Rapid and robust cloning of sgRNA expression plasmids

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Protocol status: Working
We use this protocol and it's working

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ABSTRACT

In this lab protocol, we will outline a step-by-step procedure for cloning of spCas9 sgRNAs using oligo annealing and T4 ligation. This protocol will guide you through the crucial steps of designing, annealing, ligating oligonucleotides and digested plasmids, as well as heat shock transformation procedure to clone sgRNAs expression vectors.

ATTACHMENTS

sgLenti vector for CRISPR sgRNA expression.gbk

PROTOCOL MATERIALS

Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit **Fischer Scientific Catalog #11992242**
Step 10

NucleoSpin Plasmid Transfection-grade, Mini kit for ultrapure plasmid DNA **Macherey-Nagal Catalog #740490.250**
Step 26

T4 DNA Ligase Buffer (10X) **Thermo Fisher Catalog #B69**
Step 2

AarI (2 U/µL) **Thermo Fisher Catalog #ER1581**
Step 4

T4 DNA Ligase (5 U/µL) **Thermo Fisher Catalog #EL0011**
Step 13

DNA Gel Loading Dye (6X) **Thermo Fisher Scientific Catalog #R0611**
Step 7

SAFETY WARNINGS

Wear gloves and a UV filter mask and ensure sleeves are completely covering wrists to avoid direct UV light exposure

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BEFORE START INSTRUCTIONS

Modify the ssOligo overhangs and Sanger sequencing primer according to the plasmid that you are using.

Oligo Design

1 Order **TOP** and **BOTTOM** strand oligos, where the TOP sequence is the desired 20 nt sgRNA 'spacer' sequence and the BOTTOM sequence is the reverse complement of the TOP strand. After annealing of both oligos, the 5' overhangs should result in compatible sticky ends for T4 ligation into the target vector. Shown are example sticky ends to clone into the vector sgLenti (Addgene #105996). Alternative sgRNA expression vectors may require different sticky ends.

5’-**TTGG**NNNNNNNNNNNNNNNNNNN -3’
3’-NNNNNNNNNNNNNNNNNNN**CAAA**-5’

TOP strand oligo: 5'-**TTGG**NNNNNNNNNNNNNNNNNNN-3'
BOTTOM strand oligo: 5'-**AAAC**NNNNNNNNNNNNNNNNNNN-3'

Oligo Annealing

1h 40m

2 Set up the annealing reaction: 10m

1 µL Top strand oligo [M] 100 micromolar (µM)

1 µL Bottom strand oligo [M] 100 micromolar (µM)

1 µL T4 DNA Ligase Buffer (10X) **Thermo Fisher Catalog #B69**

7 µL ddH2O

3 Anneal oligos by running the following thermocycler program: 1h 30m

1. 95 °C for 5 min
2. Ramp down at 0.1 °C /sec from 95 °C to 25 °C

Plasmid Restriction Digest

1d

4 Set up AarI plasmid digest: 10m

1 µg vector for spCas9 sgRNA expression

2 µL 10x AarI Buffer

1 µL AarI (2 U/µL) **Thermo Fisher Catalog #ER1581**

0.4 µL 50x Oligo

ddH2O to 20 µL

5 Incubate in a Thermocycler at 50 °C Overnight 16h

Digested Plasmid Purification by Agarose Gel Electrophoresis 3h

6 Prepare 1 % Agarose gel 30m

7 Add 4 µL of 6x DNA Gel Loading Dye (6X) **Thermo Fisher Scientific Catalog #R0611** 10m

to 20 µL of the restriction digestion mix.

8 Load samples on the 1 % Agarose gel and run the electrophoresis at 120 V for 50 min 1h

9 Place the gel in a UV light box 10m

Safety information

Wear gloves and a UV filter mask and ensure sleeves are completely covering wrists to avoid direct UV light exposure

10 Cut the digested plasmid band from the gel and purify the DNA using the 1h

Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit **Fischer Scientific Catalog #11992242**

11 Determine the concentration of the purified plasmid DNA (e.g. NanoDrop One) 10m

Ligation of Plasmid and Annealed Oligos 30m

12 Dilute the annealed oligos 1:200 in ddH2O 10m

13 Set up the ligation reaction: 10m

50 ng purified vector

1 µL diluted oligos

1 µL 10x T4 Ligation buffer

1 µL T4 DNA Ligase (5 U/µL) **Thermo Fisher Catalog #EL0011**

ddH2O to 10 µL

> **Note**
>
> As a control set up a ligation reaction without oligos

14 Incubate for 10 min at Room temperature 10m

Heat Shock Transformation of the Ligation Product into Chem... 1d

15 Take DH5a chemocompetent cells from -80 °C storage and thaw on ice for 30 min. Use 1 50 µL aliquot for the ligation reaction and 1 50 µL aliquot for the control reaction 30m

16 Add 2 µL of the T4 Ligation reaction and the control reaction to the cells and mix gently by flicking the tube

17 Incubate on ice for 30 min 30m

18 Incubate the tube containing the cells in a water bath at 42 °C for 45 sec and place back on ice for 2 min 2m 45s

19 Add 0.5 mL of LB medium to the cells and incubate in a thermo-block at 37 °C with shaking at 250 rpm for 1 hr 1h

20 Plate 100 µL of the cells on Room temperature LB agar plates with [M] 100 µg/mL Carbenicillin 10m

21 Incubate at 37 °C Overnight 16h

22 After the overnight incubation, count the colonies on the ligation reaction and the control plates. The ligation reaction plate should have a minimum of 10x more colonies. 10m

23 Seal the plates with parafilm and store at 4 °C for up to 2 weeks

Plasmid DNA Pr1dification

1d

24 Pick up to 3 colonies from the plate using a pipette tip and inoculate 5 mL LB medium with [M] 100 µg/mL Carbenicillin in 15 mL cell culture tubes

25 Incubate the cell culture tubes Overnight at 37 °C and 225 rpm in a shaking incubator 16h

26 Extract the plasmid DNA using the 1h 30m

NucleoSpin Plasmid Transfection-grade, Mini kit for ultrapure plasmid DNA **Macherey-Nagal Catalog #740490.250**

27 Measure the concentration of the purified plasmid DNA (e.g. NanoDrop One)

28 Send the plasmids for Sanger sequencing with the following primer to confirm the correct sequence:
GGCTTGGATTTCTATAACTTCGTATAGCA

Note

Provided primer is complementary to the 'sgLenti vector for CRISPR sgRNA expression' provided in the attachments and needs to be adjusted for alternative vectors.