Golden Gate Assembly Kit (BsmBI)

REF: EG25207-V/S

Storage Condition

Store at -20℃ for 2 years.

Components

Component	EG25207V	EG25207S
Golden Gate Mix (BsmBI)	10 μl	50 μl
10× T4 DNA Ligase Buffer	1 ml	1 ml

Description

Golden Gate Assembly Kit share the same single-step precision cloning strategy, popularly known as "Golden Gate Assembly", that relies on the unique properties of type IIs restriction enzymes to generate compatible ends on DNA fragments that are then joined together by the T4 DNA ligase. The Kit are particularly adept at assembling difficult to-clone sequences such as repetitive and very small sequences (＜ 100 bp), gene variants, and TAL (transcription activator-like) effector genes.

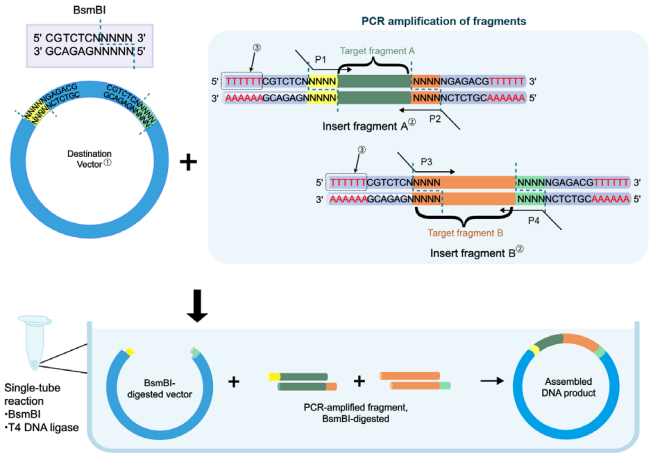
Type IIS restriction enzymes bind to their recognition sites but cut the DNA downstream from that site at a positional, not sequence specific, cut site. Thus, a single Type IIS restriction enzyme can be used to generate DNA fragments with unique overhangs.

The cloning procedure is as follows: Design recognition sites for type IIS restriction enzymes outside the cleavage sites of the target gene. 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 vector contains sticky ends complementary to those of the target gene, enabling ligation without introducing extraneous sequences, thereby achieving seamless cloning.

The Golden Gate Assembly Kit (BsmBI), based on the above principle, contains all enzymes required for restriction digestion and ligation in a ready-to-use Mix for convenient pipetting. It supports ligation of up to 16 fragments in a single reaction, fully satisfying diverse experimental requirements.

Experimental Principle

1. Example Workflow for Single DNA Fragment Insertion:



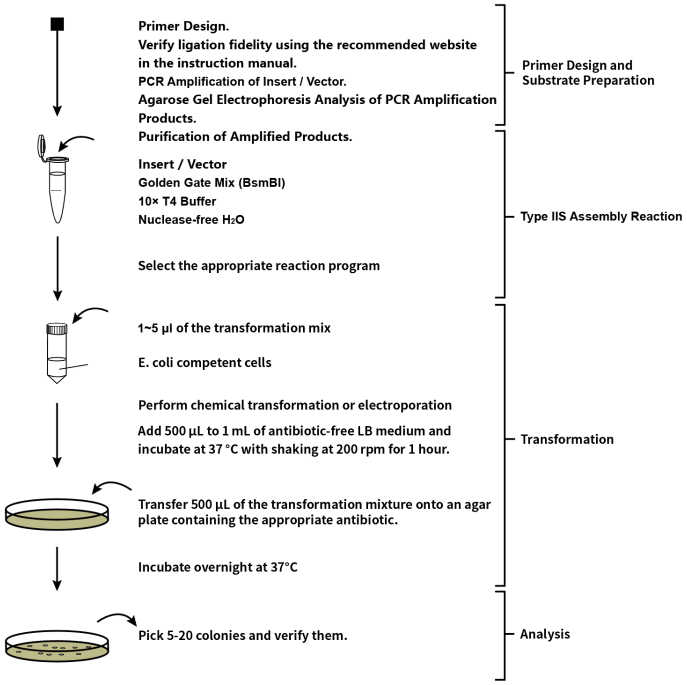
Note 1: The vector here is generated via restriction enzyme digestion (requires BsmBI sites). Alternatively, it can be PCR-amplified using primers designed as described below.

Note 2: Target inserts must be prepared by PCR with overhanging adapters added through primer design. Use high-fidelity polymerase (REF: EG24110) to ensure amplification accuracy.

Note 3: Red "TTTTTT" indicates protective bases—adjust quantity per enzyme specifications, six bases are recommended.

Note 4: While the diagram shows single-fragment assembly, multi-fragment ligation follows the same principle. Simply modify cohesive end sequences to scale up fragment count.

Workflow



Notice

1. Clone efficiency is heavily influenced by the type, quantity, and size of inserted DNA. Although experimental conditions can be optimized, cellular toxicity from certain exogenous DNA fragments and intracellular recombination cannot be entirely avoided.

2. Fragments required for ligation are typically obtained via PCR but may also come from pre-cloning or synthesis. Regardless of the method used, correct orientation of restriction sites between adjacent fragments must be ensured to enable precise end-joining after digestion.

3. The Golden Gate Assembly Kit facilitates assembly of repetitive or short difficult-to-clone sequences; however, cloning efficiency decreases progressively with increasing fragment count. When exceeding ten inserts, efficiency drops significantly. To secure target constructs, we recommend screening ~20 colonies.

4. Pre-cloned DNA fragments exhibit higher assembly efficiency than PCR-amplified ones, particularly when assembling tandem repeats with >80% identity.

5. Use a thermal cycler with appropriate programs to guarantee optimal reaction performance.

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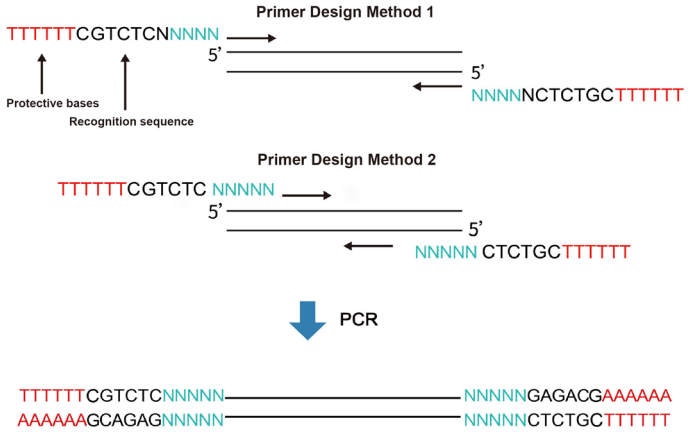
Primer Design Guide

PCR-mediated incorporation of BsmBI recognition sites is achieved by adding the sequence to the 5'-end of primers. To ensure stable binding and cleavage by the restriction enzyme on double-stranded DNA, protective bases must be appended to the terminal end of the recognition site. While the number/type of these protective bases varies (refer to Yugong's Practical Guide to Restriction Enzymes for details), we recommend using 6 bp—a length sufficient for most standard digestion protocols. As the cut site lies downstream of the recognition sequence and accommodates arbitrary flanking sequences, two common primer design strategies exist (illustrated below):



Primer Design Method 1: Protective bases + restriction site (TTTTTTCGTCTCNNNNN) are both incorporated via primers. The remaining NNNNN sequence corresponds to the insert fragment (minimum length: 15 bp). These two segments together form the complete primer.

Primer Design Method 1: Only protective bases + core recognition motif (TTTTTTCGTCTCN) are added through primers, while NNNN represents the insert sequence itself.

Note 1: Whether NNNN belongs to the insert depends on your workflow. For scarless cloning when both insert and vector derive from PCR amplification, exclusively assign NNNN to either the fragment OR vector—never both. Use distinct primer schemes accordingly.

Note 2: The NNNN overhang sequence significantly affects ligation specificity. Though theoretically 256 combinations exist, avoid palindromic sequences. We recommend NEB's Golden Gate Toolkit™ for optimized design: https://goldengate.neb.com/#!/。

Additional Primer Design Considerations:

(1) Amplicon size limit: Keep target products under 5 kb to ensure fidelity; base primer design on this constraint.

(2) Melting temperature (Tm): Maintain 58~60℃ for template-binding regions for optimal amplification efficiency.

(3) Secondary structure prevention: Avoid complementary sequences within or between primers to prevent hairpin formation.

(4) Critical quality control: Primer integrity directly impacts downstream ligation success—even single-base mutations in cohesive ends may cause failure. Use verified oligo manufacturers.

Notice for PCR

1. Optimize individually: Tailor PCR conditions per fragment to ensure singular product yield.

2. Purify multi-banded products: If multiple bands appear, gel-purify target DNA—failure to do so severely compromises assembly efficiency or causes complete failure. During excision: minimize UV exposure time; prefer blue light systems to reduce DNA damage.

3. Fragment size strategy: For amplicons >5 kb, use multiple smaller fragments instead of single large ones during ligation (larger fragments sustain more gel extraction damage).

4. Elution buffer selection: Elute DNA with ddH_2O or 10 mM Tris (pH 8.0). Avoid TE buffer to prevent inhibition of downstream ligation.

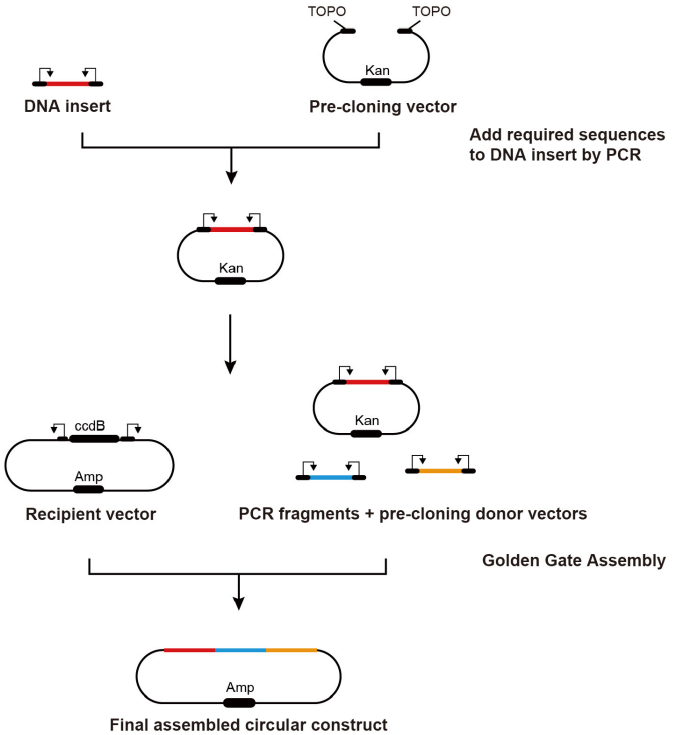
5. Purification requirements: Unpurified PCR products may work for single-fragment cloning but require purification for multi-fragment assembly.

Pre-cloning Guide

Pre-cloning involves cloning a DNA insert generated by PCR amplification into an intermediate vector ("pre-cloning donor vector", usually ~3 kb in size) and then using the pre-cloning construct carrying the insert directly in the Golden Gate Assembly Kit (BsmBI), which relies on a single-step, consolidated restriction-ligation reaction to create the final recombinant construct.

For best results, we recommend using the TOPO as your pre-cloning donor vector, the vector contains a selection gene for the screening of positive clones. The pre-cloning method is not fixed, you may choose your preferred cloning approach according to your habits.

An example pre-cloning procedure using TOPO cloning is provided below:



Note 1: Black arrows in fragment/vector diagrams indicate restriction enzyme directionality—from recognition site toward cut position.

Note 2: The ccdB gene acts as an E. coli toxicity marker; only colonies with disrupted ccdB expression (via insert integration) will survive transformation.

For sequences with >80% identity (repetitive/homologous regions), use pre-cloning.

When inserting >5 fragments, perform stepwise pre-cloning into the target vector.

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Protocol

1. Prepare the following reaction system on ice:

Component	Ligation Reaction	Negative Control[a]
Vector	0.05 pmol[b]	0.05 pmol
Insert	0.1 pmol[c]	/
10× T4 DNA Ligase Buffer	2 μl	2 μl
Golden Gate Mix (BsmBI)	1 μl	1 μl
Nuclease-free H_2O	To 20 μl	To 20 μl

a. Golden Gate Assembly typically requires no negative control. When needed, prepare a reaction without insert fragments as the negative control.

b. For a 2000 bp vector: 0.05 pmol corresponds to 60 ng; calculate amounts for other lengths proportionally based on this equivalence.

c. Use a molar ratio of insert:vector = 2:1 and maintain 1:1 stoichiometry among all inserts. Swap input quantities if any single fragment exceeds vector mass.

Note: More inserts reduce ligation efficiency/transformation success rates. Excessively long fragments or vectors also lower efficiency.

After preparing the mixture above, vortex thoroughly and run reactions in a PCR instrument using cycle programs selected below based on insert count.

2. Recommended reaction program:

Number of inserts	Reaction program
1	42℃，5 min→65℃，5 min
2~4	42℃，1 h→65℃，5 min
5~10	(42℃，1 min→22℃，1 min) ×30~60→65℃，5 min

Note: While single-step assembly of target fragments is achievable with >10 fragments, both positive rate and colony count will significantly decrease. To ensure experimental success, we recommend performing multiple assembly steps for scenarios involving 10+ fragments.

Available Isothermal Program:

Number of inserts	Reaction program
1	30℃，5 min→65℃，5 min
2~10	30℃，1~2 h→65℃，5 min

Note: Isothermal reactions do not affect efficiency in simple scenarios like single-fragment reactions. However, with multiple fragments, efficiency gradually declines as the number of fragments increases. An efficiency of over 70% can still be maintained for 2~5 fragments. When conditions permit, prioritize using the temperature-cycling program.

3. After reaction completion, it can be used directly for transformation or stored at -20℃ for later use.

4. Transformation of Recombinant Products

Take 5~10 μl of reaction solution and add to 100 μl of competent cells. Mix gently by pipetting up and down, then place on ice for 30 minutes. Apply heat shock at 42℃ for 45~60 seconds, followed by an ice bath for 5 minutes. Add 500 μl of SOC or LB medium, then shake culture at 37℃ (200 rpm) for 40~60 minutes. Evenly spread the bacterial suspension onto plates containing corresponding antibiotics, invert the plates, and incubate overnight at 37℃ .

Note 1: Final cloning positive rates may vary among different batches of competent cells; we recommend using cells with a transformation efficiency > 10^8 CFU/μg.

Note 2: Colony count depends on both quantity and purity of PCR products and linearized vectors.

Note 3: The positive control plate typically shows abundant white single colonies, while the negative control plate exhibits minimal colony growth.

5. Positive Clone Screening

Upon completion of cloning, products must undergo screening and identification—typically via restriction digestion or PCR.

Restriction Digestion Protocol: Inoculate 5~20 colonies into 1 ml of antibiotic-supplemented LB broth and culture overnight. Next day, isolate plasmid DNA, digest with selected restriction enzymes, and analyze fragments by agarose gel electrophoresis to identify target plasmids.

For standard fragment ligation, 5~10 colonies usually yield the desired product. For constructs containing repetitive/homologous sequences or >10-fragment assemblies, screen 20 colonies to ensure correct ligation.

PCR:

(1) Design appropriate detection primers with flexible amplicon length based on insert size. For single-fragment insertions, target amplicons between 500 bp~2 kb; place forward/reverse primers on vector + insert respectively. For multi-fragment insertions, position both primers entirely on the vector to amplify full-length assembled inserts.

(2) Inoculate 5~20 colonies into 1 ml antibiotic-containing LB medium overnight. Transfer 1 μl of culture directly into a 30 μl PCR reaction system per sample.

(3) Modify standard PCR protocol by extending initial denaturation from 95℃ for 3 min to 95℃ for 10 min; keep other parameters unchanged. Analyze amplified products via agarose gel electrophoresis post-PCR.

(4) Select cultures with correct amplification for plasmid isolation to obtain desired ligation products. (Recommended: Sequencing validation prevents PCR errors during amplification).

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FAQ & Troubleshooting

Problem	Possible Reason	Solution
Low transformation efficiency	Low efficiency of competent cells	Use freshly prepared or properly stored competent cells.
	Unfavorable ratio of DNA fragments	Prepare the reaction system according to the recommended optimal amounts and ratios in the instructions. Determine the concentrations of the vector and insert fragments as follows: If the linearized vector and insert fragment have been purified and show a single band on agarose gel electrophoresis, their concentrations can be measured using instruments based on spectrophotometric methods. However, the concentration value is reliable only when the A260/A280 ratio is between 1.8 and 2.0. If the linearized vector and insert fragment have not been purified, sample concentrations can be measured using agarose gel electrophoresis.
	Insufficient purity of DNA fragments	Purify the vector and insert.Since metal ion chelators such as EDTA inhibit the seamless cloning reaction, the purified products should be dissolved in ddH_2O. Do not use buffers such as Tris-EDTA.
	Excessive reaction products	In the transformation mixture, the volume of the seamless cloning reaction product should not exceed 10% of the competent cell volume.
	Fragment too long or too many fragments	Golden Gate Assembly generally enables efficient ligation of up to 10 DNA fragments. However, ligation efficiency decreases significantly when individual fragments are excessively long, and is also compromised for assemblies involving more than 10 fragments. This method is recommended for ligation applications with a total size not exceeding 10 kb.
Large numbers of clones lack the insert fragment	Incomplete linearization of the vector	When preparing linearized vectors by restriction digestion. Increase the amount of fast endonuclease used, extend reaction time, and purify digested products via gel extraction.
	Contamination with identical resistant plasmids	When performing PCR amplification of insert fragments using plasmid templates: Use pre-linearized plasmid as the amplification template; treat amplicons with methylation-sensitive endonucleases like DpnI or purify products by gel extraction.
	Insufficient antibiotic resistance on plates	Ensure correct antibiotics are used with freshly prepared antibiotic plates.
	Adapter Design Issues	Variation in ligation efficiency among different overhangs may lead to nonspecific joins if adapters are improperly designed. We recommend pre-simulating connection fidelity using bioinformatics tools to minimize off-target amplification risks.
Large numbers of clones contain incorrect insert fragments.	Non-specific PCR amplification products	Optimize the PCR system to improve amplification specificity, or perform gel extraction purification of PCR products containing overlapping sequences of primers.
	Adapter Design Issues	When connecting multiple fragments, adapter diversity significantly impacts ligation accuracy and may cause nonspecific joins. We recommend pre-simulating connection fidelity using bioinformatics tools to minimize off-target amplification risks.
	Amplification Errors in Fragments/Vectors	This method relies on 4-base overhangs, demanding high PCR fidelity. Base mutations can lead to misligation or failed ligation. Use high-fidelity polymerase for amplifying substrates.

FAQs

1. What is the maximum number of fragments per assembly reaction?

Our testing shows a single reaction can technically accommodate up to 15 fragments, though colony counts and positive rates drop significantly at this scale. To ensure experimental success, we recommend limiting each assembly to ≤5 fragments. If multi-fragment assembly fails in one attempt, perform stepwise assembly by reducing the number of fragments per reaction.

2. What is the acceptable range for insert size?

Tested insert lengths range from 20 bp to 10 kb. Cloning efficiency decreases with longer fragments; we recommend keeping the combined length of insert + vector under 13 kb for optimal results.

3. Can reaction time be extended or shortened?

Yes. For single-fragment assembly, 42℃ for 5 minutes is sufficient to generate substantial transformants. When assembling multiple fragments, we recommend running 30~60 cycles—adjust the cycle count based on ligation efficiency if needed. However, do not exceed 60 cycles, as additional cycles will not improve ligation outcomes and may increase the risk of nonspecific assembly.

4. Why do assembly reactions end with a 5 minute, 65℃ incubation step?

The final incubation step at 65℃ 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.

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

Yes, but typically only for single-fragment assembly and in volumes not exceeding 1 µl. Unpure PCR products contain residual DNA polymerase, which may fill in overhangs generated by Type IIS restriction enzyme digestion—causing nonspecific ligation. Additionally, residual polymerase competes

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with ligase, reducing connection efficiency. Furthermore, PCR amplicons often include nonspecific products or primer dimers, leading to off-target ligation events.

6. Can Golden Gate Assembly products serve as templates for subsequent PCR?

Yes. The assembled product exists as closed circular double-stranded DNA, making it suitable for downstream PCR amplification and other DNA amplification techniques such as rolling circle amplification (RCA).

7. What if there are internal BsmBI sites in my insert sequences?

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 (REF: EG24512)，BsaI (REF: EG15518)，BspQI (REF: EG23503). This kit is exclusively designed for use with BsmBI and is not compatible with other Type IIS restriction enzymes.

8. How to Select the Appropriate Golden Gate Assembly Kit?

This depends on the presence of Type IIS restriction enzyme recognition sites within your insert. Since internal sites must be removed via site-directed mutagenesis, choose a kit based on a Type IIS enzyme with no existing sites or minimal recognition sites in your target sequence.

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

This kit is specifically optimized for Golden Gate Assembly, delivering high efficiency and convenience for ligation reactions involving up to 10 fragments.

10. What affects the efficiency of Golden Gate Assembly?

Cloning efficiency and colony output in the kit are greatly influenced by the type, number, and size of the DNA fragments used. It is recommended to use fragments within 10 kb for the ligation reaction, and purified substrates should be used. We recommend using inserts within 10 kb and purified substrates for optimal results.

11. What are the advantages of pre-cloning, and when is it recommended?

Pre-cloned inserts stored in circular plasmids offer greater stability than PCR products, enabling long-term preservation of the sequence. For multiple or large inserts, stepwise assembly via pre-cloning is advised—though this extends experimental duration, it significantly improves success rates. Precloned inserts allow stable storage of inserts.

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