Eleven Tips For Optimizing Your Golden Gate Assembly Reactions

Looking to assemble multiple DNA fragments in a single reaction? Here are some tips to keep in mind when planning your Golden Gate Assembly experiment.

1 Check your sequences

Always check your assembly sequences for internal sites before choosing which Type IIS restriction endonuclease to use for your assembly. While single insert Golden Gate Assembly has such high efficiencies of assembly that the desired product is obtainable regardless of the presence of an internal site, this is not true for assemblies with multiple inserts. Options include choosing a different Type IIS restriction enzyme to direct your assembly, or eliminating internal sites through domestication. Our tutorial video on Golden Gate Assembly Domestication provides a full description of the many options available for internal site issues. Note the use of a Type IIS restriction enzyme with a 7 base recognition site, such as PaqCI, is less likely to have internal sites present in any given sequence.

2 Orient your primers

When designing PCR primers to introduce Type IIS restriction enzyme sites, either for amplicon insert assembly or as an intermediate for pre-cloning the insert, remember that the recognition sites should always face inwards towards your DNA to be assembled. Consult the Golden Gate Assembly Kit manuals or assembly videos for further information regarding the placement and orientation of the sites.

3 Choose the right plasmid

Consider switching to the versatile pGGAselect Destination Plasmid for your Golden Gate Assembly. This versatile new destination construct is included in all Golden Gate Assembly kits and can be used for BsaI-HFv2, BsmBI-v2 or BbsI directed assemblies. It also features T7 and SP6 promoter sequences flanking the assembly site, and has no internal BsaI, BsmBI or BbsI sites. The pGGAselect plasmid can also be transformed into any *E. coli* strain compatible with pUC19 for producing your own plasmid preparation if so desired.

4 Choose the right buffer

T4 DNA Ligase Buffer works best for Golden Gate Assembly with BsaI-HFv2, BsmBI-v2 and PaqCI. However, alternate buffers would be NEBuffer r1.1 for Bsa-HFv2, NEBuffer r2.1 for BsmBI-v2, or rCutSmart™ Buffer for PaqCI, if these buffers are supplemented with 1 mM ATP and 5–10 mM DTT. NEB also offers NEBridge Ligase Master Mix that has been optimized for Golden Gate Assembly with our Type IIS restriction enzymes for Golden Gate.

5 Increase your complex assembly efficiency by increasing the Golden Gate cycling levels

T4 DNA Ligase, BsaI-HFv2, BsmBI-v2 and PaqCI are very stable and continue to be active during extended cycling protocols; an easy way to increase assembly efficiencies without sacrificing fidelity is to increase the total cycles from 30 to 45–65, even when using long (5-minute) segments for the temperature steps.

6 Make sure your plasmid prep is RNA-free

For pre-cloned inserts/modules, make sure your plasmid prep is free of RNA to avoid an overestimation of your plasmid concentrations.

7 Avoid primer dimers

For amplicon inserts/modules, make sure your PCR amplicon is a specific product and contains no primer dimers. Primer dimers, with Type IIS restriction endonuclease sites (introduced in the primers used for the PCR reactions), would be active in the assembly reaction and result in mis-assemblies.

8 Avoid PCR-induced errors

Do not over-cycle and use a proofreading high fidelity DNA polymerase, such as Q5® DNA High-Fidelity Polymerase.

9 Decrease insert amount for complex assemblies

For complex assemblies involving >10 fragments, pre-cloned insert/modules levels can be decreased from 75 to 50 ng each without significantly decreasing the efficiencies of assembly.

10 Carefully design EVERY insert's overhang

An assembly is only as good as its weakest junction. 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. This ligase fidelity information can be paired with the appropriate Type IIS restriction enzyme chosen to direct your assembly to achieve high efficiencies and accurate complex assemblies. Please use the free NEBridge Golden Gate Assembly Tool to design primers for your Golden Gate Assembly reactions. Use NEBridge Ligase Fidelity Tools to predict overhang fidelity or find optimal Golden Gate junctions for long sequences.

11 If using pre-cloned proven inserts that suddenly become problematic, check for a possible mutational event in your sequence

Be aware that occasionally a pre-cloned insert/module can become corrupted by an error during propagation in *E. coli*, usually a frameshift due to slippage in a run of a single base (e.g., AAAA) by the *E. coli* DNA Polymerase. This should be suspected if previously functional assembly components suddenly become nonfunctional.

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Did you know that many of these products can be purchased in large volumes and custom formats? Learn more at **www.neb.com/customizedsolutions**

Now includes NEBridge® Ligase Master Mix

Golden Gate Assembly

50+ FRAGMENT ASSEMBLY NOW ACHIEVABLE WITH HIGH EFFICIENCY AND ACCURACY

NEW ENGLAND Biolabs®

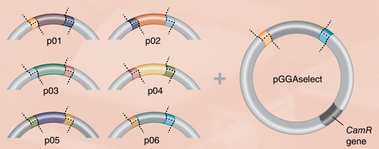
Push the limits of Golden Gate Assembly

With constant advances in both the development of new enzymes, tools and research on maximizing enzyme functionality (e.g., ligase fidelity), NEB is the industry leader in pushing the limits of Golden Gate Assembly and related methods.

Advantages:

- Clone seamlessly, with no scars remaining after assembly
- Perform single insert cloning in just 5 minutes using our fast protocols
- Generate libraries with high efficiencies
- Assemble multiple fragments (2–50[+]) in order, in a single reaction
- Experience high efficiency, even with regions of high GC content and areas of repeats
- Use with a broad range of fragment sizes (<100 bp to >15 kb)
- Simplify reaction setup with our suite of primer design and ligase fidelity tools

GOLDEN GATE ASSEMBLY WORKFLOW
FOR BOTH SIMPLE AND COMPLEX ASSEMBLIES



In its simplest form, Golden Gate Assembly requires a Type IIS recognition site, added to both ends of a dsDNA fragment. After digestion, these sites are left behind, with each fragment bearing the designed 3- or 4-base overhangs that direct the assembly.

FEATURED PRODUCTS:

Type IIS Restriction Enzymes used in Golden Gate Assembly

Type IIS restriction enzymes recognize asymmetric DNA sequences and cleave outside of their recognition sequence. Type IIS enzymes commonly used in Golden Gate Assembly are listed below. NEB currently offers over 50 Type IIS restriction enzymes.

Please visit **www.neb.com** for comprehensive table.

PaqCI

5´CACCTGCNNNNNNNN3´
3´GTGGACGNNNNNNNNN5´

PRODUCT	NEB #	SEQUENCE	SIZE
BbsI	R0539S/L	GAAGAC(2/6)	300/1,500 units
BbsI-HF	R3539S/L	GAAGAC(2/6)	300/1,500 units
BsaI-HFv2	R3733S/L	GGTCTC(1/5)	1,000/5,000 units
BsmBI-v2	R0739S/L	CGTCTC(1/5)	200/1,000 units
BspQI	R0712S/L	GCTCTTC(1/4)	500/2,500 units
BtgZI	R0703S/L	GCGATG(10/14)	100/500 units
Esp3I	R0734S/L	CGTCTC(1/5)	300/1,500 units
PaqCI®	R0745S/L	CACCTGC(4/8)	200/1,000 units
SapI	R0569S/L	GCTCTTC(1/4)	250/1,250 units

What users are saying:

"NEB has developed a reliable set of enzymes and design tools for Golden Gate Assembly that we use regularly with success. We have found the Ligase Fidelity Viewer particularly useful for screening overhang sets that are constrained by a pre-existing protein/DNA sequence. The thorough experimental basis of the tool and the availability of the underlying data are added bonuses."

– Dr. Glenna Foight,
Senior Scientist, Lyell Immunopharma

FEATURED KITS:

NEBridge Golden Gate Assembly Kits (BsmBI-v2 or BsaI-HF®v2)

The absence of internal sites in a sequence determines the choice of which Type IIS restriction enzyme to drive the assembly. For your convenience, NEB now offers two kits for Golden Gate Assembly featuring BsaI-HFv2 or BsmBI-v2. Both kits incorporate digestion followed by ligation with T4 DNA Ligase into a single reaction, and can be used to assemble 2-50[+] fragments in a single step.

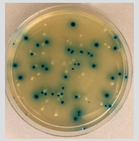
PRODUCT	NEB #	SIZE
NEBridge Golden Gate Assembly Kit (BsmBI-v2)	E1602S/L	20/100 rxns
NEBridge Golden Gate Assembly Kit (BsaI-HFv2)	E1601S/L	20/100 rxns

"...the Golden Gate Assembly paper: it revolutionized the technique."

– Dr. Edward Green,
Team Leader, Cancer Research Center (DKZ)

ONE-POT GOLDEN GATE ASSEMBLY
OF 52 FRAGMENTS INTO A DESTINATION VECTOR

A.



B.

52 FRAGMENT *lac* ASSEMBLY COLONY FORMING UNITS*

	CORRECT	INCORRECT	% CORRECT
Replicate #1	520	580	47
Replicate #2	760	740	51
Replicate #3	900	880	51
Average	727	733	49

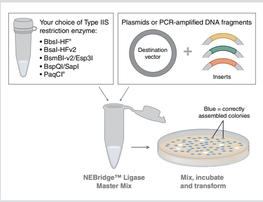
*per 100 µl of outgrowth plated

(A) Example outgrowth plate used for colorimetric scoring by reverse blue-white screening. Correctly assembled 52 insert constructs form blue colonies upon cellular transformation and incorrectly assembled constructs produce white colonies. (B) Results of the assembly reactions. This replicate experiment was carried out to quantify the number of colony-forming units harboring correct and incorrect assembly products per 100 µl of E. coli outgrowth plated (0.2 µl of the assembly reaction). On average, 49% of the observed transformants harbored correctly assembled constructs.
Pryor, J. M. et al. (2022) ACS Synth. Biol., 11, 6, 2036–2042.

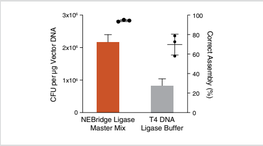
Try NEBridge® Ligase Master Mix for Added Flexibility

NEBridge Ligase Master Mix is a 3X master mix for Golden Gate Assembly. Designed for use with NEB Type IIS restriction enzymes, this master mix contains T4 DNA Ligase in an optimized reaction buffer with a proprietary ligation enhancer. Users need only choose their preferred NEB Type IIS restriction enzyme and add DNA substrates to be assembled. Low complexity single fragment insertions, as well as moderate complexity (3–6 fragment) and high complexity (7–25[+] fragment) assemblies, are all supported with this optimized reagent and accompanying protocols.

PRODUCT	NEB #	SIZE
NEBridge® Ligase Master Mix	M1100S/L	50/250 rxns



Workflow for NEBridge Ligase Master Mix



The total transformants and percentage of correct assemblies (blue colonies) were reported as the average result of three replicates with the standard deviation from the mean. The reaction with NEBridge Ligase Master Mix generated 2.2 ± 0.2 x 10⁶ correctly assembled blue colonies per µg vector DNA with 94.3 ± 1% fidelity, while the reaction with T4 DNA Ligase Buffer generated 8.3 ± 2.1 x 10⁵ correctly assembled blue colonies per µg vector DNA with 69.8 ±10.7% fidelity.

Ligase Fidelity Tools

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 to predict which overhangs have improved fidelity. This research has enabled the development of tools that enable the design of highly complex fragment assemblies with high efficiencies and >90% accuracy.

Try our suite of free online tools to design high fidelity Golden Gate Assemblies under various experimental conditions:

For help designing primers, try the **NEBridge Golden Gate Assembly Tool** at GoldenGate.neb.com

Try our **NEBridge Ligase Fidelity Tools** for the design of high-fidelity Golden Gate assemblies at ligasefidelity.neb.com

- NEBridge Ligase Fidelity Viewer® (v2) – Visualize overhang ligation preferences
- NEBridge GetSet® – Predict high-fidelity junction sets
- NEBridge SplitSet® – Split DNA sequence for scarless high-fidelity assembly

More information can be found in NEB publication, Comprehensive Profiling of Four Base Overhang Ligation Fidelity by T4 DNA Ligase and Application to DNA Assembly (3), Enabling one-pot Golden Gate Assemblies of unprecedanted complexity using data-optimized assembly design (4) or in our webinar, Listen to DAD Informatics tools and NEB enzymes to enable complex one-pot Golden Gate Assemblies.

Visit **www.neb.com/GoldenGate** to learn more and view related videos

- Golden Gate Assembly Workflow
- Golden Gate Assembly Domestication
- Golden Gate Assembly Tool Tutorial
- Listen to DAD when constructing high-complexity Golden Gate Assembly Targets

References
1. Engler, C., Kandzia, R., and Marillonnet, S. (2008) *PLoS ONE* 3, e3647.
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3. Potapov, V. et. al. (2018) *ACS Synth. Biol.* 7,1, 2665–2674.
4. Pryor, J.M. et. al. (2020) *PLoS ONE.* 15: e0238592.