Transformation of E.coli with pGem-T Easy

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1 Works for me

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A-tailing procedure (Day 1)

1 Because the Phusion polymerase will remove the A-overhangs, adding these with Taq DNA Polymerase is necessary, as this is required for cloning

Mix the following reagents:

PCR Product	1-7µl
10x Taq Buffer	1µl
ATP to 0.2mM	1µl
Taq DNA Pol 5U	1µl
dH_2O	to 10µl

1. Incubate for 30 min at 70°C
2. Ideally and if DNA conc. is high engouh (> 50ng/µl), do another quick cleanup with the Promega Kit.

Ligation (Day 1)

2 Calculate the ratio of Vector:Insert (use molar ratios of 1:1 or 3:1):

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Mix the following reagents:

Vector (50ng/µl) 3kb	50ng
Insert	xng
2x Rapid Lig. Buff.	1µl
T4 Ligase	1µl
dH_2O	to 10µl

Incubate at 4°C over night for maximum ligation efficiency or 3 hours at room temperature.

Transformation (Day 2)

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1. Thaw DH5α **on ice** (100 µl in tube)
2. Add 3µl of ligation to 50µl of cells, mix by stirring gently with tip & incubate **on ice** for 20 min
3. Heat shock in water bath at 42°C for 30 sec
4. Return tubes **to ice** for 2 min
5. Add 53µl of transformed cells to 250µl of LB and incubate at 37°C for 60 min
6. Melt LB agar in microwave and keep at 50°C in water bath
7. Prepare 50ml of LB+Agar (2 plates): Add 37.5µl Carbencillin (Stock: 100mg/ml Plate:75µg/ml), 50µl of X-Gal /IPTG (Stock: 2%/0.1M Plate: 1:1000)
8. Plate 150µl of LB+cells onto 1 plate and incubate at 37°C over night

Identify colonies (Day 3)

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1. Remove plates from 37°C the next morning and check for colonies
2. Keep in the fridge for a couple of hours or until next morning so blue/white colours become more intense
3. Prepare master mix using a primer in the insert and the M13 fwd/rev primers to verify insert. (Can use blue colonies and M13 primers as neg. control)
4. Prepare 50ml of LB+Agar (2 plates): Add 37.5µl Carbencillin (Stock: 100mg/ml Plate:75µg/ml), 50µl of X-Gal /IPTG (Stock: 2%/0.1M Plate: 1:1000) and pick verified colonies as stock
5. Grow verified colonies over night in LB + Carbencillin (5ml LB + 5µl Cabencillin (100mg/ml dilutes to 100µg/ml))

Wizard Plus Promega Mini Prep Kit (Day 4)

5 **Prepare a glycerol stock:**

1. Mix 150µl cells with 150µl glycerol
2. Store at -80°C

Production of clear lysate:

3. Pellet 5ml of overnight culture for 5 minutes at 4000rpm.
4. Thoroughly resuspend pellet with 250µl of Cell Resuspension Solution.
5. Add 250µl of Cell Lysis Solution to each sample and leave until it clears up; invert 4 times to mix.
6. Add 10µl of Alkaline Protease Solution; invert 4 times to mix. Incubate 5 minutes at room temperature.
7. Add 350µl of Neutralization Solution; invert 4 times to mix.
8. Centrifuge at top speed for 10 minutes at room temperature.

Binding of Plasmid DNA:

9. Insert Spin Column into Collection Tube.
10. Decant cleared lysate into Spin Column.
11. Centrifuge at top speed for 1 minute at room temperature. Discard flowthrough, and reinsert Column into Collection Tube.

Washing:

12. Add 750µl of Wash Solution (**ethanol added**). Centrifuge at top speed for 1 minute. Discard flowthrough and reinsert column into Collection Tube.
13. Repeat Step 1. with 250µl of Wash Solution.
14. Centrifuge at top speed for 2 minutes at room temperature.

Elution:

15. Transfer Spin Column to a sterile 1.5ml microcentrifuge tube, being careful not to transfer any of the Column Wash Solution with the Spin Column. If the Spin Column has Column Wash Solution associated with it, centrifuge again for 1 minute at top speed, then transfer the Spin Column to a new, sterile 1.5ml microcentrifuge tube.
16. Add 100µl of Nuclease-Free Water to the Spin Column. Centrifuge at top speed for 1 minute at room temperature.
17. Discard column, and store DNA at –20°C or below.

Send to sequencing (Day 4)

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1. Measure DNA concentration on plate reader and check 260/280 & 260/230 values
2. Dilute samples to 100ng/µl
3. Prepare a 1:3 dilution (to 3.2pmol/µl) of sequencing primers