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Jan 05, 2022

Cloning shRNA Oligos into pLKO.1 V.3

Addgene The Nonprofit Plasmid Repository[1]

[1]Addgene

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dx.doi.org/10.17504/protocols.io.b3hxqj7n

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This is the protocol accompanying the "pLKO.1 – TRC Cloning Vector". For information about the PLKO.1-TRC cloning vector and tips on designing shRNA oligos for pLKO.1 see Addgene's website: http://www.addgene.org/tools/protocols/plko/

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STEP MATERIALS

NEBuffer 3 - 5.0 ml **New England Biolabs Catalog #B7003S**

NEBuffer 1 - 5.0 ml **New England Biolabs Catalog #B7001S** Step 10

AgeI - 300 units **New England Biolabs Catalog #R0552S** Step 11

EcoRI - 10,000 units **New England Biolabs Catalog #R0101S** In 2 steps

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T4 DNA Ligase Reaction Buffer - 6.0 ml **New England Biolabs Catalog #B0202S** Step 26

T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #M0202S** Step 27

Annealing Oligos

1 Resuspend oligos in ddH2O to a concentration of 20 µM.

2 Add 5ul Forward oligo

5 µL

3 Add 5ul Reverse oligo

5 µL

4 Add 5ul 10X NEB Buffer 2

5 µL

5 Add 35 µL ddH2O

35 µL

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6 Incubate for 4 minutes at 95°C in a PCR machine or in a beaker of boiling water.
00:04:00

7 Incubate the sample at 70°C for 10 minutes in a PCR machine.
00:10:00

8 Slowly cool to room temperature over the period of several hours.
03:00:00

This will take a few hours, but it is important for the cooling to occur slowly for the oligos to anneal.

If using a beaker of water, remove the beaker from the flame, and allow the water to cool to room temperature.

Digesting pLKO.1 TRC Cloning Vector

9 Mix: 6 µg pLKO.1 TRC-cloning vector (maxiprep or miniprep DNA)
6 µg

10 with 5 µL 10x NEB buffer 1
5 µL
NEBuffer 1 - 5.0 ml **New England Biolabs Catalog #B7001S**

11 with 1 µL AgeI
1 µL
AgeI - 300 units **New England Biolabs Catalog #R0552S**

12 Bring up to 50 µl with ddH2O

13 Incubate at 37°C for 2 hours.
02:00:00

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14 Purify with Qiaquick gel extraction kit, eluting in 30 µL of ddH2O.

15 Digest eluate with EcoRI by mixing: 30 µL pLKO.1 TRC-cloning vector digested with AgeI

16 with 5 µL 10x NEB buffer for EcoRI
5 µL
EcoRI - 10,000 units **New England Biolabs Catalog #R0101S**

17 with 1 µL EcoRI
1 µL
EcoRI - 10,000 units **New England Biolabs Catalog #R0101S**

18 and 14 µL ddH2O
14 µL

19 Incubate at 37°C for 2 hours.
02:00:00

20 Run digested DNA on 0.8% low melting point agarose gel until you can distinctly see 2 bands, one 7kb and one 1.9kb.

When visualizing DNA fragments to be used for ligation, use only long-wavelength UV light. Short wavelength UV light will increase the chance of damaging the DNA.

21 Cut out the 7kb band and place in a sterile microcentrifuge tube.

22 Purify the DNA using a Qiaquick gel extraction kit. Elute in 30 µL of ddH2O.

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23 Measure the DNA concentration.

Ligating and Transforming into Bacteria

24 Use your ligation method of choice. For a standard T4 ligation, mix: 2 µL annealed oligo from "Annealing Oligos" section above.

2 µL

25 With 20 ng digested pLKO.1 TRC-cloning vector from the "Digesting pLKO.1 TRC Cloning Vector" section above.

20 ng

If you were unable to measure the DNA concentration, use 1 µL

26 With 2 µL 10x NEB T4 DNA ligase buffer

2 µL

T4 DNA Ligase Reaction Buffer - 6.0 ml **New England Biolabs Catalog #B0202S**

27 With 1 µL NEB T4 DNA ligase

1 µL

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

28 Bring up to 20ul with ddH2O

29 Incubate at 16°C for 4-20 hours.

04:00:00

30 Transform 2 µL of ligation mix into 25 µL competent cells, following manufacturer's protocol.

Due to the long terminal repeats found in lentiviral plasmids, we recommend using a

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strain that reduces the frequency of homologous recombination of unstable regions, such as Invitrogen Stbl3™ or NEB Stable cells. This will ensure that the repeats will be maintained and often results in a greater yield of DNA.

31 Plate on LB agar plates containing 100 µg/mL ampicillin or carbenicillin (an ampicillin analog).

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