Date: 2017/10/11
Operators: Juliette

Ligation of plasmid with DNA insert

Equipment:

- T4 DNA Ligase Reaction Buffer 10X (stored at -20°C)
- T4 DNA Ligase (stored at -20°C)
- Vector DNA = pET32a, 5900 bp (stored at -20°C)
- Insert DNA = S1, 997 bp (stored at -20°C)
- Nuclease-free water
- Pipette p10, p20, p200, p1000 and associated cones
- Microcentrifuge Eppendorf tubes (1.5 ml)

Vector DNA: pET32a, 5900 bp
Insert DNA: S1, 997 bp

M(Insert DNA : S1) = 650 x 997 = 658 020 g/mol (Average bp mass = 650 g/mol)
n(Insert DNA : S1) = $6x10^{-14}$ mol
m(Insert DNA : S1) = n x M = $6x10^{-14}$ x 658 020 g = 39 ng

Concentration of Insert DNA : S1	
3.0 ng	1 µl
39 ng	13 µl

M(Vector DNA dp : pET32a) = 650 x 5900 = 3 894 000 g/mol
n(Vector DNA dp : pET32a) = $2x10^{-14}$ mol
m(Vector DNA dp : pET32a) = n x M = $2x10^{-14}$ x 3 894 000 g = 78 ng

Concentration of Vector DNA : pET32a	
6.4 ng	1 µl
78 ng	12.2 µl

Protocol:

1) Set up the following reaction in a microcentrifuge tube on ice.
2) Mix for a ratio 1:3

Total	28.2 µl
T4 DNA Ligase reaction buffer (10X)	2 µl
Vector DNA (5900 bp)	12.2 µl = 0.06 pmol
Insert DNA (997 bp)	13 µl = 0.02 pmol
T4 DNA Ligase	1 µl

The T4 DNA Ligase reaction buffer should be thawed.
T4 DNA Ligase should be added last.

3) Gently mix the reaction by pipetting up and down and microcentrifuge briefly.
4) For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 min.

5) For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 h.
6) Heat inactivate at 65°C for 10 min.
7) Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

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Protocol for Bacteria Transformation:

Aim: Transform competent bacteria with plasmids

Equipment:

- 500 µL tube of competent bacteria (competent cells stored at -80°C) : DH5α
- Plasmid to transform
- 1.5 mL Eppendorf tubes
- P1000, P200, P10 pipettes + paired cones
- Petri dishes with LB agar/CARB for carbenicillin (an equivalent of ampicillin, resistant to temperature) → Petri dishes (LB/CARB)
- 42°C water-bath
- Incubator 37°C with or without a stirrer/agitator
- Sterile rake/scraper/comb
- Timer

Plasmid transformed
- pET32a.S1

Protocol:
Competent cells are extremely sensitive**; all manipulations must take place on ice**; always handle the tubes on ice.
You must **operate in the vicinity of the Bunsen burner** when manipulating bacterial cultures.
Name the tubes with the transformed cells: Cell type/name, plasmid (vector and composition) initials of the operator: First Name/Last Name, date.

1. Split the tube of competent cells: DH5α in aliquots of 50 µl, in 1.5 Eppendorf tubes placed on ice.

For maximum competency:

2. Add 1µl of β-mercapto-ethanol only to subcloning grade competent cells DH5α.
3. Mix by gently tapping the bottom of the tubes.
4. Add 5 µl = 4.1 ng of plasmid pET32a.S1 to transform.
5. Mix by gently tapping the bottom of the tubes.
6. Let it rest for 30 minutes on ice.

In the meantime, check that the water-bath is at 42°C, and place the SOC media at 37°C for warming.

7. Put the tubes in the floats.
8. Place the floats in the 42°C water-bath for 40 seconds, then remove the floats quickly.
9. Place the tubes on ice for 3 minutes.
10. Add 650 µl of SOC media per tube.
11. Incubate and mix the tubes at 150 rpm at 37°C for 40 minutes.

In the meantime, place the labelled LB/CARB petri dishes at 37°C.

12. Generate 2 petri dishes with each tube: one dish containing 200 µl and the other 500 µl.
13. Spread the bacteria using an inoculator.
14. Wait for the dishes to dry.
15. Store the dishes with agar side up in the incubator at 37°C for approximately 16 hours (overnight).