INSTRUCTION MANUAL

NEBridge® Golden Gate Assembly Kit (BsmBI-v2)

NEB #E1602S/L

20/100 reactions

Version 3.0_6/26

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The NEBridge Golden Gate Assembly Kit (BsmBI-v2) Includes

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

NEBridge Golden Gate Enzyme Mix (BsmBI-v2)
Contains an optimized mix of BsmBI-v2 and T4 DNA Ligase.

pGGAselect Destination Plasmid
Provides the vector backbone for assemblies.

T4 DNA Ligase Buffer (10X)
Contains buffer components optimal for BsmBI-v2 digestion and ligation of DNA

Required Materials Not Included

User-defined inserts
Competent cells
Other materials for transformation

Introduction

New England Biolabs now offers two specificities for Golden Gate Assembly; our original assembly kit (NEB #E1601) for BsaI-directed assemblies, and this assembly kit (NEB #E1602) for BsmBI-directed assemblies. This allows flexibility in choosing an appropriate kit based on the presence of internal sites in assembly components for any one Type IIS restriction enzyme. As with the previous assembly kit, optimization of enzyme amounts /ratios, buffer composition and soak/cycling parameters yields a kit of unparalleled functionality in terms of efficiency (number of transformants) and fidelity (% correct assemblies).

This assembly kit contains an optimized mix of BsmBI-v2 and T4 DNA Ligase. BsmBI-v2 has been engineered by NEB and outperforms BsmBI in Golden Gate Assemblies. Together these enzymes can direct the assembly of multiple inserts/modules and also single insert/library generation cloning with single insert(s) using the Golden Gate approach. Also provided is the pGGAselect destination plasmid, which provides a backbone for your assembly. This versatile destination construct has flanking recognition sites in the correct orientation for BsmBI-directed assemblies, and also BsaI- and BbsI-directed assemblies, enabling the destination plasmid to conveniently be used with all three of the most commonly used Type IIS restriction enzymes used for Golden Gate Assembly. It 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, BsmBI has a recognition site of CGTCTC(N1/N5), where the CGTCTC 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, and multi-site mutagenesis involved in directed evolution (7).

Please note that while general descriptions regarding Golden Gate Assembly use the BsmBI nomenclature, this kit and protocols feature the specific engineered form optimized for Golden Gate Assembly, BsmBI-v2.

Figure 1. Overview: Assembly Protocol of Golden Gate Assembly using BsmBI-v2

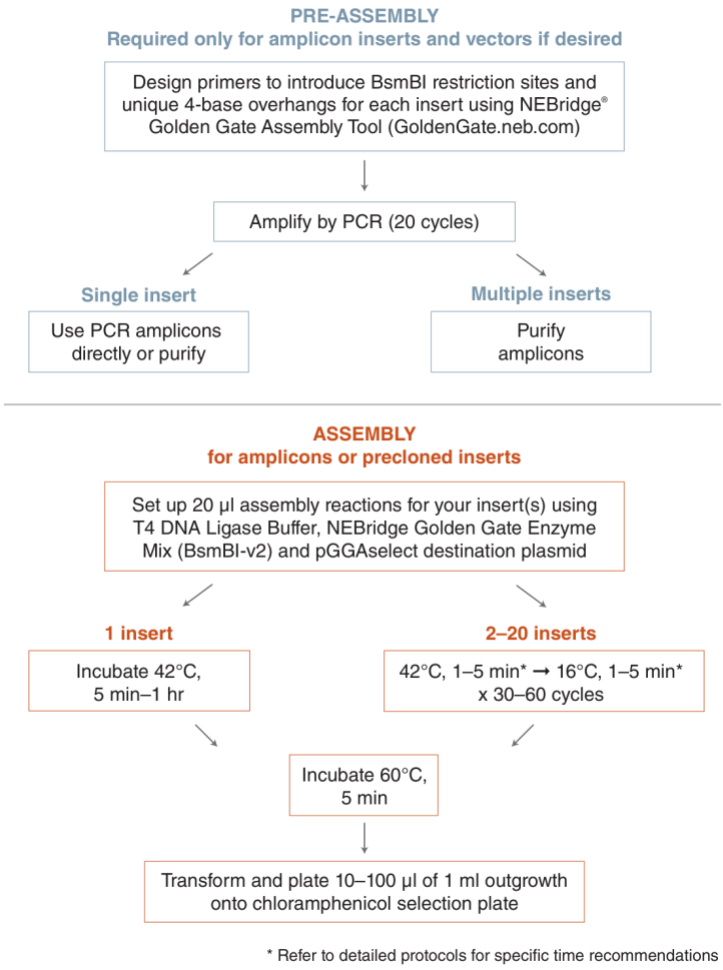
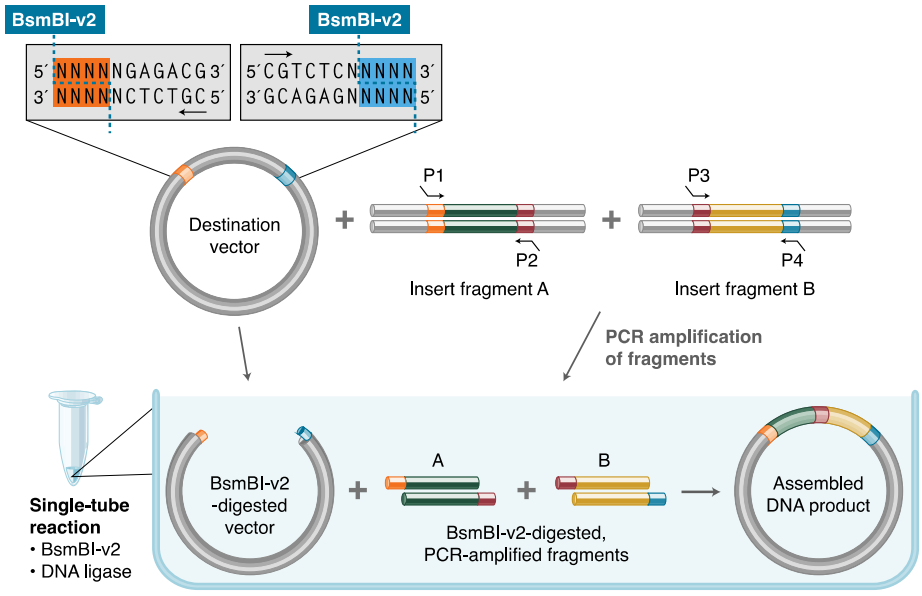


Figure 2. Golden Gate Workflow

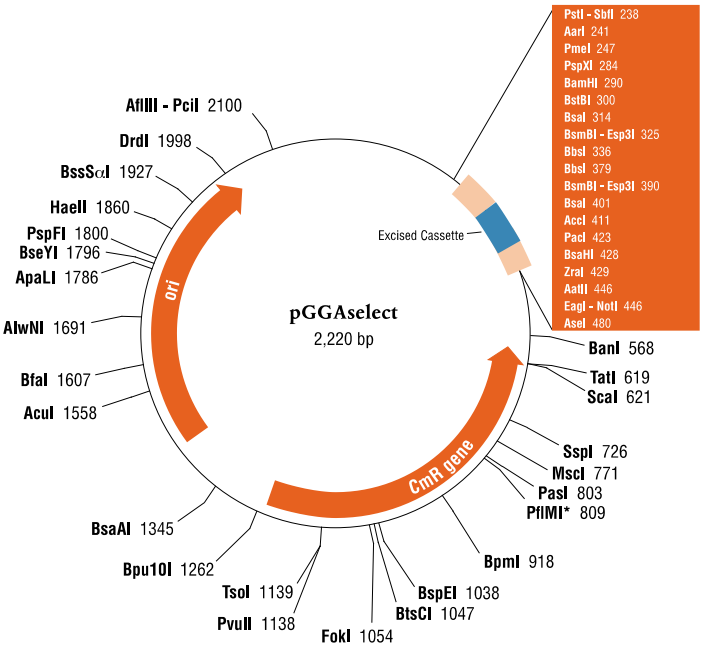


In its simplest form, Golden Gate Assembly requires a Type IIS recognition site, in this case, (CGTCTC), added to both ends of a dsDNA fragment. After digestion, these sites are left behind, with each fragment bearing the designed 4-base overhangs that direct the assembly.

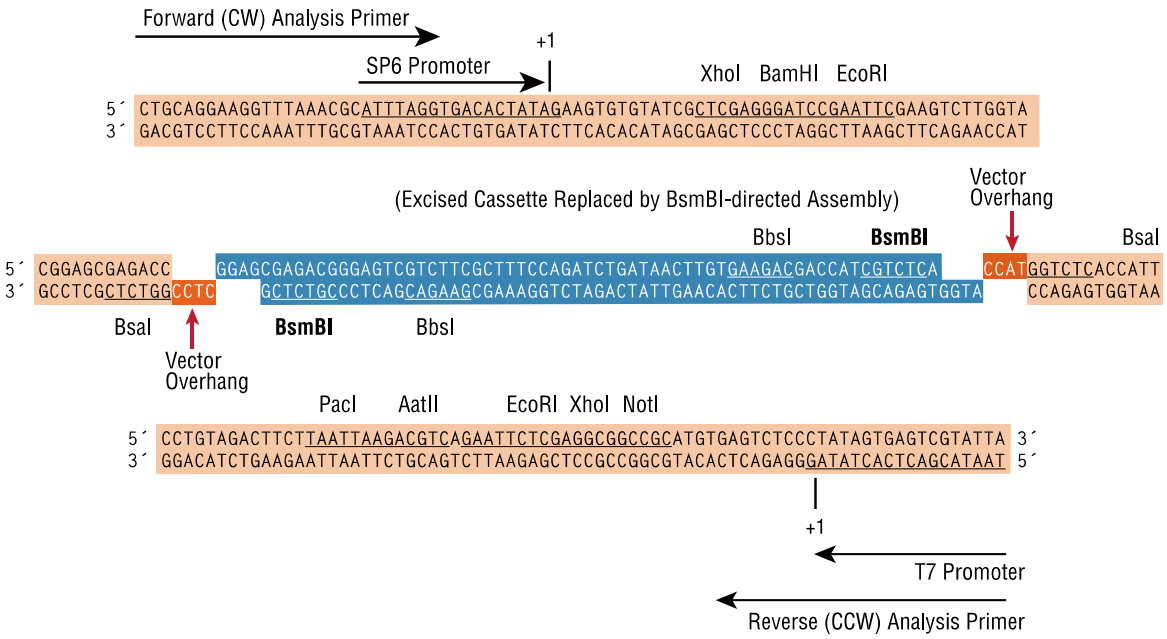
pGGAselect Destination Plasmid

pGGAselect is a 2,220 bp cloning vector useful for Golden Gate Assembly.

The plasmid contains two BsmBI, Bsa I and BbsI restriction sites; digestion with BsmBI releases a 65 bp fragment and a 2,155 bp vector backbone fragment to receive your insert or assembly.



Features within Sequence Flanking the Assembly Site



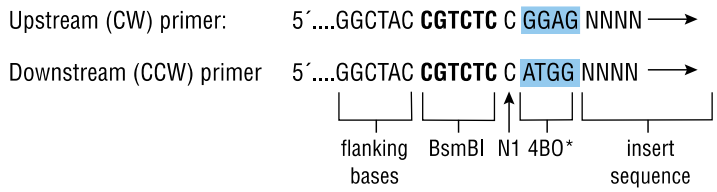
Insert Considerations

Historically, Golden Gate inserts were precloned into plasmid constructs having flanking Type IIS 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 superior for inserts < 250 bp or > 3 kb, or those containing repetitive elements that might accumulate errors during PCR amplification. Note that all sequences that will be part of the assembly must be flanked by correctly oriented BsmBI restriction sites, facing towards the insert on the top and bottom strands.

B. Amplicon Inserts: The 5′ flanking bases and BsmBI restriction enzyme recognition site are introduced through PCR primer design upstream and downstream of sequences to be assembled. In all cases, the 1:1 insert:vector backbone (2,155 bp for pGGAselect) molar ratio is suggested. For molar calculations we recommend using the NEBioCalculator (nebiocalculator.neb.com).

(a) Single Insert Cloning/Assembly Primer Design:



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

Single insert amplicons should be single specific PCR products, with no non-specific amplification or smearing present; if the amplicon is not present as a single product, optimize the PCR amplification.

While single insert amplicons can be used directly from PCR without purification under certain circumstances (see FAQ), it is always best to purify amplicons using spin columns such as the Monarch® Spin PCR & DNA Cleanup Kit (5 µg) (NEB #T1130). Use of these kits will result in purified, higher concentration DNA due to smaller elution volumes.

(b) Multiple Insert Assembly Primer Design. The first and last inserts will require the same four base overhangs diagrammed above for single insert assembly as these overhangs will ligate to the pGGAselect vector backbone. For all other inserts involved in the multiple insert assembly, we recommend using the NEBridge Golden Gate Assembly Tool (goldengate.neb.com) for the design of PCR primers to ensure the correct unique 4 base overhangs between inserts.

All amplicons should be single specific PCR products, with no non-specific amplification smearing present; if the amplicon is not present as a single product, optimize the PCR amplification.

All multiple inserts must be purified using spin columns as described above.

Detailed Protocols

Golden Gate Assembly Protocol:

1. Set up assembly reactions as follows:

REAGENT	ASSEMBLY REACTION
Destination Plasmid (1)	0.05 pmol (2) pGGAselect = 75 ng = 0.05 pmol
Inserts (user provided): -if precloned (3) - if in amplicon form (4)	 0.05 pmol each 1:1 molar ratio (insert:vector)
T4 DNA Ligase Buffer (10X)	2 µl
NEBridge Golden Gate Enzyme Mix (BsmBI-v2)	1–2 µl (5)
Nuclease-free H_2O	to 20 µl (6)

(1) User provided or pGGAselect.
(2) The NEBiocalculator® Tool (nebiocalculator.neb.com) can be used for molar calculations.
(3) Precloned inserts must possess BsmBI restriction sites at both ends of the insert sequence and in the proper orientation.
(4) Amplicon inserts must possess 5′ flanking bases (6 recommended) and BsmBI restriction sites at both ends of the amplicon and in the proper orientation.
(5) For assemblies ≤ 10 inserts, use 1 µl; for assemblies > 10 inserts, use 2 µl.
(6) Can be increased to 25 µl volume if required due to DNA component volumes; add additional 0.5 µl T4 DNA Ligase Buffer (10X)

2. Choose the appropriate assembly protocol:

INSERT	SUGGESTED ASSEMBLY PROTOCOL
For 1 Insert	42°C, 5 min (cloning) or 42°C, 1 hr (library preparation) → 60°C, 5 min
For 2–10 Inserts	(42°C, 1 min → 16°C, 1 min) x 30-60 → 60°C, 5 min
For 11–20+ Inserts	(42°C, 5 min → 16°C, 5 min) x 30-60 → 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 pGGAselect) or other appropriate antibiotic at 37°C.
2. Mix the cells thoroughly by flicking the tube and inverting, then spread 50 µl of a 1:5 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. Analysis primers are not included with the Golden Gate Assembly Kit. We recommend the following oligos be custom ordered through any oligo synthesis provider for colony PCR screening or sequencing of pGGAselect-based assemblies:

   Forward (CW) primer 65 bp upstream from assembly point:
   5′-CTGCAGGAAGGTTTAAACGCATTTAGG-3′

   Reverse (CCW) primer 62 bp downstream from assembly point:
   5′-TAATACGACTCACTATAGGGAGACTC-3′

   Note: The suggested forward primer is identical to that recommended for assemblies using pGGA as a destination plasmid (NEBridge Golden Gate Assembly Kit (BsaI-HFv2) (NEB #E1601). The reverse primers differ and are specific to pGGA or pGGAselect destination constructs.

   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) is strongly recommended.

2. Prepare plasmid mini-preps using the Monarch Spin Plasmid Miniprep Kit (NEB #T1110) and map 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. Note that larger assemblies will require internal assembly-specific primers to verify the full assembly sequence.

Frequently Asked Questions (FAQs)

For a complete list of FAQs, please visit the product page at www.neb.com

Q1. What is the mechanism for Golden Gate Assembly?

A1: Assembly utilizes two simultaneous enzymatic activities in a single reaction, Type IIS restriction endonuclease digestion and T4 DNA Ligase ligation. With optimized buffer components and enzyme ratios, a single reaction containing a destination plasmid and inserts (PCR amplicons or precloned) will result in ligation of inserts in the correct order and the accumulation of assembled product over time. The final assembly has none of the chosen Type IIS recognition sites, rendering the assembly inert to further digestion. For more information, view our online tutorial at www.neb.com/goldengate.

Q2. Which kit from NEB should I use for Golden Gate Assembly—this BsmBI-v2 kit (NEB #E1602) or the original BsaI-HFv2 kit (NEB #E1601)?

A2: It depends on whether there are any internal sites for these enzymes in your insert sequences. Since internal sites need to be eliminated by site-directed mutagenesis, choose the kit based on the Type IIS restriction enzyme that has no, or the fewest, sites in your insert sequences. If your sequences have neither BsmBI nor BsaI sites, the BsmBI-v2 kit (NEB #E1602) would be the best choice as it supports the highest complex assembly performance yet developed at NEB both in terms of efficiency (number of transformants) and fidelity (% correct assemblies). However, both kits support complex 24 fragment assemblies.

Q3. What if there are internal BsaI and BsmBI sites in my insert sequences?

A3: Either use site-directed mutagenesis to eliminate the internal sites, screen your sequences for the absence of other Type IIS restriction sites that could allow an alternative Type IIS restriction enzyme to be used such as BbsI, SapI/BspQI or BtgZI (building your assembly reactions using individual restriction enzyme and T4 DNA Ligase stocks), or consider another assembly approach such as NEBuilder® HiFi DNA Assembly if the assembly will involve 5 or less inserts.

Q4. The Type IIS restriction enzyme used in this kit is BsmBI-v2. How does it compare to the original BsmBI or Esp3I?

A4: While all 3 can perform complex (24-fragment) assemblies, BsmBI-v2 had the highest efficiency (number of transformants) and fidelity (% correctly assembled constructs) levels of the three. These comparison experiments used the individually optimized temperatures for each enzyme, 42°C for BsmBI and BsmBI-v2, and 37°C for Esp3I.

Q5. How does the performance of this kit compare to "home brews" built with individual Type IIS restriction enzymes and T4 DNA Ligase components?

A5: Building assembly reactions with separate enzyme components available at concentrations appropriate for standard digestion of DNA are limited by the need to keep glycerol levels in the reactions ideally at or less than 10%. These "home brew" assemblies will work, but will not match the efficiency and fidelity achieved with this kit that features higher enzyme levels.

Q6. What affects the efficiency of Golden Gate Assembly?

A6: 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 ~ 10-12 kb. Larger sized completed 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.

Q7. Why do many of the published Golden Gate Assembly articles feature precloned inserts as opposed to inserts generated by PCR?

A7: Precloned inserts allow stable storage of inserts while using amplicon inserts saves time. Stable storage of amplicon inserts is important and is best in a buffered solution. Single insert cloning/assemblies can use unpurified amplicons but will result in lower performance than if purified, while multiple insert amplicons should be purified, for example, by spin column protocols. We recommend the Monarch Spin PCR and DNA Cleanup Kit (5 µg, NEB #T1130S). 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 used for assembly reactions.

Q8. Using purified amplicons directly without precloning seems much easier, but is the assembly efficiency decreased?

A8: 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 are stored in the appropriate buffer (see FAQ #5). The suggested 1:1 molar ratio of amplicon inserts:destination vector has similar assembly efficiency to that of precloned inserts (using 0.05 pmol of each plasmid) for most assemblies.

Q9. Can PCR amplicons be used directly in single insert (cloning) assembly reactions without purification?

A9: Yes, as long as insert volume is 1 µl or less, although efficiencies will be decreased. Most Type IIS restriction enzymes used for Golden Gate Assembly generate 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 but will result in lower assembly performance. For multiple insert Golden Gate assemblies, purify the amplicons and if non-specific products are present, optimize the PCR or gel purify.

Q10. Why is Golden Gate Assembly also used for single insert cloning?

A10: 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 and directed evolution requiring multiple site mutagenesis.

Q11. Why do assembly reactions end with a 5 minute, 60°C incubation step?

A11: The final incubation step at 60°C favors Type IIS restriction enzyme cutting, in the absence of DNA ligation. Digesting any uncut or cut/religated destination plasmid still present in the assembly reactions reduces background.

Q12. How can I minimize PCR-generated errors in my amplicon inserts?

A12: 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.

Q13. What is an appropriate negative control for Golden Gate Assembly?

A13: Golden Gate assembly protocols do not usually call for a negative control. However if desired, a "no insert(s) added" reaction can be used.

Q14. How many cycles are optimal?

A14: Our enhanced enzyme stability allows more cycles than the traditional 30 cycles if larger numbers of transformants are desirable for complex assemblies or single insert library generation. For both our original BsaI-HFv2-based assembly kit and our BsmBI-v2-based assembly kit, efficiency increases dramatically from 30 cycles to 60-65 cycles, with no loss of fidelity.

Q15. Can I use other competent *E. coli* strains than NEB 10-beta? Can I use subcloning efficiency cells?

A15: Yes, other cell strains can be used, but large assemblies will require strains known to maintain large plasmid stability, such as NEB 10-beta competent *E. coli* (High Efficiency, NEB #C3019) or NEB Stable Competent *E. coli* (High Efficiency, NEB #C3040). NEB Stable Competent *E. coli* are also recommended for inserts containing repeat/unstable elements. For smaller assemblies other strains such as NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987), 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.

Golden Gate Assembly Tips

1. Use of the NEBridge Golden Gate Assembly Tool (GoldenGate.neb.com) is strongly recommended; this tool will check insert sequences for internal BsmBI sites and design primers to amplify your inserts for Golden Gate Assembly. The primers will feature 6 bases at the 5′ end flanking the BsmBI recognition site, the recognition site itself, plus the 4-base 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 greater accuracy (8). This ligase fidelity information can be used in conjunction with the NEBridge Golden Gate Assembly Kit (BsmBI-v2) to achieve high efficiency and accurate complex assemblies. Please visit www.neb.com/GoldenGate for more information.

3. NEB has developed ligase fidelity tools to facilitate the design of high-fidelity Golden Gate Assemblies:

   - NEBridge® Ligase Fidelity Viewer-visualize overhang ligation preferences
   - NEBridge GetSet® – predict high-fidelity junction sets
   - NEBridge SplitSet® – split DNA sequence for scarless high-fidelity assembly

   All tools are available at ligasefidelity.neb.com

4. Standard Golden Gate protocol suggests using 30 cycles, alternating between restriction and cutting. BsmBI-v2 and T4 DNA Ligase however are very stable, allowing cycling up to 60 cycles, with high efficiency and fidelity. Consider whether your workflow would be enhanced by adding more cycles.

Specifications

A 20 µl reaction containing 1X T4 DNA Ligase Buffer, 75 ng pGGAselect (Golden Gate destination plasmid, Cam^R), 75 ng each of 5 plasmids carrying fragments of a gene cassette encoding *laclZ* and 1 µl Golden Gate Enzyme Mix (BsmBI-v2) containing T4 DNA Ligase and BsmBI-v2 is incubated for 30 cycles of 42°C for 1 minute, 16°C for 1 minute, then at 60°C for 5 minutes.

Successfully assembled fragments result in functional *laclZ* gene expression in pGGAselect 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

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Ordering Information

NEB #	PRODUCT	SIZE
E1602S/L	NEBridge Golden Gate Assembly Kit (BsmBI-v2)	20/100 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
N0550S	Quick-Load® Purple 1 kb Plus DNA Ladder (0.1-10.0 kb)	125-250 gel lanes
M0494S	Q5 Hot Start High-Fidelity 2X Master Mix	100 reactions
M0493S	Q5 Hot Start High-Fidelity DNA Polymerase	100 units
M2499S/L	Q5-XT Hot Start High-Fidelity 2X Master Mix	100/500 units
N0447S	Deoxynucleotide (dNTP) Solution Mix	8 µmol of each
E0554S	Q5 Site-Directed Mutagenesis Kit	10 reactions
E0552S	Q5 Site-Directed Mutagenesis Kit (without competent cells)	10 reactions
C3019H	NEB 10-beta Competent *E. coli* (High Efficiency)	20 x 0.05 ml/tube
C2987H	NEB 5-alpha Competent *E. coli* (High Efficiency)	20 x 0.05 ml/tube
C2984H	NEB Turbo Competent *E. coli* (High Efficiency)	20 x 0.05 ml/tube
C3040H	NEB Stable Competent *E. coli* (High Efficiency)	20 x 0.05 ml/tube
C3020K	NEB 10-beta Electrocompetent *E. coli*	6 x 0.1 ml/tube
C2566H	NEB T7 Express Competent *E. coli* (High Efficiency)	20 x 0.05 ml/tube
B1500S/L	Nuclease-free Water	25/100 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	1/20
2.0	Name change NEB Golden Gate Assembly Kit (BsaI-HFv2) to NEBridge Golden Gate Assembly Kit (BsmBI-v2)	2/22
3.0	Updated reagent amounts for reaction setup. Also updated header and footer with new NEB logo and hyperlinked TOC.	6/26
3.1	Updated Figure 1.	11/26

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