Seamless cloning method

For use with IDT Gene Fragments, such as:

- gBlocks™ Gene Fragments
- gBlocks HiFi Gene Fragments
- eBlocks™ Gene Fragments

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INTRODUCTION

The seamless cloning method [1] is a great alternative to traditional restriction site cloning and offers the advantage of a single-tube reaction that assembles multiple fragments seamlessly in as little as 30 minutes (Figure 1). This method, which includes Gibson Assembly and Golden Gate, provides directional cloning without the need for specific restriction sequences. Seamless cloning relies on the use of an enzyme mixture consisting of a mesophilic exonuclease, thermophilic ligase, and high-fidelity polymerase. And because assembly requires intact sequences at both termi, this method can select against truncated sequences or sequences with errors at the ends. We recommend the seamless cloning method for assembly and most cloning applications.

For the assembly reaction, IDT Gene Fragments (e.g., gBlocks, gBlocks HiFi, and eBlocks Gene Fragments) and the vector insertion site are designed with overlapping sequences at the locations that are to be joined. At 50°C, the exonuclease digests dsDNA from the 5′ ends (Figure 2). The resulting single-stranded, complementary ends are then available to hybridize to each other, at which point the polymerase fills in missing nucleotides and the ligase covalently joins the fragments together.

Since the introduction of the seamless cloning method, several assembly kits are commercially available. Most of these kits function similarly, and the protocol description below is broadly applicable to most kits. There are several, newer Gibson Assembly kits that use multiple temperatures and allow for more fragments and larger constructs to be assembled in a single reaction, but the enzymes and steps are also generally analogous to in the isothermal process described below.

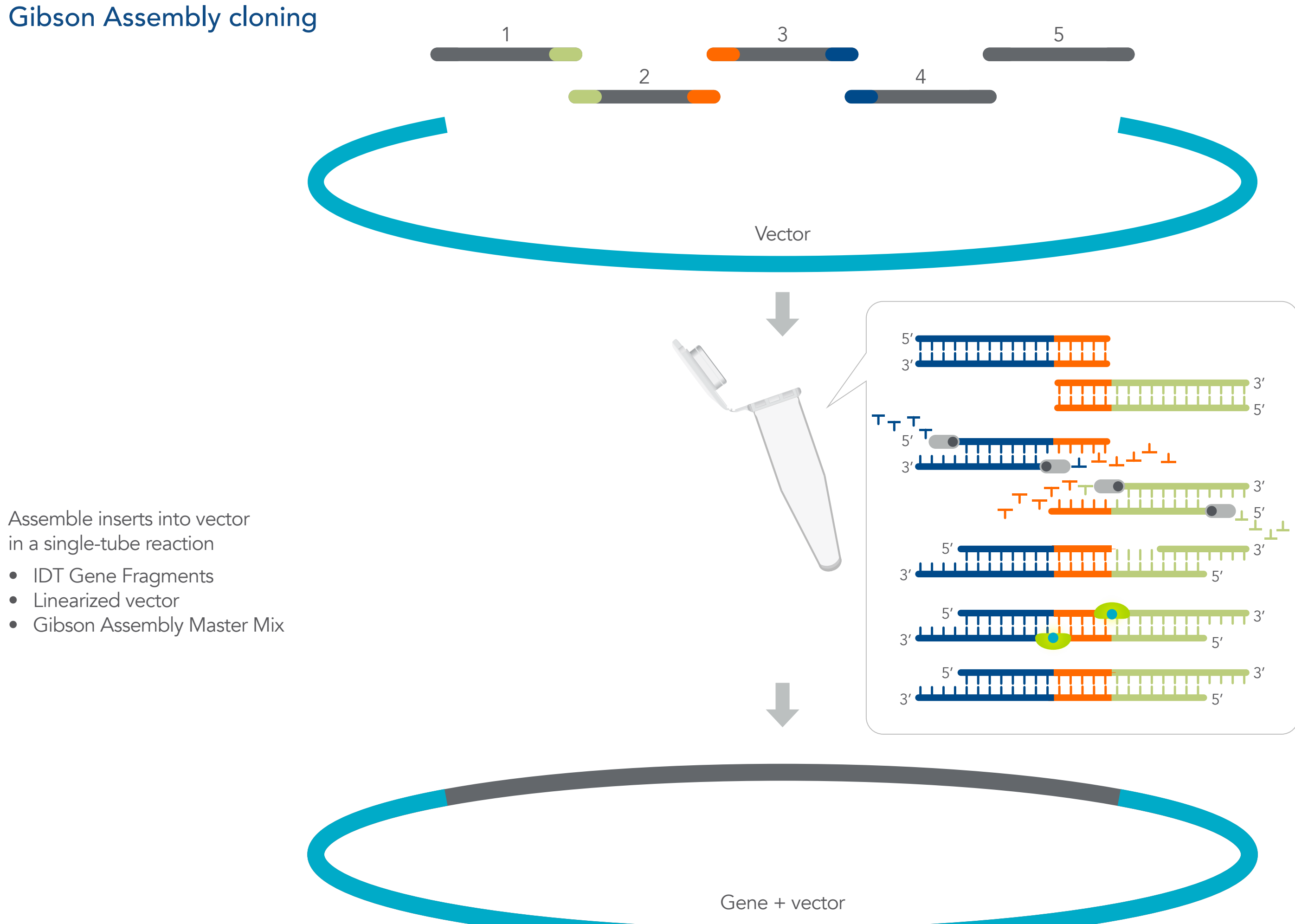

Figure 1. Overview of the Gibson Assembly cloning method.

Step 1

IDT Gene Fragments are designed with complementary 20–80 bp overlaps on the 3′ strands and used in a single 50°C reaction where the following steps occur.

Step 2

A mesophillic 5′ exonuclease briefly cleaves bases from the 5′ ends of the double-stranded DNA fragments.

Step 3

The newly generated complementary 3′ overhangs anneal.

Step 4

A high-fidelity DNA polymerase fills in any gaps resulting in completed circular plasmids or retracted free ends in linear assemblies.

Step 5

Finally, a thermophilic DNA ligase covalently joins DNA segments.

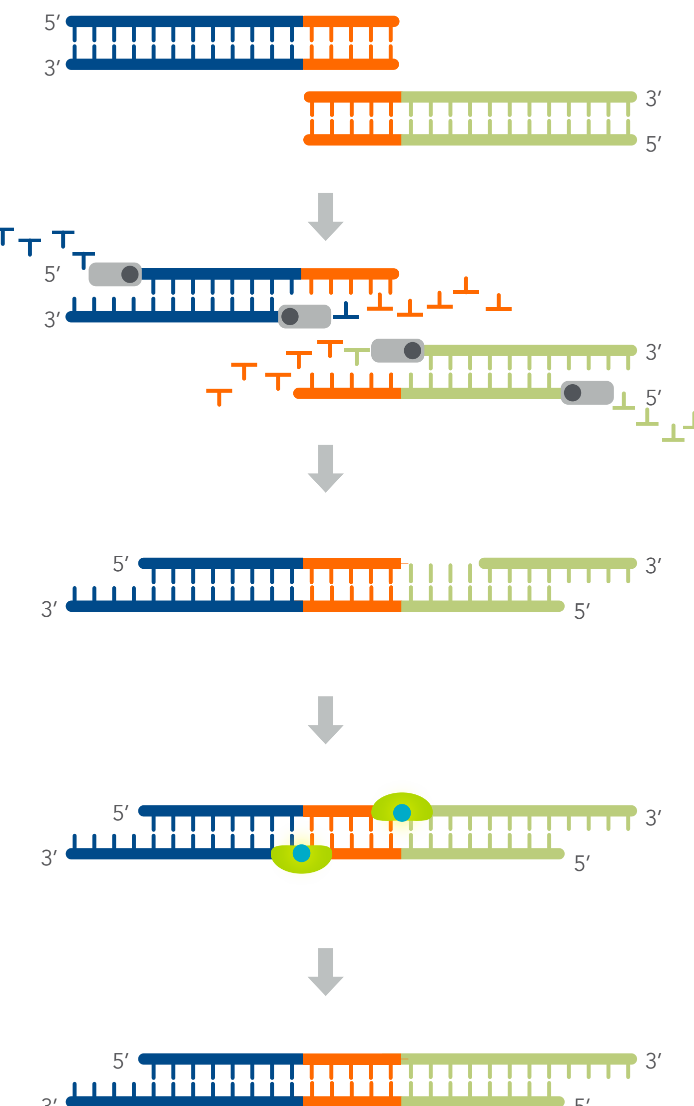

Figure 2. Process of the Gibson Assembly method with IDT Gene Fragments.

GUIDELINES

Advantages of the seamless cloning method

- Fast and efficient
- Multiple DNA elements can be assembled and cloned in a single reaction
- Allows for large gene assembly and generation of gene libraries
- Directional cloning
- Does not require restriction sites

IDT Gene Fragments

gBlocks Gene Fragments

gBlocks Gene Fragments are synthetic, double-stranded DNA (125–3000 bp), delivered dry and normalized to 250, 500, or 1000 ng, depending on length. These fragments and are ideal for gene assembly, genome editing, qPCR controls, and more.

gBlocks HiFi Gene Fragments

gBlocks HiFi Gene Fragments are between 1000–3000 bp in length, shipped dry in tubes, normalized at 1000 ng. These high-fidelity fragments are optimized for the assembly of large constructs.

eBlocks Gene Fragments

eBlocks Gene Fragments are synthetic, double-stranded DNA (300–1500 bp), normalized to 200 ng and delivered at 10 ng/μL in Nuclease-Free Water. These gene fragments are uniquely suited for high-throughput screening and can ship in as little as 1-3 business days from order confirmation.

Design considerations

For use in seamless cloning, IDT Gene Fragments must contain 20–80 bases of sequence overlap between fragments, including the flanking regions of the insert site in the desired plasmid. For most seamless cloning reactions, 20–40 bp is sufficient (~30 bp is typical), but going beyond 3 fragment assemblies and/or with very long fragments (≥8 kb), homology lengths should be increased. The portions of the sequences containing the overlaps should contain minimal secondary structure and minimal repetitive DNA sequences, which can affect alignment of the annealed sequences.

CONSUMABLES AND EQUIPMENT

Consumables—IDT

Item	Catalog #
Gene fragments with 20–80 bases of sequence overlaps, such as:	
gBlocks Gene Fragments	www.idtdna.com/gBlocks
gBlocks HiFi Gene Fragments	www.idtdna.com/gBlocks
eBlocks Gene Fragments	www.idtdna.com/eBlocks
Nuclease-Free Water	11-04-02-01
TE Buffer	Varies

Consumables—Other suppliers

Item
Vector
Option 1 for linearizing vector by restriction digestion: • Restriction enzyme • Alkaline phosphatase
Option 2 for linearizing vector by PCR amplification: • Forward and reverse primers • dNTPs • High-fidelity polymerase • $MgSO_4$ • DpnI • 10X DpnI Buffer
Gibson Assembly Master Mix
Cell transformation reagents • Competent bacteria • 14 mL polypropylene tube • SOC media • LB agar plates with appropriate selection antibiotic

Equipment

Item
PCR thermal cycler
(Optional) Heat blocks at 37°C and 80°C
Water bath at 42°C
Incubator at 37°C

PROTOCOL

Resuspend your IDT Gene Fragments

The dried down IDT Gene Fragment pellet can become displaced from the bottom of the tube during shipping, so it is important to centrifuge your tubes or plates before opening them.

1. Centrifuge the tube for 3–5 seconds at a minimum of 3000 x *g* to pellet the material to the bottom of the tube.
2. Add TE buffer to the tube for your desired final concentration.

Final stock concentration	Resuspension volume of TE buffer (µL) for IDT Gene Fragments		
	Synthesis scale		
	250 ng	500 ng	1000 ng
10 ng/µL	25	50	100
20 ng/µL	Not recommended	25	50
50 ng/µL	Not recommended	10	20

Note: Gene fragments can be resuspended in Nuclease-Free Water in some cases (see **Store your Gene Fragments**).

3. Briefly vortex, then centrifuge.

Store your Gene Fragments

gBlocks and gBlocks HiFi Gene Fragments

Store gBlocks and gBlocks HiFi Gene Fragments at –20°C in TE for up to 24 months. For short-term storage of less than 1 month, they can be stored at –20°C in Nuclease-Free Water. Aliquot resuspended gene fragments to avoid more than 2–3 freeze-thaw cycles.

eBlocks Gene Fragments

eBlocks fragments are delivered at 10 ng/µL in Nuclease-Free Water. Store eBlocks Gene Fragments at −20°C for up to 24 months. For short-term storage of less than 2 weeks, they can be stored at 4°C.

Prepare vector

Option 1: Linearize by restriction digestion

Supercoiled vector isolated from *E. coli* or purchased from a commercial vendor can be linearized using a restriction enzyme, provided the restriction site is present in the vector. In addition, the linearized vector should then be dephosphorylated using a phosphatase to prevent re-ligation of the empty vector's ends. This protocol provides an example using EcoRV (New England Biolabs) and Thermosensitive Alkaline Phosphatase (Promega).

Note: The volumes and concentrations are examples; follow the manufacturers' instructions for materials specific to your application.

1. Add the following reaction components:

Reagent	Amount
Plasmid	1 µg
10X Buffer #3	4 µL
Restriction enzyme (n U/µL)	1 µL
BSA	0.5 µL
Nuclease-Free Water	To a final **40 µL** volume

2. Follow manufacturer's recommendations for incubation, temperature, time, and heat kill.
3. Remove 5′ phosphates from vector using an alkaline phosphatase (e.g., Thermosensitive Alkaline Phosphatase).

   Important: Do not dephosphorylate the insert (i.e., IDT Gene Fragment).

   Note: Most commercially available phosphatases can be added directly at the end of the restriction digest; follow the manufacturer's instructions for your chosen phosphatase.

4. Confirm and quantify the reaction by running the product on an agarose gel with an appropriate quantification ladder.
5. To reduce background, gel purify the vector following digestion.

Option 2: Linearize by amplification and digest with DpnI

Alternatively, vectors can be amplified using primers that have their 5′ ends at the insertion site (i.e., oriented to extend away from the insertion site). This works better for smaller vectors, as large vectors can sometimes be difficult to amplify. For amplification, use a high-fidelity polymerase. To remove the PCR template, digest the reaction using DpnI, which will only digest Dam-methylated DNA isolated from *E. coli* and not the PCR-amplified DNA.

Note: The volumes, temperatures, and concentrations are examples; follow the manufacturers' instructions for materials specific to your application.

1. Set up the components (**Table 1**) for each of your vectors.

Table 1. Components of PCR.

Reagent	Amount
Supercoiled plasmid	1 ng
5 µM forward primer	1 µL
5 µM reverse primer	1 µL
2 mM dNTPs	2.5 µL
25 mM $MgSO_4$	1.5 µL
High-fidelity polymerase (2.5 U/µL)	0.5 µL
Nuclease-Free Water	To a final **25 µL** volume

2. (Optional) Confirm the linear product was generated by running 5 µL on a 0.8% agarose gel with a DNA ladder and 200 ng of uncut plasmid.

3. Digest the template from the PCR-amplified vector with DpnI by incubating the following at 37°C for 1 hour.

Reagent	Amount (µL)
PCR product	17
10X Buffer	2
DpnI (20 U/µL)	1

Tip: standard PCR primers do not contain 5′ phosphates, so there is no need to dephosphorylate after PCR.

4. Purify the PCR-amplified vector using a PCR cleanup kit.
5. Confirm the linear product was generated by running 5 µL on a 0.8% agarose gel with a DNA ladder and 200 ng of uncut plasmid.

Construct assembly (plasmid and IDT Gene Fragments)

One or more gene fragments and a linearized vector are designed with 20–80 base homologous overlaps with the adjacent sequences. This protocol provides an example using the Gibson Assembly Master Mix (Thermo Fisher Scientific).

1. Combine linearized plasmid and fragments in a tube with assembly mix as shown:

Reagent	Total
Gene fragments*	0.08 pmol each
2X Assembly Master Mix†	10 µL
Deionized H_2O	Up to 20 µL

* For large vectors with shorter insert fragments, use 50–100 ng of plasmid and a 2–3 fold excess of insert fragments.

† Enzymes are included in the master mix.

2. Incubate at 50°C for 1 hour (for simpler, 1-3 insert assemblies, this time can be shortened to 15 minutes).

Transform

Several lines of competent *E. coli* can be purchased from a variety of vendors and provide a reliable way to achieve high transformation efficiencies. Alternatively, competent cells can be prepared in the lab by following the protocols outlined in Sambrook *et al*. [2].

Below is an example protocol for transformation; follow the manufacturer's instructions for materials specific to your product.

1. Thaw cells on wet ice.
2. Add 25 µL cells to a pre-chilled 14 mL polypropylene tube on ice.
3. Add 2 µL of ligation mixture and mix gently.
4. Incubate on wet ice for 30 minutes.
5. Place in a 42°C water bath for 45 seconds.
6. Return to ice for 2 minutes.

7. Add 250 µL of SOC media to the cells and incubate shaking at 37°C for 1 hour.
8. Plate 125 µL on LB agar plates with the appropriate selection antibiotics for your vector.
9. Incubate the plates inverted in a 37°C incubator overnight.
10. Select and screen several colonies.

Tip: We recommend sequencing at least 2 times the number of IDT Gene Fragments assembled to give you the highest probability of successfully identifying your desired target. For example, if you assemble 4 gBlocks Gene Fragments into a plasmid, we recommend sequencing 8 clones to have the best chance (~95%) of obtaining your desired construct.

The resulting completed plasmid is ready for sequencing.

REFERENCES

1. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA 3rd, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*. 2009;6(5):343-345. doi:10.1038/nmeth.1318

2. Green M, Sambrook J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2012.

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