RNAi plasmid construction using pFGC5941 (Yuan Lab)

1. **Amplify insert from flower cDNA or gDNA (depending on the sequence) using Phusion PCR**

Do **2** 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 (or ideal annealing temp)
72 for 0:30
cycle 3: 72 for 5:00
cycle 4: 12 for ever

2. **Digest insert with NcoI/AscI and BamHI/XbaI and pFGC5941 plasmid with NcoI/AscI**

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

2.5 µl 10x CutSmart Buffer
4.5 µl dH2O
1.5 µl XbaI
1.5 µl BamHI
15 µl PCR product
25 µl total

Incubate 37 degrees for 1 hour
Gel purify digests and save the BamHI/XbaI digested insert for the second ligation

3. **Ligation #1**

Want an insert to vector ratio of 2:1 to 6:1
2 µl linearized pFGC5941 digested with AscI/NcoI (**~175ng; adjust volume accordingly**)
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* comp 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, unless they're sufficiently space out on the original plate
PCR across the first insert using primers on the vector to check for an insert:
An empty vector will give a band of 700bp (3082-2372=710)

8.0 ul dH20
1.0 µl 10x buffer
.125 µl dNTPs
0.5 µl pFGC5941 **2372 F**
0.5 µl pFGC5941 **3082 R**
0.05 µl Taq
10 µl total
Colony PCR Program:
cycle 1: 95 for 3:00
cycle 2: (32x) 95 for 0:15
55 for 0:15
72 for 1:00
cycle 3: 72 for 7:00
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 with the first insert, digested with BamHI/XbaI (**~175ng; adjust volume accordingly**)
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
Homemade TOP10 E. coli competent cells transformation:
1. Thaw competent cells on ice and transfer to a chilled PCR tube
2. Add your reaction to the cells (10ul if RNAi ligation, 3ul of LR reaction)
3. Incubate on ice for 5 minutes
4. Heat-shock cells for **45 seconds** at 42°C in the PCR machine
5. Immediately transfer the PCR tube to ice and incubate on ice for 5 minutes
6. Add **900ul LB** to a labeled eppie tube
7. Transfer your cells to the LB and cap the tube tightly

8. Incubate and shake the cells at 37°C for 1 hour (tape to bottom of shaker)
9. Centrifuge to spin down the cells 8000rpm for 1 minute
10. Pipet off 700ul of the LB
11. Resuspend the cells in the 200ul of LB
12. Spread all of the cells onto a LB Kan plate (or appropriate antibiotic)
13. Incubate at 37°C overnight (should visibly see colonies late morning of the next day)

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**