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RNAi Plasmid Construction using pFGC5941

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Mimulus

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EXTERNAL LINK

http://mimubase.org/FTP/Protocols/Plasmid_Construction/RNAi%20plasmid%20construction%20using%20pFGC5941.pdf

RNAi plasmid construction using pFGC5941.pdf

GUIDELINES

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

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.

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.

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

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

SAFETY WARNINGS

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

Amplifying insert from cDNA or gDNA using Phusion PCR

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

Make **TWO** **20 µl** reactions of the following in separate tubes:

Amount (µL)	Reagent
4 µL	5x Phusion Buffer
0.5 µL	10 mM dNTPs
0.6 µL	DMSO
1.0 µL	Template
0.2 µl	Phusion enzyme
11.0 µL	dH2O
1.5 µL	5 µM Forward Primer
1.5 µL	5 µM Reverse Primer
20 µL	**Total**

2 Run Phusion PCR program:

Cycle	Repeats	Temperature	Time
Cycle 1		98°C	0:30
Cycle 2	(32x)	98°C	0:10
		58°C (or the ideal annealing temperature)	0:20
		72°C	0:30
Cycle 3		72°C	5:00
Cycle 4		12°C	for ever

Digestion

3 Digest one insert with **NcoI/AscI** and the other one insert **BamHI/XbaI.**

See step 12 for BamHI/XbaI digestion.

Amount (µL)	Reagent
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**

3.1 Incubate samples for **01:00:00** at **37 °C**.

3.2 Gel purify digests and save the BamHI/XbaI digested insert for the second ligation.

First ligation

4 First Ligation (Would like insert to vector molar ratio to 2:1 to 6:1)

Amount (µL)	Component
2 µL	Linearized pFGC5941 digested with AscI/NcoI. (~175ng; adjust volume as needed)
4 µL	Insert (digested with AscI/NcoI) (~15-30ng)
2 µL	T4 Ligase Buffer
1 µL	T4 Ligase
11 µL	dH2O
20 µL	**Total**

4.1 Incubate at **Room temperature** for **00:30:00** .

4.2 Transform **10 µl** into *E. coli* competent cells (homemade) and plate on Kan plates.

Colony PCR to check for first insert

5 **Colony PCR to check for first insert**

Amount (µL)	Component
8.0 µL	dH2O
1.0 µL	10x buffer
0.125 µL	dNTPs
0.5 µL	pFGC5941 2372 F
0.5 µL	pFGC5941 3082 R
0.05 µL	Taq
10 µL	**Total**

5.1 Run Colony PCR

Cycle	Repeats	Temperature	Time
Cycle 1		95°C	3:00
Cycle 2	32x	95°C	0:15
		55°C	0:15
		72°C	1:00
Cycle 3		72°C	7:00
Cycle 4		12°C	forever

6 Circle the biggest colonies on your plate and label them 1-8.

7 Make a replica plate for your colonies.

8 PCR across the first insert using primers on the vector to check for an insert:
An empty vector will give a band of 700bp

Picking Colonies and Plasmid Prep

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

10 Incubate in **37 °C** shaker overnight .

11 The next day, do a plasmid prep (mini-prep kit) with 1 of the colonies that grew well.

Digest Plasmid with BamHI/XbaI

12

Amount	Component
5 µl	10x CutSmart Buffer
12 µl	dH2O
1.5 µl	XbaI
1.5 µl	BamHI
30 µl	Plasmid*
50 µl	**Total**

* adjust volume based on concentration; you want 2000-5000 ng of plasmid

12.1 **37 °C** for **01:00:00** .

12.2 Gel purify digest.

Ligation #2

13 **2 µl** vector that contains the first insert, digested with BamHI/XbaI (**~175 ng; adjust volume based on concentration**).

Amount	Component
4 µl	insert digested with BamHI/XbaI (done in step 3) (want ~15-30 ng)
2 µl	T4 ligase buffer
1 µl	T4 ligase
11 µl	dH2O
20 µl	**Total**

13.1 Incubate for **00:30:00** at **Room temperature**.

13.2 Transform **10 µl** into *E. coli* competent cells (homemade) and plate on Kan plates.

Colony PCR to check for second insert

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

Vector without insert will give a band of 500bp

15 Pick two correct colonies and inoculate into **3 ml LB+Kan broth**.

15.1 Incubate in **37 °C** shaker overnight.

15.2 Plasmid prep (mini-prep kit)

Check plasmid for inserts

16 PCR to check for both inserts:
2372F/3082R or RNAi_R (insert specific)
3930F/4430R or RNAi_F (insert specific)

Sequence to verify

17 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)

18 Transform into agrobacterium for infiltration.