Yuan Lab Protocols: RNAi CONSTRUCTS

Note 1: This protocol is based on the vector pFGC5941 (ABRC Stock CD3-447).

Note 2: To avoid off-target effect, make sure no other regions in the interested genome perfectly match the RNAi fragment (150-500 bp) for a contiguous block longer than 16 bp. Also, make sure there are no restriction sites for the enzymes NcoI, AscI, BamHI, or XbaI within the RNAi fragment.

Note 3: When designing primers to amplify the RNAi fragment. Add "GTTCTAGACCATGG" at the 5' end of the Forward primer and add "GTGGATCCGGCGCGCC" at the 5' end of the Reverse primer.

Note 4: Make sure you have digested the pFGC5941 vector using NcoI/AscI before the first ligation.

Protocol:

1. **Amplify insert from cDNA or gDNA (if the fragment contains no intron) using Phusion PCR;**

   Do **TWO** 20-µl reactions:
   4 µl 5x Phusion buffer
   0.5 µl 10mM dNTPs
   0.6 µl DMSO
   1.0 µl template
   0.2 µl Phusion enzyme
   11.0 µl dH2O
   1.5 µl 5 µM primer F
   1.5 µl 5 µM primer R
   20 µl total

   Phusion program:
   cycle 1: 98 for 0:30
   cycle 2: (32x) 98 for 0:10
   58 for 0:20
   72 for 0:30
   cycle 3: 72 for 5:00
   cycle 4: 12 for ever

2. **Digest insert with NcoI/AscI and BamHI/XbaI**

   2.5 µl 10x CutSmart Buffer
   4.5 µl dH2O
   1.5 µl NcoI
   1.5 µl AscI
   15 µl PCR product
   25 µl total

   same protocol for BamHI/XbaI digestion;
   incubate 37 degrees for 1 hour;
   gel purify digests and save the BamHI/XbaI digested insert for the second ligation.

3. **First ligation**
   Want an insert to vector molar ratio of 2:1 to 6:1

2 µl linearized pFGC5941 digested with AscI/NcoI **(~175ng; adjust volume based on concentration)**
4 µl insert digested with AscI/NcoI (~15-30ng)
2 µl T4 ligase buffer
1 µl T4 ligase
11 µl dH2O
20 µl total

incubate 30 minutes at room temperature;
transform 10ul into *E. coli* competent cells (homemade) and plate on Kan plates.

4. **Colony PCR to check for first insert**
Circle the biggest colonies on your plate and label them 1-8.
Make a replica plate for your colonies.
PCR across the first insert using primers on the vector to check for an insert:
An empty vector will give a band of 700bp

8.0 ul dH20	Colony PCR Program:
1.0 µl 10x buffer	cycle 1: 95 for 3:00
.125 µl dNTPs	cycle 2: (32x) 95 for 0:15
0.5 µl pFGC5941 **2372 F**	55 for 0:15
0.5 µl pFGC5941 **3082 R**	72 for 1:00
0.05 µl Taq	cycle 3: 72 for 7:00
10 µl total	cycle 4: 12 forever

5. Pick two correct colonies and inoculate into 3 mL LB+Kan broth
incubate in 37 degree shaker overnight
The next day, do a plasmid prep (mini-prep kit) with 1 of the colonies that grew well

6. Digest plasmid with BamHI/XbaI
5 µl 10x CutSmart Buffer
12 µl dH2O
1.5 µl XbaI
1.5 µl BamHI
30 µl plasmid * adjust volume based on concentration; you want 2000-5000 ng of plasmid
50 µl total

37 degrees for 1 hour
gel purify digest

7. Ligation #2
2 µl vector that contains the first insert, digested with BamHI/XbaI (**~175ng; adjust volume based on concentration**)
4 µl insert digested with BamHI/XbaI (done in step 2) (want ~15-30 ng)
2 µl T4 ligase buffer
1 µl T4 ligase
11 µl dH2O
20 µl total

incubate 30 minutes at room temperature
Transform 10ul into *E. coli* comp cells **(homemade)** and plate on Kan plates

8. Colony PCR to check for second insert

pFGC5941 **3930 F** & pFGC5941 **4430 R**

Vector without insert will give a band of 500bp

9. Pick two correct colonies and inoculate into 3 mL LB+Kan broth

incubate in 37 degree shaker overnight

Plasmid prep (mini-prep kit)

10. Check plasmid for inserts

PCR to check for both inserts:

2372F/3082R or RNAi_R (insert specific)

3930F/4430R or RNAi_F (insert specific)

11. Sequence to verify

Use 4 primers:

2372F, 3082R, 3930F, 4430R

Note: in the sequencing reaction, add DMSO to aid in the sequencing across the restriction enzyme digest sites (the chromatogram peaks usually drop off dramatically right after the digest sites; an alternative strategy is to PCR the final plasmid with 2372F&3082R for the left insert and 3930F&4430R for the right insert and then sequence the PCR product)

12. Transform into agrobacterium for infiltration

Primer sequences:

pFGC5941_2372F: CTTCATCGAAAGGACAGTAGAA
pFGC5941_3082R: CCAAACAGGCTCATAGATACT
pFGC5941_3930F: TGTACATCAGAATGTTTCTGAC
pFGC5941_4430R: CGCTCTATCATAGATGTCGCTA