

Oct 02, 2020

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

This protocol is a draft, published without a DOI.



Ofer Barnea-Yizhar<sup>1</sup>

<sup>1</sup>Shahar Taiber

AhituvLab



Ofer Yizhar-Barnea

UCSF

OPEN  ACCESS



**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>

**License:** This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), 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:** September 22, 2020

**Last Modified:** October 02, 2020

**Protocol Integer ID:** 42388



## 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and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to **protocols.io** is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with **protocols.io**, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

## Abstract

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

## Materials

### MATERIALS

⊗ QIAprep Spin Miniprep Kit **Qiagen Catalog #27104**

⊗ BbsI-HF - 1,500 units **New England Biolabs Catalog #R3539L**

⊗ pSpCas9(BB)-2A-Puro (PX459) V2.0 **addgene Catalog #62988**

⊗ 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**

### STEP MATERIALS

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

⊗ SOC Outgrowth Medium - 100 ml **New England Biolabs Catalog #B9020S**

⊗ SOC Outgrowth Medium - 100 ml **New England Biolabs Catalog #B9020S**


⊗ QIAprep Spin Miniprep Kit **Qiagen Catalog #27104**

⊗ BbsI **New England Biolabs Catalog #R0539S**




## Protocol materials


 QIAprep Spin Miniprep Kit **Qiagen Catalog #27104** In Materials, Materials, Step 24

 pSpCas9(BB)-2A-Puro (PX459) V2.0 **addgene Catalog #62988** Materials

 BbsI-HF - 1,500 units **New England Biolabs Catalog #R3539L** Materials

 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**

### Materials

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

### Materials, Step 9

 SOC Outgrowth Medium - 100 ml **New England Biolabs Catalog #B9020S** In Materials, Materials and [2 steps](#)

 BbsI **New England Biolabs Catalog #R0539S** Materials, Step 1




## Vector digest

- 1 Digest  1 µg of an empty vector with

 BbsI **New England Biolabs Catalog #R0539S**

(the enzyme is kept in -80 REVC0). Comprise the reaction as follows:

Vector (derived from CRISPRon)	1-1.5 ug ( X uL)
BbsI enzyme	1 uL
NEB buffer 2.1	5 uL
ddH2O	up to 50 uL
<b>TOTAL</b>	<b>50 uL</b>

- 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 oligos

- 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
<b>TOTAL</b>	<b>10 uL</b>

- 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
ddH <sub>2</sub> O	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

### Note

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 **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

### Note

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

#### Note

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 **New England Biolabs Catalog #B9020S**

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

01:00:00

#### Note

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)

#### Note

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 **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



## 21 Incubate overnight at 37°C

 15:00:00

### Note

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 **Qiagen Catalog #27104**

Send to sequencing using either the px459-U6-SEQ primer or the pLK05.1 primer

pLK05.1 SEQ primer **gactatcatatgcttaccgt**

px459-U6-SEQ primer **caaggctgttagagagataa**