Blunt-end 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 cloning of blunt-ended DNA fragments, especially those created by polymerase chain reaction (PCR), presents a challenge due to the inefficient ligation of blunt ends by T4 DNA ligase. This protocol demonstrates how to use blunt-end cloning to ligate double-stranded DNA (dsDNA) into a plasmid where both the insert and linearized plasmid have no overhanging bases at their ends.

Blunt-end cloning is a relatively simple method of cloning gene fragments, such as gBlocks, gBlocks HiFi, and eBlocks Gene Fragments. This method requires no specific sequences near the ligation site or additional gene fragment preparation. However, blunt-end cloning can be less efficient than cohesive-end restriction site cloning and is not directional, so the gene fragment will be inserted randomly in either orientation. In most cases, screening several colonies after transformation will identify vectors containing the desired insert orientation, but if there is selection against the correctly orientated sequence, identification of a correct colony may require extensive screening. In addition, a lot of plasmid re-circularization occurs, resulting in many empty vectors. Therefore, when using this method, you should screen by restriction digestion or PCR, before sequence verification.

Blunt-end cloning

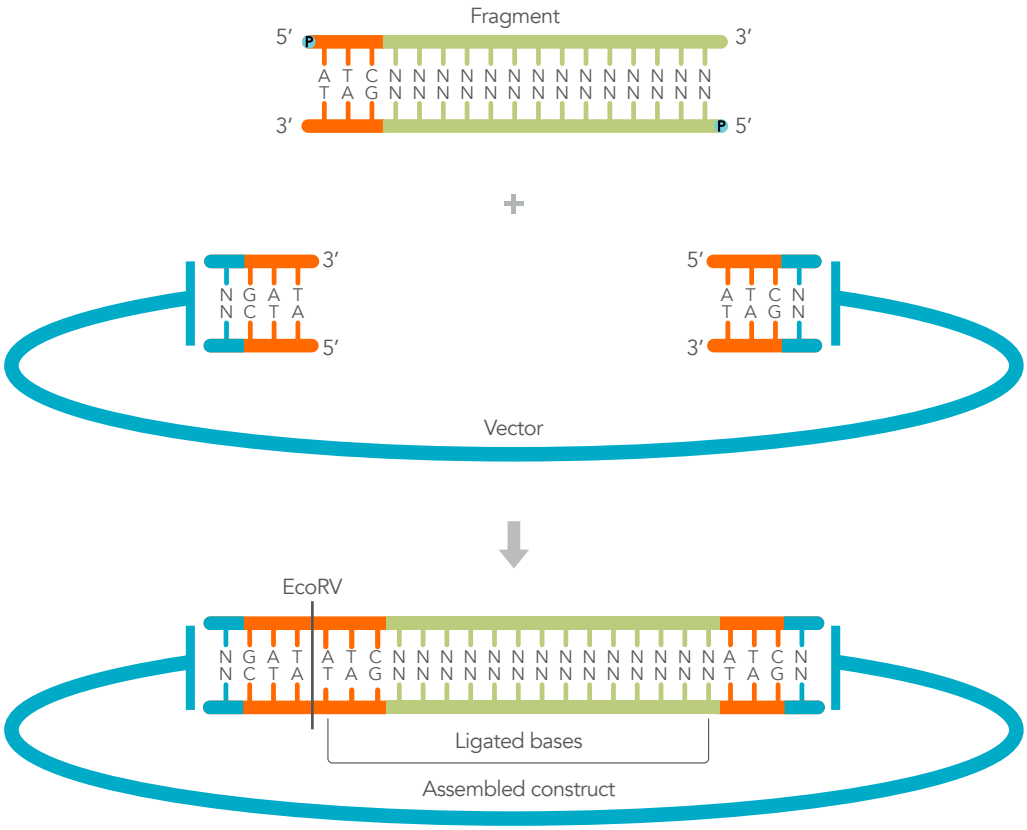

Figure 1. Blunt-end cloning using dephosporylated vector. In this example, the vector is linearized by EcoRV to produce blunt ends. The insert could be an IDT Gene Fragment or could be derived from 2 hybridized DNA oligonucleotides with optional bases to reproduce the EcoRV on one end (orange). The required 5′ phosphates are shown as blue circles. The ligated bases form a single, new EcoRV site that can be re-cut at one end. The other end has only half of the original restriction recognition site and can no longer be digested by EcoRV. Because the EcoRV site is palindromic, there is a 50% chance that the recreated site occurs at either end of the multiple cloning site (MCS).

GUIDELINES

Advantages of the blunt-end cloning method

- Fast and simple cloning method
- Versatile because insert and vector have fewer sequence limitations
- Restriction sites are unnecessary

Limitations to the blunt-end cloning method

- Insertion limited to one fragment
- Often preferentially clones in a single and possibly undesirable orientation

IDT Gene Fragments

gBlocks Gene Fragments

gBlocks Gene Fragments are chemically synthesized, double-stranded DNA (125–3000 bp), delivered dry and normalized to 250, 500, or 1000 ng, depending on length. These fragments 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 chemically synthesized, 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

IDT Gene Fragments are synthesized with blunt ends. When used in blunt cloning applications, they should be synthesized with 5′ phosphates to facilitate ligation, as this a complimentary modification and generally results in a higher level of phosphorylation than when adding phosphates with T4 kinase. In general, gene fragments with low G/C content near the ends (e.g., ≤30% within 25 bp) clone less efficiently than those with higher G/C content.

Important: To prevent plasmids from recircularizing without an insert, linearized plasmid should be dephosphorylated with alkaline phosphatase, which itself must be removed or deactivated from the plasmid prep to prevent further dephosphorization of the gene fragments during ligation.

CONSUMABLES AND EQUIPMENT

Consumables—IDT

Item	Catalog #
Gene fragments:	
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

Consumables—Other suppliers

Item
Vector with a cloning site that contains a sequence for a blunt-cutting restriction enzyme (e.g., EcoRV)
Option 1 for linearizing vector by restriction digestion: • Blunt-cutting restriction enzyme [e.g., EcoRV (400 U/µL)] • Alkaline phosphatase
Option 2 for linearizing vector by PCR amplification: • Forward and reverse primers • dNTPs • High-fidelity polymerase • $MgSO_4$ • DpnI • T4 ligase • 10X KOD Buffer
Cell transformation reagents • Competent bacteria • 14 mL BD Falcon® polypropylene tube (Corning) • SOC media • LB plates

Equipment

Item
PCR thermal cycler
(Optional) Heat blocks at 37° 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 to reach 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 up to 24 months. For short-term storage of less than one 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 up to 24 months. For short-term storage of less than two 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 blunt cutting 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 religation of empty vector ends. This protocol provides an example using EcoRV (New England Biolabs) and Thermosensitive Alkaline Phosphatase (Promega).

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

1. Add these reaction components together:

Reagent	Amount
Vector	1 µg
10X Buffer #3	4 µL
EcoRV (400 U/µL)	1 µL
BSA	0.5 µL
Nuclease-Free Water	Up to 40 µL

2. Incubate at 37°C for 1 hour, followed by 80°C for 20 minutes.
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 after 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). For amplification, use a high-fidelity polymerase that leaves blunt ends on the products. 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 listed below are examples, follow the manufacturer's instructions for materials specific to your application.

1. Set up the components (Table 1), then amplify (Table 2) 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
10X KOD Buffer	2.5 µL
25 mM $MgSO_4$	1.5 µL
High-fidelity polymerase (2.5 U/µL)	0.5 µL
Nuclease-Free Water	To final 25 µL volume

Table 2. Cycling conditions.

Step	Cycles	Time	Temperature (°C)
Initial denaturation	1	3 min	98
Amplification:			
Denaturation	30	15 sec	98
Annealing		15 sec	60
Extension		30 sec per kb	72
Final extension	1	30 sec	72
Hold, if needed	1	—	4

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
PCR product	17 µL
10X Buffer	2 µL
DpnI (20 U/µL)	1 µL
Total volume	**20 µL**

Tip: Standard PCR primers do not contain 5′ phosphates so you do not need to dephosphorylate after PCR.

4. (Optional) 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.

Ligate vector with gene fragment

Two common DNA ligases are available from a variety of vendors: T4 Ligase and Taq Ligase. T4 ligase is functional at low temperatures (16–20°C) and can efficiently ligate blunt-ended DNA. In contrast, Taq ligase is functional at elevated temperatures and is inefficient at ligating blunt-ended DNA, therefore it should be avoided for blunt-end cloning applications.

T4 Ligases are available in standard or "quick" versions. In general, the quick versions contain a crowding agent such as PEG that increases the ligation kinetics and decreases reaction time. Quick versions are preferred for day-to-day uses.

To ligate your IDT Gene Fragment into the vector efficiently, use the optimum molar ratio of vector to gene fragment. The ratio we recommend is 1:5–1:12 vector to gene fragment.

Tips:

- High salt concentrations from DNA can inhibit ligation activity, so the use of DNA cleanup kits can often improve reaction efficiencies.
- T4 DNA ligases require buffers containing ribo ATP. Always use fresh buffers that have not been repeatedly frozen and thawed to ensure the ATP is active.
- Be careful to not heat inactivate ligase reactions with buffers containing PEG, as this decreases transformational efficiency.

1. Set up the reaction by combining these items:

Item	Amount
Linearized vector	50 ng
Gene Fragment*	5–12X molar excess
2X ligase buffer	10 µL
T4 DNA Ligase	1 µL
Nuclease-Free Water	Up to 20 µL

* Molar ratios of the gBlocks, gBlocks HiFi or eBlocks Gene Fragment (IDT) can be converted to ng using the following formula:

$$50\ \text{ng} \times \text{desired molar ratio} \times \frac{\text{IDT Gene Fragments length (bp)}}{\text{Plasmid length (bp)}} = \text{ng gene fragment needed}$$

2. (Optional) Store ligation at –20°C.

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. [1].

Example protocol

Here 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 BD Falcon polypropylene tube on ice.
3. Add 2 µL of ligation mixture, then gently mix.
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, then incubate shaking at 37°C for 1 hour.
8. Plate 125 µL on LB plates with the appropriate selection reagents for your vector.
9. Incubate the plates inverted in a 37°C incubator overnight.
10. Select and screen several colonies.

REFERENCES

1. Green M, Sambrook J. **Molecular Cloning: A Laboratory Manual: A Laboratory Manual (Fourth Edition)**. Cold Spring Harbor Laboratory Press; 2012.

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