Over-expression and Rescue Constructs (Yuan Lab)

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	
F primer (5µM)	1.0 µl	***add "CACC" at beginning of forward primer**
R primer (5µM)	1.0 µl	
template	1.0 µl	
Phusion enzyme	0.2 µl	ADD THIS LAST
TOTAL	20.0 µl	

PCR Program:

1. 98ºC for 30 sec
2. 98ºC for 10 sec
3. 60ºC for 15 sec — *anneal temperature depends on the primers
4. 72ºC for 30 sec-2min — *depends on the length of the fragment (30 sec/1kb)
5. go to step #2, 33x
6. 72ºC for 10 min
7. 12ºC forever

Run PCR product on a 1% gel to check for size and a single band. If the PCR product is a clean, single band, it can be PCR purified. If there are multiple bands, gel purify the fragment. Elute PCR product in 25µl dH_2O.

2. pENTR-D TOPO cloning reaction

dH_2O	1.0µl	
PCR product	1.0µl	*adjust the volume of PCR product and dH_2O based on concentration**.**
salt solution	0.5µl	
Topo vector	0.5 µl	
	3.0 µl	

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 µl S.O.C. to the tube (125 µl 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 µl of the transformation on pre-warmed selective plates and incubate overnight at 37º C

3. Colony PCR to check for the insert

Use **M13F (or T7F)** primer and an insert specific primer **(cdsR)** to check for the size and direction of the insert – **Important!** Sometimes the insert could be inserted in the opposite direction
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 in the correct orientation.

4. Culture two colonies with the correct insert and isolate the plasmid = entry clone

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

dH_2O	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 37º 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

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 two colonies and isolate the plasmid = final plasmid

Culture two 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 verify the final plasmid