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Cloning into pSL2680 CRISPR Plasmid - iGEM IISER Pune 2021

misaal.bedi [1]

[1]Indian Institute of Science Education and Research, Pune

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misaal.bedi

ABSTRACT

This protocol can be used to clone the guide RNA and Homologous Repair Template into the pSL2680 plasmid which was a gift from Himadri Pakrasi (Addgene plasmid # 85581 ; http://n2t.net/addgene:85581 ; RRID:Addgene_85581).

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Cpf1 Is A Versatile Tool for CRISPR Genome Editing Across Diverse Species of Cyanobacteria. Ungerer J, Pakrasi HB. Sci Rep. 2016 Dec 21;6:39681. doi: 10.1038/srep39681. 10.1038/srep39681 PubMed 28000776

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MATERIALS TEXT

pSL2680 was a gift from Himadri Pakrasi (Addgene plasmid # 85581 ; http://n2t.net/addgene:85581 ; RRID:Addgene_85581)

Restriction enzymes: AarI, KpnI + their buffers
Antibiotics: Kanamycin
LB media
Distilled Water
Plasmid Miniprep Kit
Gel Purification Kit
Gel electrophoresis set up
Ligase buffer
PNK Enzyme
NEB Gibson Assembly Kit

Cloning the gRNA

1 The gRNA is to be cloned before the cassette into the plasmid.

Day 1

2 Take **30 mL** of LB media with **0.05 µg/µl** Kanamycin and scratch off some solid culture from the vial and add to the media in a flask. Grow this **Overnight** at **37 °C**

Day 2

3 Perform miniprep on the entire **30 mL** culture (since the pSL2680 plasmid is of a low copy number). Spin it down and resuspend it in **500 µl** resuspension buffer in the fridge. Divide it into two sets and follow the rest of the miniprep protocol. In the end, elute from the column with **50 µl** of distilled water.

3.1 Digest the **50 µl** of miniprep with AarI using the following reaction mix:

AarI buffer - **10 µl**

plasmid prep - **50 µl**

50X AarI oligo - **2 µl**

AarI enzyme - **4 µl**

Distilled water - **34 µl**

Incubate at **37 °C** for 4 hours.

3.2 Gel extract the digest. Run the entire digest in a double or triple lane on a 0.7-1% agarose gel. Excise the largest band and place it in a **2 mL** Eppendorf tube. Melt at **55 °C** (Tip: Inverting the tube every five minutes helps in faster melting)

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3.3 Perform gel purification using a gel purification kit and follow its protocol.

3.4 In the end, elute with **20 µl** of water (ensure that a small amount of water is placed in the center of the membrane).

Day 3

4 Anneal the gRNA oligos with the following reaction mixture:

100 Micromolar (µM) stock of the gRNA left primer - **10 µl**

100 Micromolar (µM) stock of the gRNA right primer - **10 µl**

Ligase buffer - **5 µl**

Distilled water - **25 µl**

PNK enzyme - **1 µl**

Use the following thermocycler program:

Heat to **95 °C** for 5 minutes, and then ramp to **4 °C** at **0.1 °C** /second.

4.1 Dilute the annealed oligos 1:50. Not a lot is required so **2 µl** with **98 µl** of water can be used.

Ligate the oligos into pSL2680 which was digested the previous day with the following reaction mix:

pSL2680 gel extract - **8 µl**

Annealed oligos that were dilute 1:50 - **0.5 µl**

Ligase buffer - **1 µl**

Ligase - **0.5 µl**

Incubate **Overnight** at **16 °C**

Day 4

5 Transform the entire **10 µl** ligation reaction into the E. coli XL1 - blue or Top10 strains as follows:

Thaw cells **On ice** for 10 minutes.

Add **10 µl** ligation reaction to cells and stir. Don't pipette up and down.

Incubate **On ice** for another 10 minutes.

Heat shock for 45 seconds at **42 °C** .

Place **On ice** for 2 minutes.

Add **500 µl** of liquid LB media.

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Incubate at **37 °C** for 1 hour.

5.1 Centrifuge the reaction mixture after incubation and remove the supernatant. Add the pellet to **50 µl** of the media.

Plate transformants on LB media with **0.05 µg/µl** Kanamycin and add IPTG+X-gal.

Spread **40 µl** of **20 Milimolar (mM)** IPTG and **20 mg/ml** X-gal on a plate with LB media with **0.05 µg/µl** Kanamycin and let it soak in for at least an hour.

Prepare the plates before starting the transformation.

Day 5

6 Pick 8 white colonies from the transformants and patch them on LB media with **0.05 µg/µl** Kanamycin.

Start **10 mL** cultures of 2 colonies off the plate in LB media with **0.05 µg/µl** Kanamycin.

Day 6

7 Isolate the plasmid from the 2 cultures incubated the previous day. Determine the concentration and send for sequencing. Once the correct sequence is confirmed, prepare stocks.

Cloning of cassette

8 After the gRNA has been cloned into the plasmid, we can begin cloning the cassette to make modifications in the S. elongatus UTEX 2973 genome.

Day 1

9 Synthesize the repair template using high-fidelity PCR.

Gel extract the PCR reactions and purify them, and elute in **25 µl** of distilled water.

Digest the plasmid carrying the gRNA using KpnI and make sure to dephosphorylate it, to prevent recircularization of the backbone.

Use the following reaction mixture:

Buffer - **15 µl**

Plasmid Prep - **75 µl**

KpnI restriction enzyme - **3 µl**

FastAP (dephosphorylates vector) -

Distilled Water - **53 µl**

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Incubate for 4 hours at **37 °C**

Gel extract the digest and elute in **25 µl** of distilled water.

Day 2

10 Concentrate the gel extractions and perform Gibson Assembly and concentrate the DNA.
Allow samples to dry for 2.5-3 hours.

For Gibson Assembly, exactly **5 µl** of correctly mixed and highly concentrated DNA is required. Use **200 ng** of the vector and a 2X molar ratio of each fragment to be assembled by Gibson Assembly.

Note: Use molar ratios of the fragments, not weight.

The vector and PCR fragments should be mixed using the

Prepare the mixture in a PCR tube with a total volume of **5 µl** .

Add **15 µl** of the Gibson master mix to the **5 µl** of DNA.

Mix them together and PCR at **50 °C** for an hour and then hold at **4 °C**

Day 3

11 Transform at least **10 µl** of the Gibson reaction in XL1-blue (or Top10) using the same protocol as described in step 5 of Day 4 in the 'Cloning the gRNA' section of this protocol.
Plate on LB media with **[M]0.05 µg/µl** Kanamycin

Day 4

12 Check the transformed colonies by PCR.
Run the reactions on a gel and select two positive colonies.

Start with **10 mL** of each colony in LB media with **[M]0.05 µg/µl** Kanamycin for plasmid isolation.

Day 5

13 Isolate plasmids from the two colonies isolated the previous day and elute in **50 µl** .
Sequence the insert in the plasmid. If the sequencing is correct, this protocol has been successfully completed and all that remains is to transform the plasmid into cyanobacteria.

Note: Don't forget to freeze the recombinant E.coli in permanent stock.

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