Oct 02, 2020

CRISPRon-GFP (based on pX459 plasmid) cloning of guides

Ofer Barnea-Yizhar[1]

[1]Shahar Taiber

1 *Works for me* This protocol is published without a DOI.

AhituvLab

Ofer Yizhar-Barnea

ABSTRACT

This protocol was written in accordance in accordance with the Zheng Lab protocol and the following lessons learned from the following papers.

PROTOCOL CITATION

Ofer Barnea-Yizhar 2020. CRISPRon-GFP (based on pX459 plasmid) cloning of guides. **protocols.io** https://protocols.io/view/crispron-gfp-based-on-px459-plasmid-cloning-of-gui-bmmuk46w

CREATED

Sep 22, 2020

LAST MODIFIED

Oct 02, 2020

PROTOCOL INTEGER ID

42388

MATERIALS

NAME	CATALOG #	VENDOR
QIAprep Spin Miniprep Kit	27104	Qiagen
BbsI-HF - 1,500 units	R3539L	New England Biolabs
pSpCas9(BB)-2A-Puro (PX459) V2.0	62988	addgene
10 U/µl NEB T4 PNK, 3 U/µl NEB T4 DNA polymerase I , 5 U/µl NEB DNA polymerase I, Large (Klenow) Fragment		New England Biolabs

STEPS MATERIALS

NAME	CATALOG #	VENDOR
NEB 5-alpha Competent E.coli (High Efficiency) - 20x0.05 ml	C2987H	New England Biolabs
SOC Outgrowth Medium - 100 ml	B9020S	New England Biolabs
QIAprep Spin Miniprep Kit	27104	Qiagen
BbsI	R0539S	New England Biolabs

DISCLAIMER:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety

ABSTRACT

This protocol was written in accordance in accordance with the Zheng Lab protocol and the following lessons learned from the following papers.

Vector digest

1 Digest **1 µg** of an empty vector with

BbsI
by New England Biolabs
Catalog #: R0539S

(the enzyme is kept in -80 REVCO). Comprise the reaction as follows:

Vector (dervided from CRIPSRon)	1-1.5 ug (X uL)
BbsI enzyme	1 uL
NEB buffer 2.1	5 uL
ddH2O	up to 50 uL
TOTAL	**50 uL**

2 Run for 1hr at **37 °C** , and another **00:20:00** at **65 °C** for inactivation

3

OPTIONAL - run a sample from the digested product on 1% agarose gel with an UNCUT control

Phosphorylate and anneal each pair of oligosVector digest

4 In a 0.2ml PCR tube prepare the annealing and phosphorylation reaction as follows:

FWD oligo (100uM)	1 uL
REV oligo (100uM)	1 uL
10X T4 ligation buffer (NEB)	1 uL
T4 PNK (NEB)	0.5 uL
ddH2O	6.5 uL
TOTAL	**10 uL**

5 Run the reaction in a thermocycler according to the following parameters

37 °C 20min

95 °C 5min

then ramp down to 25oC at the rate of 5oC per min

[see program OFER/**CRISPRAN** in the thermocycler]

Ligation reaction

6 In a 0.2ml PCR tube prepare the ligation reaction as follows:
[no need to check the concentration of the gel purified digested vector, just take a fix volume]

BbsI digested vector from previous step	5 uL
Phosphorylated and annealed oligo duplex from previous step (1:250 dilution)	1 uL
10X T4 ligation buffer (NEB)	2 uL
ddH2O	11 uL
T4 Ligase (NEB)	1 uL

7 It is possible to prepare a stock of the T4 Ligase, buffer water and digested plasmid and aliquot 19ul to each 0.2ml PCR tube.

8 Put the reaction in a thermocycler set at **25 °C** for 10-15min

Transformation

9 Thaw a tube of NEB 5-alpha Competent E. coli cells on ice for 10 minutes.
00:10:00

Cells are best thawed on ice and DNA added as soon as the last bit of ice in the tube disappears. Cells can also be thawed by hand, but warming above 0°C will decrease the transformation efficiency.

NEB 5-alpha Competent E.coli (High Efficiency) - 20x0.05 ml
by New England Biolabs
Catalog #: C2987H

10 Add 1-5 µl containing 1 pg-100 ng of plasmid DNA to the cell mixture.

11 Carefully flick the tube 4-5 times to mix cells and DNA. **Do not vortex.**

12 Place the mixture on ice for 30 minutes. Do not mix.
00:30:00

For maximum transformation efficiency, cells and DNA should be incubated together on ice for 30 minutes. Expect a 2-fold loss in transformation efficiency for every 10 minutes this step is shortened.

13 Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.

00:00:30

Both the temperature and the timing of the heat shock step are important and specific to the transformation volume and vessel. Using the transformation tube provided, 30 seconds at 42°C is optimal.

14 Place on ice for 5 minutes. Do not mix.

00:05:00

15 Pipette 950 µl of room temperature SOC into the mixture.

950 µl

SOC Outgrowth Medium - 100 ml
by New England Biolabs
Catalog #: B9020S

16 Place at 37°C for 60 minutes., shaking vigorously (250 rpm) or rotating.

01:00:00

Outgrowth at 37°C for 1 hour is best for cell recovery and for expression of antibiotic resistance. Expect a 2-fold loss in transformation efficiency for every 15 minutes this step is shortened. SOC gives 2-fold higher transformation efficiency than LB medium; and incubation with shaking or rotating the tube gives 2-fold higher transformation efficiency than incubation without shaking.

17 Warm selection plates to 37°C. (Ampicilin)

Selection plates can be used warm or cold, wet or dry without significantly affecting the transformation efficiency. However, warm, dry plates are easier to spread and allow for the most rapid colony formation.

18 Mix the cells thoroughly by flicking the tube and inverting.

SOC Outgrowth Medium - 100 ml
by New England Biolabs
Catalog #: B9020S

19 Perform several 10-fold serial dilutions in SOC.

20 Spread 50-100 µl of each dilution onto a selection plate

Incubate overnight at 37°C

21 **15:00:00**

Alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours.

Sequence analysis

22 Pick up to 4 colonies for each sgRNA clone (oligo pair) and touch a REPLICA plate and insert into 2ml liquid LB (in a 2phase 13ml tube) with the right selectable marker antibiotic (ampicillin or kanamycin)
grow 2 colonies per gRNA, keep 4 on replica plates. Do not throw away the transformation plates until a positive clone is confirmed

23 Grow in arotating incubator for 6-18hrs (until the media opaque)

grow 2 colonies per gRNA, keep 4 on replica plates. Do not throw away the transformation plates until a positive clone is confirmed

24 Use plasmid miniprep kit

QIAprep Spin Miniprep Kit
by Qiagen
Catalog #: 27104

Send to sequencing using either the px459-U6-SEQ primer or the pLKO5.1 primer

pLKO5.1 SEQ primer **gactatcatatgcttaccgt**
px459-U6-SEQ primer **caaggctgttagagagataa**