CYCV20-CHANGYU pTOPO-ENTR/D Directional Cloning Kit

Contents	20 rxns (CYCV2001)
pTOPO-ENTR/D Vector (30 ng/μl)	20 μl
10× Enhancer	20 μl

Note: Store at -20 °C; stable for at least 12 months. Competent cells are not included.

Description

This kit uses directional topoisomerase cloning to place a gene of interest (a blunt-end fragment amplified with a high-fidelity polymerase) into a Gateway entry vector in one step within 5 minutes. The resulting entry clone can be recombined with Gateway destination vectors in an LR reaction to give expression clones, so the gene can easily be moved between prokaryotic, mammalian, yeast and insect expression systems.

1. Simple and fast: the entry clone is made in 5 minutes at room temperature.

2. Directional cloning: more than 90% of inserts are in the correct orientation, reducing screening time.

3. The vector contains no prokaryotic Shine-Dalgarno (SD) sequence. For prokaryotic expression, choose a destination vector with an SD sequence for the LR reaction, or include an SD sequence in front of the insert when designing primers.

Note: pTOPO-ENTR/D (2580 bp) carries a **kanamycin** resistance gene. Sequencing can be done with the M13F/M13R primers given in this manual.

❖ Procedure

1. Primer design.

(1) For directional cloning, add four extra bases, CACC, to the 5' end of the forward primer. The 5' end of the PCR product then pairs with the GTGG overhang of the vector. Example - sequence to express: 5'-ATG GGA TCT GAT AAA ...; forward primer: 5'-CACC ATG GGA TCT GAT AAA ...

(2) If no C-terminal fusion with the vector is planned, the reverse primer should start with a stop codon (as the reverse complement; for stop codon TGA, the reverse primer starts with TCA).

(3) If a C-terminal fusion is planned, the reverse primer must not contain a stop codon.

(4) The 5' end of the reverse primer must not contain CACC; otherwise the 3' end of the PCR product can also pair with the GTGG overhang and insert in the wrong orientation.

2. Preparation. PCR primers must not be phosphorylated. Amplify with a high-fidelity polymerase that gives blunt-end products (such as *Pfu*, Vent, KOD or Phusion DNA polymerase). Gel purification of the PCR product is recommended.

3. Ligation. Set up the following 10 μl reaction at room temperature (20-30 °C):

Purified PCR product	0.5-8 μl
pTOPO-ENTR/D Vector	1 μl
10× Enhancer	1 μl
Sterile water	X μl
Final Volume	10 μl

Mix gently by pipetting or flicking, then spin briefly to collect the liquid. Set up this step at room temperature (20-30 °C), **not on ice**.

Insert size (bp)	Optimal amount (ng)
100-1000	20-40
1000-2000	30-70
2000-5000	40-100

Incubate at room temperature (20-30 °C) for 5 minutes; in many cases 2-3 minutes already gives enough transformants. The reaction can be transformed directly into cloning competent cells (such as DH5α or TOP10) or stored at -20 °C; if the cells are not ready, keep it on ice for a short time.

4. Transformation.

1) Thaw 50-100 µl competent cells at room temperature (about 1 minute) and resuspend by flicking.

2) Add 5 µl of the reaction (up to all of it, as long as it is no more than 1/10 of the cell volume), mix gently and leave at room temperature for 5 minutes. With commercial competent cells, ice incubation and heat shock are not needed; with home-made or low-efficiency cells, follow the standard heat-shock protocol.

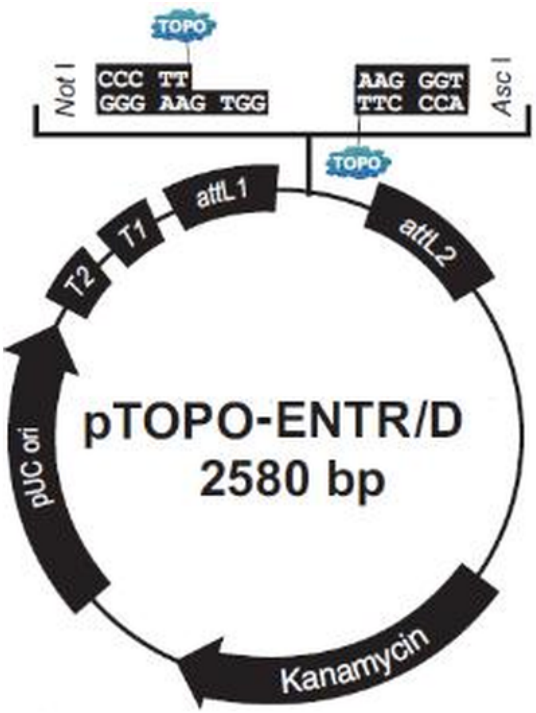
3) Add 300-500 µl LB or SOC medium without antibiotics (pre-equilibrated to room temperature) and shake at 37 °C, 180 rpm for 60 minutes. The medium can be added directly to the 1.5 ml tube, which is fixed horizontally in the shaker.

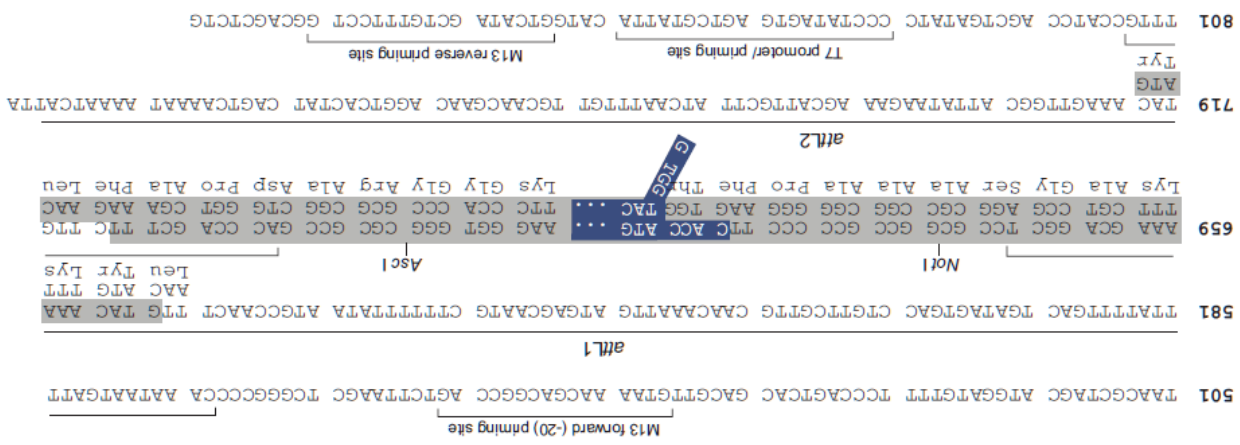
4) Spread 200 µl on LB agar containing **kanamycin** and incubate overnight. If few transformants are expected, centrifuge at 4,000 rpm for 1 minute, remove part of the supernatant, resuspend in 100-150 µl and plate all of it.

Note: This vector is kanamycin-resistant. Do not use ampicillin plates.

5. Screening: identify positive clones by colony PCR or plasmid restriction digestion, and confirm by sequencing with the M13 primers below.

Map of pTOPO-ENTR/D and cloning site sequence





Sequencing primers

Primer	Sequence (5' to 3')
M13F	CTGTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC

Vector features

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
attL1: bases 569-668 (c)
TOPO recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO recognition site 2: bases 689-693
attL2: bases 705-804
T7 Promoter/priming site: bases 821-840 (c)
M13 reverse priming site: bases 845-861
Kanamycin resistance gene: bases 974-1783
pUC origin: bases 1904-2577

(c) = complementary sequence

This product is furnished for LABORATORY RESEARCH USE ONLY. Not for diagnostic or therapeutic use.