Yuan Lab Protocols: Over-expression and Rescue Constructs

Note 1: This protocol is based on the pEarleyGate vectors (ABRC Stock CD3-683 – CD3-695, CD3-724)

1. **PCR insert and purify**

   use a high-fidelity enzyme (Phusion) to amplify your insert

PCR	1x	
dH_2O	12.0 μl	
5x Phusion buffer	4.0 μl	
dNTPs (10mM)	0.4 μl	
DMSO 100%	0.6 μl	
5' primer (5μM)	1.0 μl	***add "CACC" at beginning of forward primer**
3' primer (5μM)	1.0 μl	
template	1.0 μl	
Phusion enzyme	0.2 μl	ADD THIS LAST
TOTAL	20.0 μl	

   PCR Program:

   1. 98º C for 30 sec
   2. 98º C for 10 sec
   3. 60º C for 15 sec *may change depending on the primers you are using
   4. 72º C for 30 sec-2min *depends on the length of the fragment (30 sec/1kb)
   5. go to step #2, 33x
   6. 72º C for 10 min
   7. 12º C forever

   Run PCR product on a 1% gel to check for size and a single band. If the PCR is a clean, single band it can be PCR purified. If there are multiple bands, gel purify the fragment. Elute PCR product in 25ul dH2O.

2. **pENTR-D TOPO cloning reaction**

dH2O	1.0ul	***depending on the concentration of your PCR product, often you don't**
PCR product	1.0ul	***need add water, just use 2.0 ul of PCR product.**
salt solution	0.5ul	

Topo vector	<u>0.5 ul</u>
	3.0 ul

Mix and incubate at room temperature for at least 30 minutes, then put on ice.
Transform entire reaction into *E.coli* competent cells and plate all on Kan plates.

Transforming One Shot Top10 E. coli competent cells:

a. Take out S.O.C. from -20 freezer to thaw
b. Thaw 1 vial of One Shot cells on ice (1 vial can do 3 or 4 transformations, but it cannot be re-frozen) and aliquot the cells into separate PCR tubes, if necessary
c. Add product to be transformed into One Shot cells and mix gently (DO NOT mix by pipetting up and down)
d. Incubate on ice for 5 minutes
e. Heat shock the cells for 30 seconds at 42º C in the PCR machine
f. Immediately transfer the tubes to ice
g. Add 250 ul S.O.C. to the tube (125 ul if the cells were split into 2 or 3 tubes)
h. Shake the tube horizontally at 37º C for 1 hour
i. Spread 50-200 ul of the transformation on pre-warmed selective plates and incubate overnight at 37º C

3. <u>Colony PCR to check for the insert</u>

Make a Master Mix using the PCR-10x buffer protocol.
M13F (or T7F) primer and an insert specific primer **(cdsR)** to check for direction of the insert – **Important!**
Making a replica plate and adding template:

a. Circle and number the colonies you wish to colony PCR (8-16 colonies)
b. Get a new selective plate and make a grid and number each cell = replica plate
c. Using a P20 pipet tip, gently touch the side of a SINGLE colony, gently touch the corresponding grid on the replica plate, then place the pipet tip in the PCR tube containing your Master Mix
d. Incubate replica plate at 37º C for a few hours or overnight

Run PCR product on a gel to check for the presence of an insert. If using M13F/R, a fragment with no insert is ~300bp. Note which colonies have the insert.

4. <u>Culture 2 colonies with the insert and isolate the plasmid = entry clone</u>

In a labeled 15mL Falcon tube, pipet 3 mL of LB broth.
Add 3 uL of Kan to each tube
Pick a colony with the insert with a P20 tip and eject the tip into the Falcon tube.

Incubate at 37ºC with shaking overnight (try to start your cultures in the afternoon and take them out of the shaker in the morning – if left to grow too long, it will start to die).

Isolate the plasmid from the culture using a Plasmid Mini-prep Kit. Elute plasmid in 70 uL of dH2O. **note: elute the plasmid twice (35 + 35) works much better than once (70).**

5. **PCR using M13 F/R primers to amplify fragment for LR reaction**
   Necessary since the entry clone and the destination vector both have Kan resistance.
   Use the Phusion protocol (from Step 1) and M13F/R primers to amplify the fragment.
   Use only 28 cycles since the PCR should be very strong
   Gel or PCR purify the fragment.

6. **LR Recombination reaction**

dH2O	2.5 ul	
entry clone PCR fragment	1.0 ul	
Destination vector	0.5 ul	*select desired vector p100, p103, p302, etc.
LR clonase	1.0 ul	*vortex 2 sec. twice and spin down before adding
	5.0 ul	

   Mix well and incubate at room temperature (25ºC) for 2 hours.
   Add 1 ul proteinase K to terminate the reaction and vortex briefly.
   Incubate at 37° for 10 minutes.
   Transform 3 ul of the reaction into *E. coli* competent cells and plate 20-100 uL on Kan plates.

7. **Colony PCR using insert specific primers to check for insert**
   Make a Master Mix using the PCR-10x buffer protocol.
   Use insert specific primers to check for insert or a primer on the vector if available (attR2 for example)
   Make a replica plate.
   Run PCR product on a gel to check for the presence of an insert.

8. **Culture 2 colonies and isolate the plasmid = final plasmid**
   Culture 2 colonies in 3 mL of LB broth + Kan overnight at 37ºC with shaking.
   Isolate the plasmid using a Plasmid Mini-prep Kit. Elute in 70 uL (35 + 35) of dH2O.

9. **Sequence the final plasmid to check for mutations**
   See sequencing protocol.