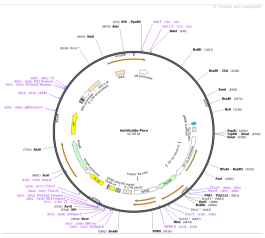
Aug 13, 2020

Target Guide Sequence Cloning Protocol Version 2



Forked from Target Guide Sequence Cloning Protocol

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Protocol status: Working
We use this protocol in our workspace and it is working. This protocol is a revised version of the first Target Guide Sequence Cloning Protocol and is much easier to follow.

Created: August 13, 2020

Last Modified: August 13, 2020

Protocol Integer ID: 40411

Keywords: Lentivirus vector, cloning, vector digestion, oligo annealing, CRISPR,

Disclaimer

This protocol is a modified version of the Zhang Lab's *GeCKOv2* Target Guide Sequence Cloning Protocol attached below based off of Joung, J., Konermann, S., Gootenberg, J.*et al.* Genome-scale CRISPR-Cas9 knockout and transcriptional activation screening.*Nat Protoc***12,**828–863 (2017).https://doi.org/10.1038/nprot.2017.016

Other protocols modified/used in this protocol:
Bacterial Transformation, Addgene: **https://www.addgene.org/protocols/bacterial-transformation/**

More information about the specific lentiGuide-puro plasmid can be found here: **https://www.addgene.org/52963/**.

Abstract

Create single gRNA vectors for targeted cloning utilizing CRISPR or CRISPR-based systems.

Attachments

Lentivirus_Protocol.... 2.3MB

Addgene_ Protocol - ... 164KB

Image Attribution

https://www.addgene.org/52963/

Materials

MATERIALS

- NEBuffer 3.1 - 5.0 ml **New England Biolabs** **Catalog #**B7203S
- T4 DNA Ligase - 20,000 units **New England Biolabs** **Catalog #**M0202S
- Agar
- lentiGuide-Puro **addgene** **Catalog #**52963
- double distilled water (ddH2O)
- SOC Media
- 1X TAE Buffer
- 10X NEB T4 DNA ligase buffer **New England Biolabs**
- 10X T4 PNK Reaction Buffer **New England Biolabs**
- ethanol
- 10X PCR Buffer **Life Technologies** **Catalog #**10966-034
- LB-Broth Miller (= LB mix) **Formedium** **Catalog #**LMM0104
- One Shot™ TOP10 Chemically Competent E. coli **Thermo Fisher** **Catalog #**C404010
- BsmBI-v2 **New England Biolabs** **Catalog #**R0739L
- HotStarTaq Plus DNA Polymerase (1000) **Qiagen** **Catalog #**203605
- dNTP Set (100mM each A C G T) **Ge Healthcare** **Catalog #**95038-256

STEP MATERIALS

-

-

- lentiGuide-Puro **addgene** **Catalog #**52963
- double distilled water (ddH2O)
- 10X NEB T4 DNA ligase buffer **New England Biolabs**
- 10X T4 PNK Reaction Buffer **New England Biolabs**
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One Shot™ TOP10 Chemically Competent E. coli **Thermo Fisher** **Catalog #**C404010

ethanol

10X PCR Buffer **Life Technologies** **Catalog #**10966-034

dNTP Set (100mM each A C G T) **Ge Healthcare** **Catalog #**95038-256

HotStarTaq Plus DNA Polymerase (1000) **Qiagen** **Catalog #**203605

ddH20

LB-Broth Miller (= LB mix) **Formedium** **Catalog #**LMM0104

SOC Media

lentiGuide-Puro**: RRID:Addgene_52963**
Sigma-Aldrich**: RRID:SCR_008988**

Protocol materials

- double distilled water (ddH2O)
- lentiGuide-Puro **addgene** **Catalog** #52963
- ddH20
- LB-Broth Miller (= LB mix) **Formedium** **Catalog** #LMM0104
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SOC Media
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lentiGuide-Puro **addgene** **Catalog** #52963
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lentiGuide-Puro **addgene** **Catalog** #52963
Agar
1X TAE Buffer
double distilled water (ddH2O)
10X NEB T4 DNA ligase buffer **New England Biolabs**
10X T4 PNK Reaction Buffer **New England Biolabs**
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10X PCR Buffer **Life Technologies** **Catalog** #10966-034
dNTP Set (100mM each A C G T) **GE Healthcare** **Catalog** #95038-256
HotStarTaq Plus DNA Polymerase (1000) **Qiagen** **Catalog** #203605
LB-Broth Miller (= LB mix) **Formedium** **Catalog** #LMM0104
SOC Media

Before start

Design and order gRNA oligos from **Sigma-Aldrich** (RRID:SCR_008988).

Lentiviral vector digestion

1 Digest and dephosphorylate

lentiGuide-Puro **addgene Catalog #52963**

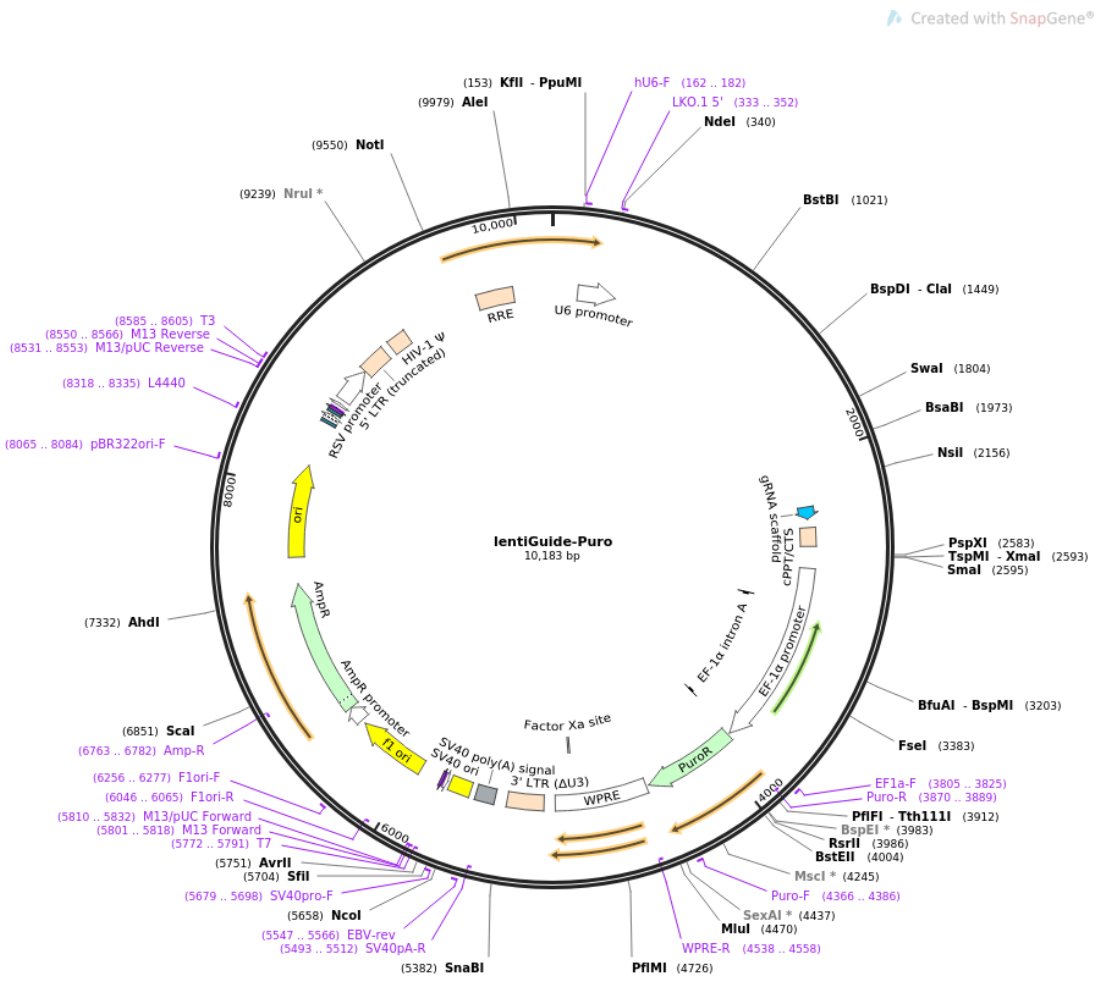

Image attribution: https://www.addgene.org/52963/

1.1 Add 40 µL

ddH20

to a 1.5 mL

Equipment

Snap Cap Microcentrifuge Tube or equivalent	NAME
Polypropylene Microcentrifuge Tube	TYPE
Corning Costar Snap Cap Microcentrifuge Tube	BRAND
07200210	SKU
https://www.fishersci.com/shop/products/costar-microcentrifuge-tubes-6/07200210	LINK
2 mL snap cap polypropylene micro tube	SPECIFICATIONS

.

1.2 Add 5 µL of
NEBuffer 3.1 - 5.0 ml **New England Biolabs Catalog #**B7203S
to solution.

1.3 Add 3 µL of
BsmBI-v2 **New England Biolabs Catalog #**R0739L
to solution.

Note

Note: we used BsmBI Cat #R0580, NEB, but this one has been discontinued. #R0739L is considered as an effective replacement.

1.4 Add 2 µL of
lentiGuide-Puro **addgene Catalog #**52963
to solution.

1.5 Close cap on microcentrifuge tube and place in

Equipment

Mini-centrifuge

NAME

Centrifuge

TYPE

Fisher

BRAND

S67601B

SKU

https://www.fishersci.com/shop/products/fisherbrand-standard-mini-centrifuge-standard-mini-centrifuge/s67601b

LINK

Any standard mini centrifuge with adapters for different tube sizes will suffice

SPECIFICATIONS

for 00:00:10 on until all of the solution is at the bottom of the tube.

1.6 Place microcentrifuge tube with vector digestion mixture in a

Equipment

Oven	NAME
Oven forced-air convection	TYPE
Fisher Isotemp	BRAND
15-103-0510	SKU
http://www.fishersci.ca/shop/products/fisher-scientific-isotemp-general-purpose-heating-drying-ovens/151030510?keyword=true	LINK

on 55 °C

1.7 Close lid and set a timer for 01:00:00

Gel purify the digested plasmid from Step 1

2 Prepare gel

2.1 Create gel concentration of 1.2-1.5%
Agar
for 100 mL of
1X TAE Buffer
solution.

3 Run gel

3.1 Isolate 2kb and 8kb band. Collect 8kb (8318 bp) band for gel purification.

Preparing the gRNAs

4 Design gRNA sequence for CRISPR strategy using **CRISPR direct**.

5 Order oligos from **Sigma-Aldrich** (RRID:SCR_008988).

6 Create a dilution from stock oligos at a 1:10 ratio in
double distilled water (ddH2O)
.

> Note
>
> Diluted oligo concentration should be [M] 10 micromolar (µM) .

Phosphorylate and anneal each pair of oligos

7 Prepare phosphorylation/annealing reaction

7.1 Add 6.5 µL
double distilled water (ddH2O)
to a microcentrifuge tube.

7.2 Add 1 µL each of [M] 10 micromolar (µM) Oligo 1 (F), [M] 10 micromolar (µM) Oligo 2 (R), and
10X NEB T4 DNA ligase buffer **New England Biolabs**

7.3 Add 0.5 µL
10X T4 PNK Reaction Buffer **New England Biolabs**

7.4 Vortex and microcentrifuge

8 Place the phosphorylation/annealing reaction in a

Equipment

SimpliAmp Thermal Cycler	NAME
PCR	TYPE
Applied Biosystems	BRAND
A24811	SKU
https://www.thermofisher.com/order/catalog/product/A24811	LINK
Any standard PCR thermocycler will suffice	SPECIFICATIONS

Note

Settings should be as follows: 37 °C for 00:30:00 , 95 °C for 00:05:00 , and then ramp down to 25 °C at 5 °C / 00:01:00 .

Setting up and incubating the ligation reaction

9 Making the ligation reaction

9.1 Place 4.8 µL of double distilled water (ddH2O)

in a microcentrifuge tube.

9.2 Add 2.2 µL

BsmBI-v2 **New England Biolabs Catalog #**R0739L

9.3 Add 1 µL each of

10X NEB T4 DNA ligase buffer **New England Biolabs**

, diluted oligo duplex from go to step #7 , and

T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #**M0202S

.

9.4 Lightly vortex and microcentrifuge

10 Incubate the ligation reaction at room temperature for 02:00:00 - 03:00:00

Transformation into E. coli bacteria

11 Prepare LB Agar plates

11.1 Wipe down your bench with at least 70%

ethanol

and light a bunsen burner.

11.2 Remove Agar Ampicillin Plates 250 µL from 4 °C and let warm up to room temperature.

12 E. coli competent cell transfection

12.1 Take competent cells

One Shot™ TOP10 Chemically Competent E. coli **Thermo Fisher Catalog #**C404010

out of -80 °C and thaw on ice (00:20:00 - 00:30:00).

12.2 Add 100 µL of E.coli cells to 10 µL of DNA in a microcentrifuge tube next to the bunsen burner.

Note

When working with the E. coli, be very diligent and make sure you are working in the sterile area of your bench near the bunsen burner flame.

12.3 Gently flick tube a few times with your finger to mix.

12.4 Incubate the competent cell/DNA mixture on ice for 00:30:00 .

12.5 Place transformation tube(s) into water bath at 42 °C for 00:00:30 - 00:01:00 to heat shock E. coli cells.

12.6 Place the transformation tube(s) back on ice for 00:02:00 .

12.7 Add 250 µL
LB-Broth Miller (= LB mix) **Formedium Catalog #**LMM0104
(without antibiotic) or
SOC Media
to the tube(s).

12.8 Place tube(s) in 37 °C shaking incubator for 00:45:00 - 01:00:00 .

12.9 Plate all of transformation onto LB agar plate(s) with ampicillin. Incubate plates at 37 °C overnight.

Picking Colony for Suspension Growth

13 Before you get started

Note

Start this whole section (picking colony and suspension growth) sometime in the late afternoon (~4-5pm), so that you can run PCR for the bacterial plasmids you collect the next morning (~16 hours later). More than 24 hours of incubation can cause other non-ampicillin resistant bacteria to grow in the suspension tubes.

Note

Set up an aseptic area for handling the bacterial colonies.

14 Picking colony

14.1 Pick 10-15 colonies from each agar plate to suspend in an LB solution.

Note

We originally selected only 4 colonies from each plate, but did not have a successful PCR. To increase chances of successfully amplifying the plasmid vector, we suggest picking 10-15 colonies.

14.2 Place each colony in a tube with 3 mL of LB.

15 Culture the bacteria

15.1 Place tubes into shaking incubator at 37 °C overnight.

Run PCR to Identify Positive Clones

16 Prepare Master PCR Mix by adding each of the below reagents to a microcentrifuge tube.

Note

Before you start: multiply each volume below by the same amount (x 10, 15, etc.) according to how much master mix you need to run your sets.

16.1
15.875 µL
ddH20

16.2
2.5 µL
10X PCR Buffer **Life Technologies Catalog #**10966-034

16.3
0.5 µL
[M] 10 millimolar (mM)
dNTP Set (100mM each A C G T) **GE Healthcare Catalog #**95038-256

16.4
2 µL
[M] 10 micromolar (µM)
Forward Primer hU6-02

Note

Forward Primer hU6-02 sequence: TAATTAGAATTAATTTGACT
Ordered from **Sigma-Aldrich** (RRID:SCR_008988).

16.5
2 µL
[M] 10 micromolar (µM)
Reverse Primer (gRNA reverse primer oligo)

16.6
0.125 µL
HotStarTaq Plus DNA Polymerase (1000) **Qiagen Catalog #**203605

17 Loading and Prepping PCR wells

17.1 Aliquot equal proportions of master mix to each PCR well (we used 23 µL).

17.2 Add 2 µL of each bacterial suspension sample from go to step #15 to each PCR well.

Note

Total volume of Master Mix and Sample should be 25 µL in each well.

17.3 Seal off PCR wells tightly with a clear plastic cover or tube tops.

17.4 Centrifuge 1200 rpm, 00:01:00 .

18 Place PCR reaction in thermocycler and run PCR.

Note

Settings should be as follows:
1) 95C for 5mins (ramp up).
2) 95C for 30sec.
3) 55C for 45 sec.
4) 72C for 45 sec.
- x35 cycles steps 2-4.
5) 72 for 10mins.

Running Gel for PCR Product Verification

19 Prepare a gel of 2% concentration. Run gel and examine bands. Desired band length is about 200bp with the gRNA insertion.

Congrats!

20 You have successfully transformed a lentiviral vector with your gRNA sequence of interest! For confirmation, feel free to sequence your vector.