VERSION 2

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OPEN ACCESS

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<http://www.addgene.org/tools/protocols/plko/>

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

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🌐 Cloning shRNA Oligos into pLKO.1 V.2

Addgene The Nonprofit Plasmid Repository¹

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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/>

MATERIALS

STEP MATERIALS

- ⊗ NEBuffer 3 - 5.0 ml New England Biolabs Catalog #B7003S
- ⊗ NEBuffer 1 - 5.0 ml New England Biolabs Catalog #B7001S
- ⊗ Agel - 300 units New England Biolabs Catalog #R0552S
- ⊗ EcoRI - 10,000 units New England Biolabs Catalog #R0101S
- ⊗ EcoRI - 10,000 units New England Biolabs Catalog #R0101S
- ⊗ T4 DNA Ligase Reaction Buffer - 6.0 ml New England Biolabs Catalog #B0202S
- ⊗ T4 DNA Ligase - 20,000 units New England Biolabs Catalog #M0202S

Annealing Oligos

- 1 Resuspend oligos in ddH₂O to a concentration of 20 μ M.
- 2 Add 5ul Forward oligo
5 μ L
- 3 Add 5ul Reverse oligo
5 μ L
- 4 Add 5 μ L 10x NEB buffer 2
5 μ L
NEBuffer 3 - 5.0 ml New England Biolabs Catalog
#B7003S
- 5 Add 35 μ L ddH₂O
35 μ L
- 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

Note

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

Note

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 Agel

1 µL

Agel - 300 units New England Biolabs Catalog
#R0552S

- 12 bring to 50 µL ddH₂O

50 µL

- 13 Incubate at 37°C for 2 hours.

02:00:00

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

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

16 with 5 μ L 10x NEB buffer for EcoRI

5 μ L

EcoRI - 10,000 units New England Biolabs Catalog
#R0101S

17 with 1 μ L EcoRI

1 μ L

EcoRI - 10,000 units New England Biolabs Catalog
#R0101S

18 and 14 μ L ddH₂O

14 μ L

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.

Note

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 μ L of ddH₂O.

23 Measure the DNA concentration.

Ligating and Transforming into Bacteria

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

2 μ L

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

20 ng

Note

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

26 With 2 μ L 10x NEB T4 DNA ligase buffer

2 μ L

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

27 With 1 μ L NEB T4 DNA ligase

1 μ L

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 DH5 alpha cells, following manufacturer's protocol.
 - 31** Plate on LB agar plates containing 100 $\mu\text{g/mL}$ ampicillin or carbenicillin (an ampicillin analog).