1.i) UTR Preparation

If UTRs arrive as single-stranded oligonucleotides, then follow this protocol to get double-stranded UTRs which can be cloned. Note that the following protocol is applicable for those UTRs which contain a promoter upstream to them, i.e., a P-UTR (Promoter-UTR)

- Vortex the tubes containing the UTRs for a few seconds and add the required quantity of water (as specified by the document) to get the concentration of 100 µmol.
- To a PCR tube, add 15 µL of the forward and the reverse strands in a PCR tube.
- Heat the PCR tube at 95C for 10 minutes using a Thermocycler
- Transfer the Oligonucleotide solution to a heating block at 95C and allow it to cool gradually to RT. Double-stranded UTRs are now ready.
- The following reagents are added to the PCR tube containing the UTRs as follows
    - 30 µL of UTRs (already present)
    - 4 µL 10X fast digestion buffer
    - 2 µL XbaI
    - 2 µL PstI
    - 2 µL Distilled water.
- Once the reagents are added, the mixture is inoculated for 4 hours at 37C in a water bath.

1. ii) Plasmid Preparation (Modified protocol of thermos-Fischer kit)

- A 10 ml LB culture of E.coli containing chloramphenicol (10 µL) is prepared.

- Inoculate 10 µL of glycerol stock solution of the E.coli in 10 ml of LB containing chloramphenicol (10 µL). Allow the cells to grow overnight (generally, 12 -16 hours is sufficient). The next day, Pellet down the culture at 11,000 rpm for 45s.
- Resuspend the pelleted cells in 250 µL of the Resuspension Solution. Transfer the cell suspension to a microcentrifuge tube. The bacteria should be resuspended completely by pipetting until no cell clumps remain.
- Add 250 µL of the Lysis Solution and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear.
- Add 350 µL of the Neutralization Solution and thoroughly mix the tube by inverting it 4-6 times.
- Centrifuge for 10 min at 10,000 rpm to pellet cell debris and chromosomal DNA.
- Transfer the supernatant to the supplied GeneJET spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate.
- Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
- Add 500 µL of the Wash Solution (diluted with ethanol prior to first use) to the GeneJET spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
- Repeat the wash procedure (previous step) using 500 µL of the Wash Solution.
- Discard the flow-through and centrifuge for 3 min to remove residual Wash Solution. This step is essential to avoid residual ethanol in plasmid preps.

- Transfer the GeneJET spin column into a fresh 1.5 mL microcentrifuge tube. Add 50 µL of the milli-Q autoclaved water to the centre of the GeneJET spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 1 min at room temperature and centrifuge for 3 min for elution.
- Discard the column. The purified plasmid is now ready for digestion
- **Restriction digestion** of plasmid pSB1C3.
    - 50 µL of the purified plasmid
    - 2 µL of SpeI
    - 2 µL of PstI
    - 6 µL of 10X fast digestion buffer
- Once the reagents are added, incubate the mixture overnight for 6-8 hours at 37C in the water bath.

2.) Ligation

The PUTRs can now be ligated into the plasmid. Ligation should be carried out in the 3:1 ratio (insert : vector)

- Combine the following in a PCR or Eppendorf tube
    - PCR Purified digested vector.
    - Digested P-UTR.
    - Ligase Buffer (1µL/10µL reaction for 10X buffer)
    - 1µL T4 DNA Ligase
    - H_2O to a total of 10µL
- Incubate overnight at 16C using a thermomixer (0 rpm)

3.) Plates Preparation

LB plates containing chloramphenicol are prepared on the day before the transformation step. Around two plates are required per transformation, i.e., two plates for 1 UTR. The plates are then stored under 4C for the next day.

4.) Preparation of Competent E.coli cells

- Inoculate 10 µL of the E.coli cells (DH5α) from glycerol stock to 10 ml LB broth and incubate the cells overnight at 37C @ 180rpm.
- The next day, use 100 µL from the overnight culture and inoculate into a new LB broth of 10ml.
- Incubate the cells for 3 hr (to achieve an OD of 0.05) at 37C @ 180rpm. After incubation, centrifuge the broth at 10,000 rpm for 45s to obtain the pellets containing the cells. Resuspend the pellets using 1 ml ice-cold CaCl2.2H2O and allow it to stand for 30 minutes on ice.
- After 30 minutes, spin the centrifuge tubes at 10,000 rpm for 1min and discard the supernatant. After that, add 100 µL of CaCl2.2H2O (ice cold) and mix using a pipette slowly. The competent cells are now ready.

5.) Transformation

- Remove the stored plates and allow them to come to room temperature
- Add 100 µL of the competent cell solution to a microcentrifuge tube and add 2-4 µL of the ligation mix containing the plasmid. Mix the contents by flicking the bottom of the tube gently.
- Incubate the mixture on ice for 30 mins.
- After incubation, Heat-shock the tube by placing it into a 42C water bath for 90s.
- Put the tubes back on ice for 5 min.
- Add 800 µL of LB media (devoid of chloramphenicol) to the tube and allow the cells to grow in 37C shaking incubator for 2 hours.
- Plate 100 µL of the above mixture onto an agar plate containing chloramphenicol and another plate containing 200 µL of chloramphenicol. Use a triangular glass rod to spread the colonies throughout the agar.
- Incubate the plates at 37°C overnight. The next day, the colonies transformed successfully (fluorescence) are picked and patched onto a new agar plate containing chloramphenicol antibiotic for pure colony isolation.

6.) Fluorescence Evaluation

- Remove the patched plates, pick out the individual colonies, and place them in a 10ml LB tube containing chloramphenicol. The tube is incubated overnight at 37C at 180 rpm.
- The next day, measure the optical density at 600nm (OD_{600}) of the solution using a UV spectrophotometer and based on the reading, add an appropriate amount of LB to get a standard OD_{600} of 0.05
- Now, check the fluorescence of the cells at 0, 6, 12 and 24 hr intervals and note the results (Red fluorescence protein excitation at 532nm and emission at 605 nm).

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