

Jun 13, 2019

RNAi Plasmid Construction using pFGC5941

DOI

dx.doi.org/10.17504/protocols.io.2w2gfge



Yaowu Yuan¹

¹University of Connecticut

Mimulus



Andrea Sweigart

University of Georgia

OPEN  ACCESS



DOI: dx.doi.org/10.17504/protocols.io.2w2gfge

External link:

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

Protocol Citation: Yaowu Yuan 2019. RNAi Plasmid Construction using pFGC5941. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.2w2gfge>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: May 16, 2019

Last Modified: June 13, 2019

Protocol Integer ID: 23226

Keywords: RNAi, Plasmid Construction, Plasmids, Mimulus, pFGC594, Constructs

Attachments



RNAi plasmid constru...

110KB

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, Ascl, 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/Ascl before the first ligation.

Primer sequences:


pFGC5941_2372F: CTTTCATCGAAAGGACAGTAGAA

pFGC5941_3082R: CCAAACAGGCTCATAGATACT

pFGC5941_3930F: TGTACATCAGAATGTTTCTGAC

pFGC5941_4430R: CGCTCTATCATAGATGTCGCTA


Safety warnings

 For Safety Warnings and Hazard Information please refer to the SDS (Safety 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	dH ₂ O
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/Ascl** and the other one insert **BamHI/XbaI**.

Note

See step 12 for BamHI/XbaI digestion.



Amount (μL)	Reagent
2.5 μL	10x CutSmart Buffer
4.5 μL	dH ₂ O
1.5 μL	NcoI
1.5 μL	Ascl
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 Ascl/NcoI. (~175ng; adjust volume as needed)
4 μL	Insert (digested with Ascl/NcoI) (~15-30ng)
2 μL	T4 Ligase Buffer
1 μL	T4 Ligase
11 μL	dH ₂ O
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	dH ₂ O
1.0 μL	10x buffer
0.125 μL	dNTPs
0.5 μL	pFGC 5941 2372 F
0.5 μL	pFGC 5941 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	dH ₂ O
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	dH ₂ O
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

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.

