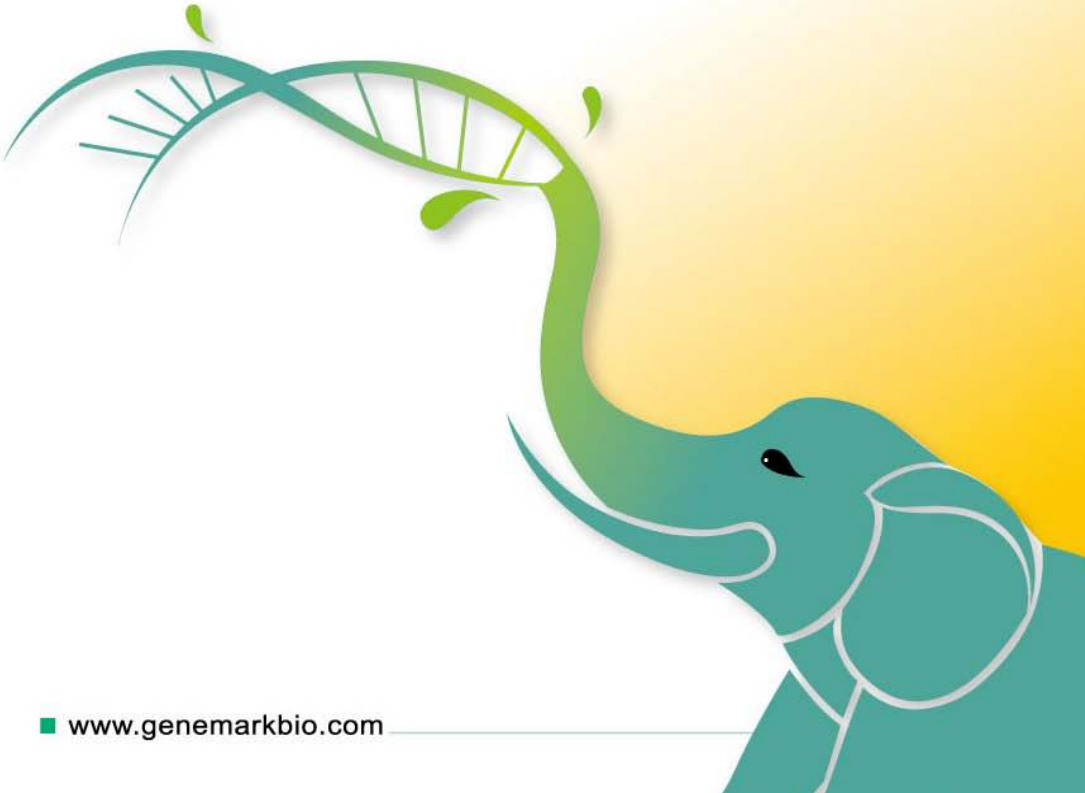
GeneMark

TOPBlunt PCR Cloning Kit

Cat. No.: **GTOP02-20 / GTOP02-40**

GTOP02-V20 / GTOP02-V40

For research use only



Introduction

Description：

TOPBlunt PCR Cloning Kit is suitable for cloning Pfu polymerase amplified PCR products with blunt ends at room temperature in 5min. The vector provided in this kit contains topoisomerase covalently coupled to its ends, which allows efficient cloning of blunt-ended DNA fragments to the vector without using a regular ligase.

Characteristics：

- Short reaction time (5 min)
- Highly efficient cloning

Important Notes：

1. It is necessary to use the control fragment during the transformation process so as to be used for debugging problems encountered during the experiment.
2. It is suggested to keep some ligation products just in case if there are any unexpected problems.
3. The volume of bacteria added to the antibiotic-containing LB agar plate can be adjusted according to the experimental results. Reduce the bacteria volume to 100 μl if there is too much of transformed DNA; contrarily, the bacteria volume can be increased to 200~300 μl. If little number of clones is observed, centrifuge the bacteria at 4,000 rpm for 2 min and remove some culture medium to concentrate the bacteria sample before streaking. Store the remaining sample at 4°C for later use.

Kit Contents and Storage

TOPBlunt PCR Cloning Kit (Including competent cell)

Cat. No.: GTOP02-20 / GTOP02-40

Contents	GTOP02-20	GTOP02-40
TOPBlunt Vector (10 ng/μl)	**20 μl**	**20 μl X 2**
6X TOPBlunt™ buffer	**20 μl**	**20 μl X 2**
Control insert DNA(Blunt) (1kb)	**5 μl**	**10 μl**
DH5α Competent Cell (100 μl/tube)	**21 tubes**	**21 X 2 tubes**
Control Plasmid	**5 μl**	**5 μl**
M13 Control primer mix	**400 μl**	**400 μl X 2**
SOC medium	**7.5 ml**	**7.5 ml X 2**
Sterile water	**1 ml**	**1 ml X 2**

Competent cells must be stored at -70°C, all other reagents should be stored at -20°C. Subdivision could be taken to avoid repeated freezing and thawing.

DH5α Genotype：

*SupE*44 △*lacU*169 (φ80*lacZ*△M15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1

Kit Contents and Storage

TOPBlunt PCR Cloning Vector (Not including competent cell)
Cat. No.: GTOP02-V20 / GTOP02-V40

Contents	GTOP02-V20	GTOP02-V40
TOPBlunt Vector (10 ng/μl)	**20 μl**	**20 μl X 2**
6X Blunt^{TM} buffer	**20 μl**	**20 μl X 2**
Control insert DNA (1kb)	**5 μl**	**10 μl**
M13 Control primer mix	**400 μl**	**400 μl X 2**

Store TOPBlunt PCR Cloning Vector and control insert DNA at -20°C. Divide into small aliquots to prevent contamination due to repeated freezing and thawing of product.

Protocol

Ligation：

All procedures must be done in asepsis environment.

1. The PCR products should be analyzed on agarose gel before use in the ligation reaction to make sure that the PCR reaction did produce the desired product. The PCR product to be ligated can be gel-purified or purified directly from the PCR amplification using PCR clean up & gel extraction system. Clean-up of reactions prior to ligation is recommended to remove any undesired reaction subproducts such as primer dimers, and to improve ligation efficiency.
2. The vector vial must be placed on ice to thaw. Do not freeze and thaw repeatedly. Briefly centrifuge the vial to remove drops from inside the lid.
3. Add all components to the asepsis tube as following.

   The molar ratio of vector and insert fragment must be controlled within 1:3 ~ 1:8. Too much DNA fragment may disturb ligation reaction.

Component	Reaction conditions	
Contents in Ligation system	Reaction system	Control system
PCR Product (blunt-end)	X μl	—
Control insert(Blunt) (1kb, 50 ng/μl)	—	1 μl
TOP Blunt Vector(10 ng/μl)	1 μl	1 μl
6X TOP Blunt™ buffer	1 μ	1 μ
Sterile water	Up to 6 μl	Up to 6 μl

4. Gently mix the solution in the tube and centrifuge briefly.
Incubate at room temperature (25°C) for 5 min.
Transform the ligated DNA into competent *E. coli* cells.

Transformation：

1. Prepare agar plates for transformation.

   Add 80 µl IPTG (50 mM) and 80 µl X-gal (20 mg/ml) on agar plate surface containing ampicillin (50 µg/ml). Spread uniformly using a sterile bent glass rod or a specialized spreader, then place the plate at 37°C until the surface seemed appropriate for use.

2. Transformation step

   a. Thaw the competent cells on wet ice. Carefully add the ligation-reaction mixture to 50~100 µl competent cells. The volume of ligation-reaction mixture added should be less than one tenth of competent cell volume. Gently flick the tubes to mix and place on ice for 30 min.

      Transform the control plasmid to competent cells at the same time to detect transformation efficiency. Add 1 µl of control plasmid to a new tube with proper volume of competent cell, and follow the transformation step as above.

   b. Heat-shock the cells for 45 sec in a water bath at exactly 42°C (do not shake), and immediately incubate the tubes to ice for 2~3 min (do not shake).

   c. Add 250 µl of SOC medium (brought to room temperature) per tube (without antibiotics), and then incubate in shaker (~225 rpm) at 37°C for 1 hr.

   d. Mix the transformants thoroughly and plate 150 µl transformants onto LB agar plate containing antibiotics to ensure good separation of colonies for subsequent single-colony isolation. Incubate the plate at 37°C for 12~16 hrs after the surface of plate has dried.

Detection：

a. **Quick detection:** to detect whether the DNA fragment is correctly inserted by direct bacterium PCR.

i. Calculate the number of clones that are to be screened. Prepare a PCR master mix (25 µl for each reaction, e.g. for 10 colonies prepare 275 µl of master mix (25 µl x 11).

For each 25 µl PCR mixture:

Component	Volume
Water	18.3 µl
10X Reaction Buffer with $MgCl_2$	2.5 µl
2.5 mM dNTP	2 µl
M13 Control primer mix (5 µM)	2 µl
5 u/µl Taq DNA Polymerase	0.2 µl

Or use **PCR Master Mix Kit (Cat# RP02)**

Component	Volume
Water	18 µl
5X PCR master mix	5 µl
M13 Control primer mix (5 µM)	2 µl

ii. Dispense 25 µl aliquots of the master mix into the appropriate number of PCR tubes.

iii. Use a 10 or 200 µl, sterile pipet tip (or a toothpick) and gently touch each colony, wash the pipette tip in 25 µl master mix.

Note: Do not use too much bacteria from each colony or add agar to the master mix, which may inhibit the PCR reaction.

iv. Perform the following PCR program:

Cycles	Temperature	Time
1	94°C	2 min
30	94°C	30 sec
	56°C	20 sec
	72°C	1 min (1 min/kb)
1	72°C	5 min

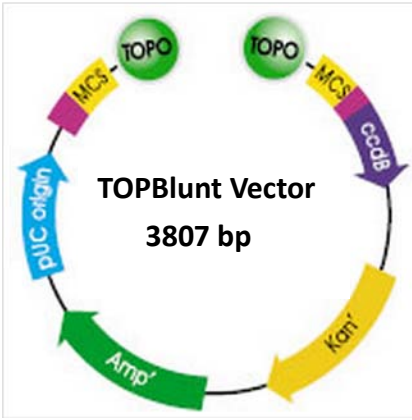
v. Load 2~5 µl of PCR products on 1.5**~**2% agarose gel for electrophoresis analysis. For positive clones, the molecular weight should be 202 bp with additional molecular weight of inserted DNA, e.g. for 300 bp PCR positive control, the PCR product should be about 502 bp. For self-ligation, the PCR product is about 202 bp.

b. **General detection:** select the colonies and inoculate in 1~5 ml liquid LB culture medium (containing 50~100 µg/ml ampicillin), and culture at 37°C overnight with shaking. Use either commercial kit (GeneMark Cat# DP01) or in house protocol to purify plasmid DNA. The inserted DNA sequence can be cut off from vector by *Eco*R I digestion or by other multiple cloning enzymes if inserts contain *Eco*R I site. See the restriction map below of multiple cloning site for appropriated enzyme selection.

c. **Sequencing:** DNA sequencing of inserted fragment after general or quick detection.

TOPBlunt Vector Map

A molecule of topoisomerase (TOPO) is covalently coupled to the 3 blunt duplex ends (nt positions 294 and 295) of TOPBlunt vector. Therefore, TOPBlunt vector can form a covalent bond with blunt-ended PCR products obtained by Pfu DNA polymerase.



Comments for TOPBlunt Vector (3807 nucleotides)

Lac promoter/operator：95-216

M13 Reverse Primer binding site：205-221

LacZα ORF：217-534

MCS, Multiple Cloning Sites：234-357

M13(-20) Forward Primer binding site：391-406

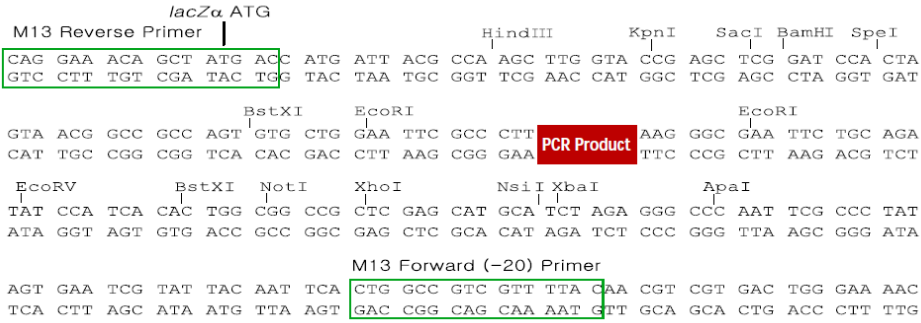
ccdB ORF：544-846

Kan^r gene：1057-1989

Amp^r gene：2007-2867

pUC origin：3012-3685

TOPBlunt Vector Cloning Site



Troubleshooting Guide

Problem	Comments and Suggestions
Few or no colonies	
a) Incorrect antibiotic concentration	Use appropriate antibiotic concentration.
b) Insufficient DNA in ligation mixture	Increase the amount of vector/ insert used for ligation.
c) The cells have lost competency	Use high-efficiency competent cells (≥1 × 10^8cfu/µg DNA) Calculation of transformation efficiency : 1. Add 1 µl of positive control plasmid (100 pg) into one tube of competent cells. 2. Follow the transformation protocol. **Transformation efficiency formula (cfu/µgDNA):** colonies on plate/ng of DNA plated X 1000 ng/µg
Low number or no white colonies containing PCR product	
a) Improper dilution of the Ligation buffer	The 6X TOPBlunt™ buffer is provided at a concentration of 6X. Use 1 µl in a 6 µl reaction.

b) Ligation reaction has failed	1) Avoid multiple freeze-thaw cycles. 2) Optimize insert : vector ratio. 3) Ligation incubation is incorrectly performed and temperature needs optimization.
c) The PCR product is inserted, but have not disrupted the lacZ gene	Screen both blue and pale blue colonies to avoid false-negative results
d) Primer-dimers contamination	The PCR fragment should be purified using clean up methods.
e) Incorrect amounts of X-gal and IPTG plate	Ensure the correct X-gal/IPTG concentration is used.

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