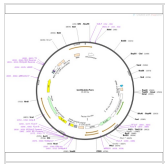

Aug 13, 2020

Target Guide Sequence Cloning Protocol Version 2

Forked from Target Guide Sequence Cloning Protocol

Skye Waterland[1], Yang Li[1]

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1 Works for me dx.doi.org/10.17504/protocols.io.bjp3kmqn

Skye Waterland

ABSTRACT

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

ATTACHMENTS

Lentivirus_Protocol.pdf

Addgene_ Protocol - Bacterial Transformation.pdf

DOI

dx.doi.org/10.17504/protocols.io.bjp3kmqn

PROTOCOL CITATION

Skye Waterland, Yang Li 2020. Target Guide Sequence Cloning Protocol Version 2. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

FORK FROM

Forked from Target Guide Sequence Cloning Protocol , Skye Waterland

KEYWORDS

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

IMAGE ATTRIBUTION

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

CREATED

Aug 13, 2020

LAST MODIFIED

Aug 13, 2020

PROTOCOL INTEGER ID

40411

MATERIALS

NAME	CATALOG #	VENDOR
NEBuffer 3.1 - 5.0 ml	B7203S	New England Biolabs
T4 DNA Ligase - 20,000 units	M0202S	New England Biolabs
Agar		
lentiGuide-Puro	52963	addgene

NAME	CATALOG #	VENDOR
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	10966-034	Life Technologies
LB-Broth Miller (= LB mix)	LMM0104	Formedium
One Shot™ TOP10 Chemically Competent E. coli	C404010	Thermo Fisher
BsmBI-v2	R0739L	New England Biolabs
HotStarTaq Plus DNA Polymerase (1000)	203605	Qiagen
dNTP Set (100mM each A C G T)	95038-256	Ge Healthcare

STEPS MATERIALS

NAME	CATALOG #	VENDOR
ddH20		
NEBuffer 3.1 - 5.0 ml	B7203S	New England Biolabs
lentiGuide-Puro	52963	addgene
double distilled water (ddH2O)		
10X NEB T4 DNA ligase buffer		New England Biolabs
10X T4 PNK Reaction Buffer		New England Biolabs
BsmBI-v2	R0739L	New England Biolabs
Agar		
1X TAE Buffer		
T4 DNA Ligase - 20,000 units	M0202S	New England Biolabs
One Shot™ TOP10 Chemically Competent E. coli	C404010	Thermo Fisher
ethanol		
10X PCR Buffer	10966-034	Life Technologies
dNTP Set (100mM each A C G T)	95038-256	Ge Healthcare
HotStarTaq Plus DNA Polymerase (1000)	203605	Qiagen
LB-Broth Miller (= LB mix)	LMM0104	Formedium
SOC Media		

MATERIALS TEXT

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

EQUIPMENT

NAME	CATALOG #	VENDOR
Snap Cap Microcentrifuge Tube or equivalent	07200210	
Mini-centrifuge	S67601B	
Oven	15-103-0510	
SimpliAmp Thermal Cycler	A24811	

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

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

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/.

BEFORE STARTING

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

Lentiviral vector digestion

1 Digest and dephosphorylate

lentiGuide-Puro
by addgene
Catalog #: 52963

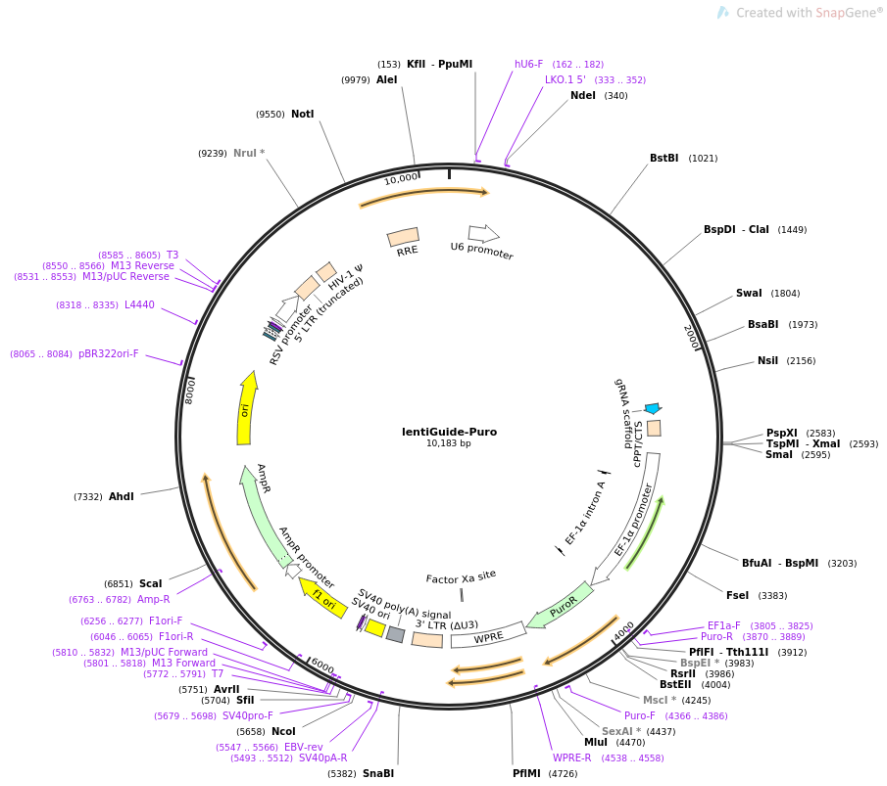

Image attribution: https://www.addgene.org/52963/

1.1 Add **40 µl**

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

ddH20

to a **1.5 mL**

Snap Cap Microcentrifuge Tube or equivalent
Polypropylene Microcentrifuge Tube
Corning Costar Snap Cap Microcentrifuge Tube
2 mL snap cap polypropylene micro tube

07200210

.

1.2 Add **5 µl** of

NEBuffer 3.1 - 5.0 ml
by New England Biolabs
Catalog #: B7203S

to solution.

1.3 Add **3 µl** of

BsmBI-v2
by New England Biolabs
Catalog #: R0739L

to solution.

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

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

lentiGuide-Puro
by addgene
Catalog #: 52963

to solution.

1.5 Close cap on microcentrifuge tube and place in

Mini-centrifuge
Centrifuge
Fisher S67601B
Any standard mini centrifuge with adapters for different tube sizes will suffice

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

Oven
Oven forced-air convection
Fisher Isotemp 15-103-0510

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%

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

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)

.

Diluted oligo concentration should be **10 Micromolar (µM)** .

Phosphorylate and anneal each pair of oligos

7 Prepare phosphorylation/annealing reaction

7.1 Add **6.5 µl**

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

double distilled water (ddH2O)

to a microcentrifuge tube.

7.2 Add **1 µl** each of **10 Micromolar (µM)** Oligo 1 (F), **10 Micromolar (µM)** Oligo 2 (R), and

10X NEB T4 DNA ligase buffer
by New England Biolabs

7.3 Add **0.5 µl**

10X T4 PNK Reaction Buffer
by New England Biolabs

7.4 Vortex and microcentrifuge

8 Place the phosphorylation/annealing reaction in a

SimpliAmp Thermal Cycler
PCR
Applied Biosystems A24811
Any standard PCR thermocycler will suffice

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** .

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

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
by New England Biolabs
Catalog #: R0739L

9.3 Add **1 µl** each of

10X NEB T4 DNA ligase buffer
by New England Biolabs

, diluted oligo duplex from **go to step #8** , and

T4 DNA Ligase - 20,000 units
by 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

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

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
by 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.

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** .

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

12.7 Add **250 µl**

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

(without antibiotic) or

SOC Media
View

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

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.

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.

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.

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

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.

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
by Life Technologies
Catalog #: 10966-034

16.3 **0.5 µl** **10 Milimolar (mM)**

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

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

16.4 **2 µl** **10 Micromolar (µM)** Forward Primer hU6-02

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

16.5 **2 µl** **10 Micromolar (µM)** Reverse Primer (gRNA reverse primer oligo)

16.6 **0.125 µl**

HotStarTaq Plus DNA Polymerase (1000)
by 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.

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.

Settings should be as follows:

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn

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.

Citation: Skye Waterland, Yang Li (08/13/2020). Target Guide Sequence Cloning Protocol Version 2. https://dx.doi.org/10.17504/protocols.io.bjp3kmqn