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 “GTGGATCCGCGCGG” at the 5’ end of the Reverse primer.

**Note 4:** Make sure you have digested the pFGC5941 vector using Ncol/Ascl 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 Ncol/Ascl and BamHI/XbaI**
   
   2.5 µl 10x CutSmart Buffer
   4.5 µl dH2O
   1.5 µl Ncol
   1.5 µl Ascl
   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 Ascl/NcoI (~175ng; adjust volume based on concentration)

4 μl insert digested with Ascl/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
1.0 μl 10x buffer
t.125 μl dNTPs
cycle 1: 95 for 3:00
0.5 μl pFGC5941 2372 F
0.5 μl pFGC5941 3082 R
0.05 μl Taq
cycle 2: (32x) 95 for 0:15
55 for 0:15
72 for 1:00
cycle 3: 72 for 7:00
10 μl total
cycle 4: 12 for ever

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