

Subcloning Protocol

For subcloning DNA fragments into pGEM-3 blue vector

1. Digest 1 µg-1.5 µg pGEM-3 blue DNA with restriction enzyme. Phosphatasing reaction can be performed at the same time by adding 1 unit of CIP to the restriction reaction mixture, incubate at 370C, 1-2 hr as needed for complete digestion. Phosphatasing reaction can also be performed after restriction digest by adding 1 unit CIP to the mixture and incubate at 370C, 30 min. For blunt end DNA, phosphatasing can be done as described above if CIP is good, or for safety, as follows: after digestion, add 1 unit CIP to the mixture, 370C 15 min, 550C, 15 min. Add 1 unit CIP, 370C 15 min, 550C 15 min. CIP works well in TA buffer. The restriction digest volume is 10-20 µl.

2. Stop the phosphatasing reaction by adding 1µl 0.5M EDTA, heat at 680C 15 min. Add TE to 60 µl, extract 3 times with phenol, once with chloroform. Add %M ammonium acetate to final 2M, add 2 volumes ethanol, -700C, 20 min - 1 hr. Spin at 14,000 rpm, 40C, 10 min. Wash pellet (almost invisible) with 70% ethanol, spin 5 min 12,000 rpm, 40C. Dry pellet in vacuum, resuspend in 10 µl TE. Recovery is about 500 ng DNA.

3. Ligation reaction:

Mix the following in an eppendorf tube(all sterile):

~200 ng plasmid DNA

~200 ng DNA fragment

2 µl 5x BRL ligase buffer (has PEG)

1 unit BRL T4 DNA ligase

Total volume 10 µl. Stay at room temperature for 1 hr. Add 1x BRL ligase buffer to 50µl, add 2 unit BRL ligase. Incubate at 140C overnight. Store at -200C until use.

4. Preparation of E.coli DH5α competent cells:

DH5α cells are cultured in a test tube in LB overnight. Inoculate 0.5 ml to 50 ml LB in a 250 ml flask. Shake at 370C ~200 rpm, after 2 hr, start taking samples and check OD₆₀₀ every 10 min, until OD₆₀₀ reaches 0.3. Chill on ice, transfer 30 ml to a sterile, clean 30 ml screw cap centrifuge tube, spin 5,000 rpm 5 min, 40C. Resuspend pellet in 15 ml ice cold 100mM CaCl₂, 10mM Tris pH 8.0. Get ~300 µl competent cells. At this point, the cells can be used immediately or stored on ice for up to 24 hr with transformation efficiency go up to ~5 fold. But sometimes cells will lyse. For each sample, 100 µl cells(equal to 10 ml OD 0.3 culture) are needed.

5. Transformation:

100 µl competent cells were put in an ice cold 4 ml glass test tube or eppendorf tube. Add 100 ng plasmid DNA (~25 µl ligation mixture). Shake gently to mix. Leave on ice 30 - 60 min. Heat at 370C or 420C for 2 min (VERY IMPORTANT). Add 1 ml room temperature LB. Shake slowly at 370C, 1 hr. Plate 50-100 µl on each selection plate. After incubation at 370C overnight, recombinant give big white colonies (1 mm diameter), nonrecombinants give small blue colonies.

Transformation efficiency of DH5α cells is ~5,000,000/µg supercoil plasmid DNA.

Preparation of X-gal plates: Cool LB-agar(1.5%) to 55°C, add 60µg/ml ampicillin, 0.5mM IPTG, 40 µg/ml X-gal. Pour plates immediately. Plates were poured 2 hr before use, do not store long.

Preparation of X-gal: prepare 40mg/ml solution in dimethylformamide. Store at -200C, no need to sterilize.

Preparation of IPTG: prepare 0.1M solution in water, filter sterilize, store at -200C.

If cells aggregate upon addition of DNA, they are probably lysed due to dirty centrifuge tube.

If phosphatase reaction works well, >50% of transformants will be recombinants.

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