Smart-Join™ Blunt-end PCR Cloning Kit

Cat. No. IC007 (20 reactions)
IC008 (100 reactions)

User Manual

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USER MANUAL

Smart-Join™ Blunt-end PCR Cloning Kit

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I. Introduction

The Smart-Join™ Blunt-end PCR Cloning Kit offers a convenient method for high efficiency cloning of blunt-end PCR products (or any blunt-end DNA fragment). The blunt-end linear vector in the kit contains the Barnase gene from *Bacillus Amyloliquefaciens*, which express Barnase, a small RNase that degrades RNA and is toxic to cells. In the Smart-Join™ Blunt-end PCR Cloning Kit, successful ligation of a blunt-end PCR fragment to the linear vector disrupts the expression of Barnase, permitting growth of only positive recombinants upon transformation. Meanwhile, cells with self-ligated vectors are killed by Barnase. Therefore, only the cells with target fragments ligated to the plasmids can grow. The kit provides high successful cloning rates and a more straightforward way to eliminate false positive transformant colonies that makes blue/white screening unnecessary.

II. Applications

1. Cloning of blunt-end PCR fragments

GeneCopoeia recommends using high-fidelity polymerase, such as Pfu, which can be found in the IndelCheck™ Target Site PCR kit (GeneCopoeia Cat#IC003), to obtain blunt-end PCR products. Blunt-end PCR products can be recovered without purification and directly ligated to the blunt-end linear vector in the kit to obtain recombinant plasmids with high frequency. If there are non-specific bands in the PCR products, Genecopoeia recommends gel purifying the products before the ligation.

2. Cloning and sequencing of other blunt-end fragments

With Smart-Join™ Blunt-end PCR Cloning Kit, other blunt-end fragments can be cloned in the same way, such as blunt-end products generated by restriction endonuclease digestion. The resulting plasmids can then be used as templates for DNA sequencing.

3. Cloning and sequencing of point mutation fragments

After point mutation of a target fragment, GeneCopoeia recommends using the IndelCheck ™ T7 Endonuclease I Assay Kit (GeneCopoeia Cat#IC005) to detect whether the mutation has been successfully integrated (Figure 1). Fragments with point mutations are amplified using high-fidelity polymerase, and cloned into the blunt-end linear vector for sequencing.

4. IndelCheck™ insertion or deletion detection system

The Smart-Join™ Blunt-end PCR Cloning Kit is a key component of the IndelCheck™ detection system from GeneCopoeia, and can be used to confirm mutations introduced by CRIPSR. Using this kit, the target PCR product can be cloned into the blunt-end linear vector for sequencing to detect the targeted genomic modifications.

For more information, please visit our website at http://www.genecopoeia.com/product/indelcheck-detection-system/.

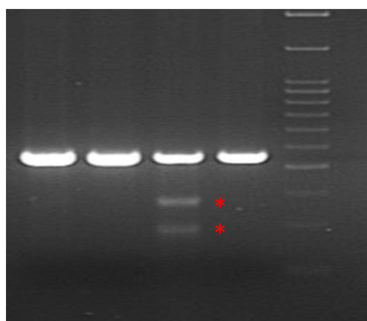


Figure 1. Results of T7 Endonuclease I Assay Kit
* The mismatched PCR products digested by T7 endonuclease I form smaller bands on agarose gel, indicating successful point mutation.

III. Contents and Storage

Contents and storage recommendations for the Target Site PCR Cloning Kits (Cat. No. IC007 and IC008) are provided in the following table.

Catalog Number	Content	Quantity 20 reactions 100 reactions
IC007-01 IC008-01	5 × Ligase Buffer	40 µl 40 µl×5
IC007-02 IC008-02	T4 DNA Ligase (200 U/µl)	20 µl 20 µl×5
IC007-03 IC008-03	Blunt-end linear vector (20 ng/µl)	20 µl 20 µl×5
IC007-04 IC008-04	Control Insert (40 ng/µl)	20 µl 20 µl×5
IC007-05 IC008-05	Forward sequencing primer (20 µM)	250 µl 250 µl×5
IC007-06 IC008-06	Reverse sequencing primer (20 µM)	250 µl 250 µl×5

Shipping and Storage:

The Smart-Join™ Blunt-end PCR Cloning Kits are shipped on dry ice and ice pack.
The Smart-Join™ Blunt-end PCR Cloning Kits are stable for at least 12 months in storage at -20 °C. Be sure to avoid repeated freezing and thawing.

IV. Notes

1. PCR products

The PCR products used for the ligation reaction must be blunt-ended. Do not use Taq polymerase to produce your PCR products as it will not produce blunt-end fragments. GeneCopoeia recommends using high-fidelity polymerase, such as Pfu, which can be found in the IndelCheck™ Target site PCR kit (GeneCopoeia Cat#IC003) to obtain blunt-end PCR products.

2. Amplification of target fragments from cell lysates

If cell lysate is directly used as a PCR template, GeneCopoeia recommends using the IndelCheck™ Target Site PCR kit (GeneCopoeia Cat#IC003) to amplify the target fragment. The IndelCheck™ Target Site PCR kit contains lysis buffer and PCR Mix, providing convenience and high-specificity. GeneCopoeia also provides design and synthesis services for target site PCR primers. For details, please visit our website at http://www.genecopoeia.com/product/indelcheck-detection-system/.

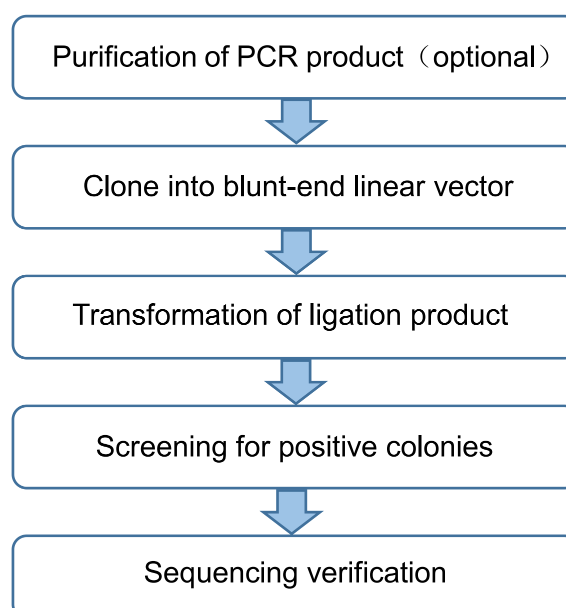
3. Ligation of long PCR products

If the PCR product is long (>1500 bp) and there are no non-specific products in the agarose gel electrophoresis, GeneCopoeia recommends quantifying the PCR products before ligation to ensure that the minimum amount of the PCR product in the ligation reaction will not be less than 30 ng. If non-specific product is present, GeneCopoeia recommends gel-purifying and quantifying the products before the ligation.

4. Storage

Store the kits at -20°C. Place the required components on ice before use, especially T4 DNA Ligase because of its poor heat-resistance (T4 DNA ligases is inactivated at 68°C). In addition, a white precipitate may appear after thawing of 5×Ligase Buffer. This precipitate does not affect performance, but can re-dissolved by vortexing.

V. Procedures



1. **Purification of PCR products (optional)**

Confirm the quality of PCR products by agarose gel electrophoresis, which should show a single band with no non-specific products (Figure 2). Remove 5-10 μl from each PCR reaction for agarose gel electrophoresis to verify the quality and quantity of your PCR products. GeneCopoeia recommends gel-purifying the products before ligation if the PCR products do not appear as single bands on an agarose gel.

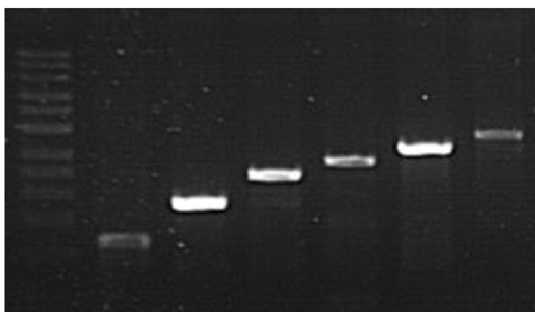


Figure 2. Agarose gel electrophoresis of blunt-end PCR products.

2. **Cloning into the blunt-end linear vector**

Set up the following 10 μl ligation reaction:

Reagent	Volume	Final Concentration
5× Ligase Buffer	2 μl	1×
Blunt-end linear vector	1 μl	20 ng/μl
PCR product	1 μl*	≥ 30 ng/μl*
ddH_2O	5 μl*	——
T4 DNA Ligase	1 μl	200 U/μl
Total	**10 μl**	

**Note: Add the PCR product according to the concentration in the table. If the agarose gel electrophoresis band is not bright enough, GeneCopoeia recommends quantifying the PCR product first, then increasing the amount of the target fragment to ensure the minimum amount of the PCR product is not less than 30 ng.*

Place the ligation reaction in a 25°C water bath for 1 hr.

3. **Transformation of ligation products**

- Thaw competent *E. coli* cells on ice. Place the required number of microcentrifuge tubes on ice and dispense 100 μL of competent cells into each tube.
- Gently mix 2 μL of each ligation reaction with the competent cells (the expected transformation efficiency is 1×10^9). Incubate on ice for 30 min.
- Heat shock cells by placing the tubes into a 42 °C water bath for 45 s. Immediately place the tubes on ice for 3 min.
- Add 400 μL of SOC (or LB) medium and incubate at 37 °C for 1 hr with shaking at 200 rpm.
- Plate 200 μL of each transformation onto LB plates containing ampicillin (recommended concentration is 100 μg/mL). Incubate plates overnight (12 to 16 hr) at 37 °C.

4. Screening for positive colonies

Randomly pick approximately 10 colonies from each plate for PCR verification. Use the Forward sequencing primer (20 μM)/Reverse sequencing primer (20 μM) mix in the kit to detect the positive colonies. Perform PCR reactions according to the protocol provided by the manufacturer of your PCR kit.

An example PCR reaction is shown below

Reagent	Volume	Final Concentration
2× PCR buffer	12.5 μl	1×
Forward sequencing primer (20 μM)	1 μl	0.8 μM
Reverse sequencing primer (20 μM)	1 μl	0.8 μM
dNTP(25 mM)	0.2 μl	0.2 mM
Enzyme(5 U/μl)	0.2 μl	1 U/μl
ddH_2O	10.1 μl	——
colony	——	——
Total	**25 μl**	

Step	Temperature	Time	Cycles
1	94°C	5 min	1
2	94°C	30 s	30
	58°C	30 s	
	72°C	1 min*	
3	72°C	5 min	1

**The extension time should be adjusted according to the length of the fragment. The extension rate of Taq DNA polymerase is approximately 1000 bp/min.*

5. Sequencing verification

- Inoculate a single positive colony in 5 mL of LB liquid media containing ampicillin (recommended concentration is 100 μg/mL). Incubate overnight at 37°C with shaking at 220 rpm.
- Extract plasmids according to the protocol provided by the manufacturer of your extraction kit.
- Use the Forward sequencing primer (20 μM)/Reverse sequencing primer (20 μM) mix in this kit to perform sequencing verification.

Partial vector sequence and primer sequence information for blunt-end linear vector are shown below:

```
TTAATACGACTCACTATAGGGCTAGCGATCGCCATGGAATAAGTAAAGGAATCACATGGCACAGGTTATCAACACGTTTGACGGGGTTGC
AATTATGCTGAGTGATATCCCGATCGCTAGCGGTACCTTATTCATTTCCTTAGTGTACCGTGTCCAATAGTTGTGCAAACTGCCCCAACG
 T7 promoter

                                                                   Forward sequence primer
                                                                    AGAAGCACAAGCCCTCGG
GGATTATCTTCAGACATATCATAAGCTACCTGATAATTACATTACAAAATCAGAAGCACAAGCCCTCGGCTGGGTGGCATCAAAAGGGAA
CCTAATAGAAGTCTGTATAGTATTCGATGGACTATTAATGTAATGTTTTAGTCTTCGTGTTCGGGAGCCGACCCACCGTAGTTTTCCCTT

CCTTGCAGACGTCGCTCCGGGGAAAAGCATCGGCGGAGACATCTTCTCAAACAGGGAAGGCAAACTCCAGGGCAAAAGCGGACGAACATG
GGAACGTCTGCAGCGAGGCCCCTTTTCGTAGCCGCCTCTGTAGAAGAGTTTGTCCCTTCCGTTTGAGGTCCCGTTTTCGCCTGCTTGTAC

GCGTGAAGCGGAT  Blunt PCR product  ATCAACTATACATCAGGCTTCAGAAATTCAGACCGGATTCTTTACTCAAGCGA
CGCACTTCGCCTA                    TAGTTGATATGTAGTCCGAAGTCTTTAAGTCTGGCCTAAGAAATGAGTTCGCT

CTGGCTGATTTACAAAACAACGGACCATTATCAGACCTTTACAAAAATCAGATAATGTTTAA   3'
GACCGACTAAATGTTTTGTTGCCTGGTAATAGTCTGGAAATGTTTTTAGTCTATTACAAATT   5'
               GTTGCCTGGTAATAGTCTG
               Reverse sequence primer
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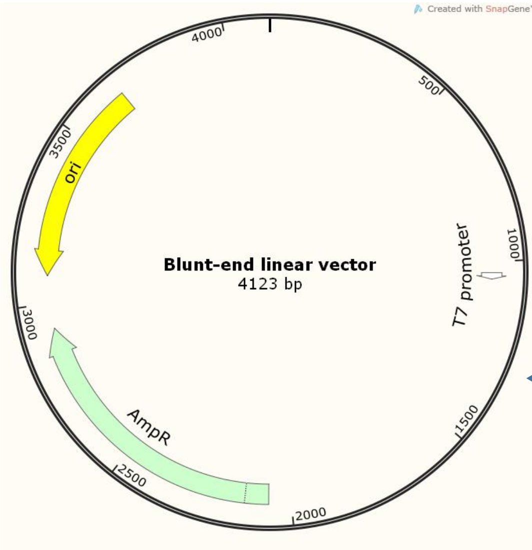

Forward Sequencing Primer: 5'-agaagcacaagccctcgg-3'
Reverse Sequencing Primer: 5 '-gtctgataatggtccgttg-3'

6. Ligation reaction of control insert (positive control)

If this is the first time you are using the Target Site PCR Cloning kit, GeneCopoeia recommends performing the control insert (40 ng/μl) reaction to help you evaluate your results.

Reagent	Volume	Final Concentration
5× Ligase Buffer	2 μl	1×
Blunt-end linear vector	1 μl	20 ng/μl
Control Insert	1 μl	40 ng/μl
T4 DNA Ligase	1 μl	200 U/μl
ddH$_2$O	5 μl	——
Total	**10 μl**	

The ligation reaction of control insert is performed as follows.

- Place ligation reaction in a 25°C water bath for 1 hr.
- Thaw competent *E. coli* cells on ice. Place the required number of microcentrifuge tubes on ice and dispense 100 μl of competent cells into each tube.
- Gently mix 2 μl of each ligation reaction with the competent cells (the expected transformation efficiency is 1×10^9).
- Refer to steps V.3 and V.4 for transformation and screening procedures.

VI. Troubleshooting Guide

Observation	Cause	Solution
Few or no colonies obtained	1.Loss of PCR product during purification. 2.The quality of the insert in the ligation reaction is too low. 3.Low transformation efficiency of competent cells. 4.A polymerase that adds 3´ A-overhangs is used (e.g. Taq polymerase). 5.Too much PCR product is added to the ligation.	1.Ensure that the concentration of PCR product is not too low, and minimize the loss of fragments during gel-purification. 2.If the minimum amount of the PCR product is not less than 30 ng, perform the control insert ligation reaction. 3.The transformation efficiency of competent cells is very important for transformants; competent cells should yield ~1 × 10^9 transformants/μg DNA. 4.If you used an enzyme mix containing Taq polymerase, you will need to blunt-end your PCR product. 5.The high salt content of PCR reactions can inhibit ligation. Do not use more than 4 μL of the PCR mixture in the ligation reaction.
Low positive rate	1.PCR product is not specific, and has not been gel-purified. 2.Nuclease contamination in reagents. 3.Inserts are special fragments (e.g. GC-rich).	1.Non-specific PCR products will reduce the positive rate of ligation. If the product is not specific, gel purify the product and quantify before the ligation. 2.Standardize operation and ensure cleanliness of reagents in use. 3.For special fragments, special treatment is needed, such as adding the appropriate amount of DMSO in the PCR reactions, or using Pfu polymerase to amplify the target fragments.
Low plasmid yield	1.Medium is not sufficiently rich enough for optimal growth. 2. Medium does not contain antibiotic 3. Bacterial incubation time is too short.	GeneCopoeia recommends inoculating a single positive colony into 5 mL of liquid LB media containing ampicillin (recommended concentration is 100 μg/mL), and incubating overnight at 37°C with shaking at 220 rpm.

VII. Limited Use License and Warranty

Limited Use License

The following terms and conditions apply to use of all Smart-Join™ Blunt-end PCR Cloning Kit (the Product). If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. The product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The Product must not be resold, repackaged or modified for resale, or used to manufacture commercial products without prior written consent from GeneCopoeia. This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the Product constitutes acceptance of the above terms.

Limited Warranty

GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. GeneCopoeia's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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