Cloning shRNA Oligos into pLKO.1

Caroline LaManna

Abstract

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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Protocol

Annealing Oligos

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

Annealing Oligos

Step 2.
Add 5ul Forward oligo

AMOUNT
5 µl Additional info:

Annealing Oligos

Step 3.
Add 5ul Reverse oligo

AMOUNT
5 µl Additional info:

Annealing Oligos

Step 4.
Add 5 µL 10x NEB buffer 2

AMOUNT
5 µl Additional info:

REAGENTS
NEBuffer 3 - 5.0 ml B7003S by New England Biolabs

Annealing Oligos

Step 5.
Add 35 µL ddH2O

AMOUNT
35 µl Additional info:

Annealing Oligos

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

DURATION

00:04:00

Annealing Oligos

Step 7.

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

DURATION

00:10:00

Annealing Oligos

Step 8.

Slowly cool to room temperature over the period of several hours.

DURATION

03:00:00

NOTES

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

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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

Step 9.

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

AMOUNT

6 µg Additional info:

Digesting pLKO.1 TRC Cloning Vector

Step 10.

with 5 µL 10x NEB buffer 1

AMOUNT

5 µl Additional info:

REAGENTS

NEBuffer 1 - 5.0 ml B7001S by New England Biolabs

Digesting pLKO.1 TRC Cloning Vector

Step 11.

with 1 µL AgeI

AMOUNT

1 µl Additional info:

REAGENTS

AgeI - 300 units R0552S by New England Biolabs

Digesting pLKO.1 TRC Cloning Vector

Step 12.

bring to 50 µL ddH2O

AMOUNT

50 µl Additional info:

Digesting pLKO.1 TRC Cloning Vector

Step 13.

Incubate at 37°C for 2 hours.

DURATION

02:00:00

Digesting pLKO.1 TRC Cloning Vector

Step 14.

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

Digesting pLKO.1 TRC Cloning Vector

Step 15.

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

Digesting pLKO.1 TRC Cloning Vector

Step 16.

with 5 µL 10x NEB buffer for EcoRI

AMOUNT

5 µl Additional info:

REAGENTS

EcoRI - 10,000 units R0101S by New England Biolabs

Digesting pLKO.1 TRC Cloning Vector

Step 17.

with 1 µL EcoRI

AMOUNT

1 µl Additional info:

REAGENTS

EcoRI - 10,000 units R0101S by New England Biolabs

Digesting pLKO.1 TRC Cloning Vector

Step 18.

and 14 µL ddH2O

AMOUNT

14 µl Additional info:

Digesting pLKO.1 TRC Cloning Vector

Step 19.

Incubate at 37°C for 2 hours.

DURATION

02:00:00

Digesting pLKO.1 TRC Cloning Vector

Step 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.

NOTES

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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.

Digesting pLKO.1 TRC Cloning Vector

Step 21.

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

Digesting pLKO.1 TRC Cloning Vector

Step 22.

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

Digesting pLKO.1 TRC Cloning Vector

Step 23.
Measure the DNA concentration.

Ligating and Transforming into Bacteria

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

AMOUNT
2 µl Additional info:

Ligating and Transforming into Bacteria

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

AMOUNT
20 ng Additional info:

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

Ligating and Transforming into Bacteria

Step 26.
With 2 µL 10x NEB T4 DNA ligase buffer

AMOUNT
2 µl Additional info:

REAGENTS
T4 DNA Ligase Reaction Buffer - 6.0 ml B0202S by New England Biolabs

Ligating and Transforming into Bacteria

Step 27.
With 1 µL NEB T4 DNA ligase

AMOUNT
1 µl Additional info:

REAGENTS
T4 DNA Ligase - 20,000 units M0202S by New England Biolabs

Ligating and Transforming into Bacteria

Step 28.
Bring up to 20ul with ddH2O

Ligating and Transforming into Bacteria

Step 29.
Incubate at 16°C for 4-20 hours.

DURATION
04:00:00

Ligating and Transforming into Bacteria

Step 30.
Transform 2 µL of ligation mix into 25 µL competent DH5 alpha cells, following manufacturer's protocol.

Ligating and Transforming into Bacteria

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