SOP: Cloning of PCR Product

By

Richard Yu

香港城市大學 City University of Hong Kong

Research fellow

AREA OF EXCELLENCE M E R I T

Centre for Marine Environmental Research and Innovative Technology (MERIT)
Department of Biology and Chemistry
City University of Hong Kong
(Email: bhrichyu@cityu.edu.hk)

A. PCR amplification

1. Below is an example for setting up a PCR reaction using iTaq DNA Polymerase (BioRad):

	Vol. per rnx (µl)
10× iTaq buffer	5.0
50 mM $MgCl_2$	1.5
10 mM dNTP	1
10 µM Forward primer	1
10 µM Reverse primer	1
cDNA template (RT product)	3
Sterile water	37.25
iTaq DNA Polymerase	0.25
Total:	50

2. Thermal profile:

1 cycle of	3 min, 95°C
35 cycle of	30 sec, 95°C 30 sec, 55–60°C 30 sec – X min, 72°C (1 min for 1 kb product)
1 cycle of	10 min, 72°C
1 cycle of	4°C, forever

Note:
If *Pfu* is used for PCR amplification, perform a 10-min 72°C extension with dATP and *Taq* on the purified PCR product just before gel purification.

B. Gel electrophoresis and DNA extraction from agarose gel

1. Electrophorese 5–25 µl PCR product (0.2-1.0 µg) on a 1× modified TAE gel (40 mM Tris-acetate, pH 8.0, 0.1 mM Na_2EDTA).
2. Upon completion, cut out the gel slice containing the band of interest with a razor blaze under long-length UV (do it as fast as possible).
3. Place the gel slice into an Ultrafree-DA column (Millipore, Cat. 42600) and seal the cap. Spin at 5,000 ×g for 10 min to elute DNA from the gel slice.
4. Collect the filtrate (containing DNA) from the bottom of the tube.

C. Ligation using the pGEM-T Easy Vector System I (Promega, Cat. A1360)

1. Prepare a ligation reaction at RT (don't put the tube on ice) as follows:

pGEM-T vector (50 ng)	1 µl
Purified PCR product (filtrate)	3 µl
2× Rapid Ligation Buffer	5 µl (vortex before each use, place at RT)
T4 DNA Ligase (3 Weiss units/µl)	1 µl
Total volume:	10 µl

2. Mix the reaction by gentle pipetting. Incubate the reaction at room temperature for 2 h.

D. Transformation

1. Transfer 5–10 µl ligation product to just thawed DH5α competent cells (vol.=100 µl; thawing normally takes about 5 min).
2. Gently flick the tube to mix and place it on ice for 15 min.
3. Heat-shock the cells for 1 min in a 42°C water bath.
4. Immediately return the tube to ice. Keep it for 2 min.
5. Add 1 ml room-temperature LB broth (without ampicillin) to the tube (placed on a rack now) and incubate the cells for 1–1.5 hour in a 37°C water bath.
6. Collect the cells by spinning at 4,000 rpm for 5 min.
7. Spread the cells onto a LB+Ampicillin (150 µg/ml, Sigma, Cat. A-9518) agar plate pre-spread with 20 µl X-Gal (50 mg/ml, Promega, Cat. V394A) and 100 µl IPTG (100 mM, Invitrogen, Cat. 15529-019).
8. Invert and incubate the plate overnight at 37°C.

E. Check clones

1. Transfer 5 single clones with autoclaved toothpicks to tubes containing 30 µl LB+Ampicillin broth. Mix gently.
2. Dispense 2 µl of the cell culture to each PCR tube containing 18 µl sterile water.
3. Boil the cells for 10 min with a thermal cycler.
4. Add 5 µl of PCR master mix to the boiled cells (20 µl). Mix gently.

PCR master mix:

	Vol. per rnx (µl)	5 rnx (×6 vol.)
10× PCR SuperTherm buffer	2.5	15
10 mM dNTP	0.5	3
10 µM pGEMT-F primer	0.5	3
10 µM pGEMT-R primer	0.5	3
Sterile water	0.85	5.1
iTaq DNA Polymerase	0.15	0.9
	5	30

5. Perform the following profile with a thermal cycler:

1 cycle of	2 min, 94°C
35 cycle of	30 sec, 94°C 30 sec, 60°C 30 sec – X min, 72°C (1 min for 1 kb product)
1 cycle of	4°C, forever

6. Run PCR products on a 1.2% agarose gel stained with ethidium bromide or SYBR Green Safe Dye for 30 min.

```
Primer sequences:
pGEMT-F: 5′-ATTGGGCCCGACGTCGCATG-3′;
pGEMT-R: 5′-CAAGCTATGCATCCAACGCG-3′

Size of flanking region = 131 bp
```