/100 reactions
COMPANION PRODUCTS		
Quick-Load® Purple 2-log DNA Ladder (0.1-10.0 kb)	N0550S	125-250 gel lanes
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S	100 reactions
Q5 Hot Start High-Fidelity DNA Polymerase	M0493S	100 units
Deoxynucleotide (dNTP) Solution Mix	N0447S	8 µmol of each
Q5 Site-Directed Mutagenesis Kit	E0554S	10 reactions
Q5 Site-Directed Mutagenesis Kit (without competent cells)	E0552S	10 reactions
NEB 10-beta Competent *E. coli* (High Efficiency)	C3019H	20 x 0.05 ml/tube
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987H	20 x 0.05 ml/tube
NEB Turbo Competent *E. coli* (High Efficiency)	C2984H	20 x 0.05 ml/tube
NEB Stable Competent *E. coli* (High Efficiency)	C3040H	20 x 0.05 ml/tube
NEB 10-beta Electrocompetent *E. coli*	C3020K	6 x 0.1 ml/tube
NEB T7 Express Competent *E. coli* (High Efficiency)	C2566H	20 x 0.05 ml/tube
NEB PCR Cloning Kit	E1202S	20 reactions
NEB PCR Cloning Kit (Without Competent Cells)	E1203S	20 reactions
Nuclease-free Water	B1500S/L	25/100 ml

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