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Over Expression and Rescue Constructs

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Mimulus

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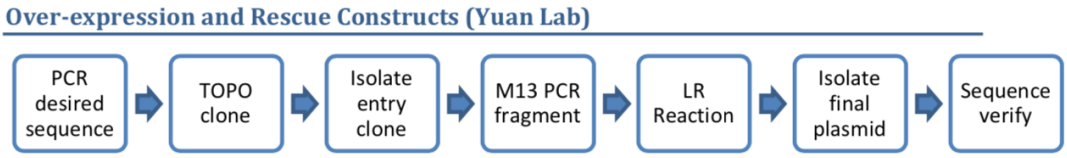
EXTERNAL LINK

http://mimubase.org/FTP/Protocols/Plasmid_Construction/Over-expression%20and%20Rescue%20Constructs.pdf

PDF

Over Expression and Rescue Constructs (Yuan Lab(.pdf

GUIDELINES



SAFETY WARNINGS

For Safety Warnings and Hazard Information please refer to the SDS (Satety Data Sheet).

PCR Insert and Purify

1 Use a high-fidelity enzyme (Phusion) to amplify your insert.

PCR	1x
dH2O	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
R Primer (5µM)	1.0 µL
Template	1.0 µL

Phusion Enzyme**	0.2 µL
Total	**20 µL**

*F Primer - Add "CACC" at beginning of forward primer.
**Add Phusion Enzyme last.

PCR Program

1.1 PCR Program

1. **98 °C** for **00:00:30**
2. **98 °C** for **00:00:10**
3. **60 °C** * for **00:00:15**
4. **72 °C** for **00:00:30** - **00:02:00** **
5. go to step #2, 33x
6. **72 °C** for **00:10:00**
7. **12 °C** forever

*Anneal Temperature depends on primers.
**Depends on the length of the fragment (30 sec/1 kb)

1.2 Run PCR product on 1% gel to check for size and a single band.

If the PCR product is clean, single band, it can be purified.

If there is multiple bands, gel purify the fragment. Elute PCR product in **25 µl dH2O**.

pENTR-D TOPO Cloning Reaction

2 Mix and incubate at room temperature for at least **00:30:00**, then put on ice. Transform entire reaction into *E. coli* competent cells and plate all on Kan plates.

dH2O	1.0 µL
PCR Product*	1.0 µL
Salt Solution	0.5 µL
Topo Vector	0.5 µL
Total	**3.0 µL**

*adjust the volume of PCR product and dH2O based on concentration.

Transforming One Shot Top10 *E. Coli* competent cells

3 Take out S.O.C from **-20 °C** freezer to thaw.

4 Thaw 1 vial of One Shot cells on ice and aliquot the cells into separate cells into separate PCR tubes, if necessary.

1 vial can do 3 or 4 transformations, but it cannot be refrozen.

5 Add product to be transformed into One Shot cells and mix gently.

DO NOT mix by pipetting up and down.

6 Incubate on ice for **00:05:00**.

7 Heat shock the cells for **00:00:30** at **42 °C** in the PCR machine.

8 Immediately transfer the tubes to ice.

9 Add **250 µl** S.O.C to the tube.

Add **125 µl** if the cells were split into 2 or 3 tubes.

10 Shake the tube horizontally at **37 °C** for **01:00:00**.

11 Spread **50 µl**-**200 µl** of the transformation on pre-warmed selective plates and incubate overnight at **37 °C**.

Colony PCR to check for the Insert

12 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

12.1 Circle and number the colonies you wish to colony PCR (8-16 colonies).

12.2 Get a new selective plate and make a grid and number each cell = Replica Plate

12.3 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.

12.4 Incubate replica plate at **37 °C** for a few hours or overnight.

12.5 Run PCR product on a gel to check for the presence of an insert in the correct orientation.

Culture two colonies with the correct insert and isolate the plasmid (entry clone)

13 In a labeled 15 mL Falcon tube, pipet **3 ml LB broth**.

14 Add **3 µl** of Kan to each tube.

15 Pick a colony with the insert using a P20 tip and eject into falcon tube.

16 Incubate at **37 °C** 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.

17 Isolate the plasmid from the culture using a Plasmid Mini-Prep Kit. Elute plasmid in **70 µl dH2O**.

Elute the plasmid twice (**35 µl** + **35 µl**). Works much better than once (**70 µl**).

PCR using M13 F/R primers to amplify fragment for LR reaction

18 Necessary since the entry clone and the destination vector both have Kan resistance.
Use the Phusion protocol from Step 1 **go to 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.

LR Recombination reaction

19 Mix well and incubate at **Room temperature** (**25 °C**) for **02:00:00**.

dH2O	2.5 µL
Entry clone PCR fragment	1.0 µL
Destination Vector*	0.5 µL
LR Clonase**	1.0 µL
Total	5.0 µL

*Selected Desire vector p100, p103, p302, etc.
** Vortex 2 sec. twice and spin down before adding

20 Add **1 µl proteinase K** to terminate the reaction and vortex briefly.

21 Incubate at 37 °C for 00:10:00 .

22 Transform 3 µl of the reaction into *E.Coli* competent cells and plate 20-100 uL on Kan plates.

Colony PCR using insert specific primers to check for insert

23 Use insert specific primers to check for insert or a primer on the vector if available (attR2 for example).

24 Make a replica plate.

25 Run PCR product on a gel to check for the presence of an insert.

Culture two colonies and isolate the plasmid (final plasmid)

26 Culture two colonies in 3 ml **LB broth + Kan** overnight at 37 °C with shaking.

26.1 Isolate the plasmid using a Plasmid Mini-Prep Kit. Elute in 70 µl **dH2O**.

Elute the plasmid twice (35 µl + 35 µl). Works much better than once (70 µl).

Sequencing

27 Sequence verify the final plasmid.