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shRNA Design, Subcloning, and Lentivirus Production

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We use this protocol and it's working

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Abstract

This protocol provides a detailed method for designing, subcloning, and producing shRNA-expressing lentivirus for gene silencing experiments in primary cultured hippocampal neurons.

Materials

- shRNA sequences:

1. **shRNA targeting DDHD2:** TRCN0000346842, ATAGTTTAGGTTCGCTTATAT
2. **shRNA targeting CPT2 #1:** GACCCAAAGTCTGAGTATAAT
3. **shRNA targeting CPT2 #2:** ACTAACTCAGCTGTATTTATT

- pLKO-TRC vector (Addgene, 191566) expressing shRNA under U6 promoter and BFP under hPGK promoter
- pLKO.1 - TRC mTagBFP2 **addgene Catalog #**191566
- QIAquick Gel Extraction Kit (250) **Qiagen Catalog #**28706
- Packaging plasmids:

1. psPAX2 **addgene Catalog #**12260
2. pMD2.G **addgene Catalog #**12259

- jetPRIME® **POLYPLUS TRANSFECTION SA Catalog #**101000027
- Lenti-X™ Concentrator **Takara Bio Inc. Catalog #**631231
- Lenti-X GoStix Plus **Takara Bio Inc. Catalog #**631280

- Restriction enzymes: AgeI and EcoRI (All acquired from NEB)

- Stbl3 competent cells **Thermo Fisher Scientific Catalog #**C7373-03 or
- NEB Stable Competent E.coli (High Efficiency) - 20×0.05 ml **New England Biolabs Catalog #**C3040H
- 293FT Cell Line **Thermo Fisher Catalog #**R70007

- Virus Production Media: ultraCULTURE Serum-free Medium (Lonza, 12-725F) with
  Sodium Pyruvate (100 mM) **Thermo Fisher Scientific Catalog #**11360070 ,
  Sodium Bicarbonate 7.5% solution **Thermo Fisher Catalog #**25080094 ,
  Sodium butyrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**B5887 and
  Penicillin-Streptomycin-Glutamine (100X) **Thermo Fisher Catalog #**10378016

Equipment:

- 0.45 µm syringe filter set (VWR Cat. No. 28145-481)
- PCR machine
- Heat block or thermal cycler for annealing
- Tabletop centrifuge
- Biosafety cabinet

Procedure

16h 15m

1 **shRNA Design:**

1.1 Use the GPP Web Portal (https://portals.broadinstitute.org/gpp/public/) to design shRNAs targeting the consensus coding sequence of the gene of interest.

1.2 Ensure shRNA oligonucleotides include the following regions: Sense strand, Hairpin loop, Antisense strand, and Restriction sites (AgeI and EcoRI) for subcloning.

2 **Annealing of shRNA Oligonucleotides:**

2.1 Mix equal concentrations of complementary shRNA oligonucleotides in annealing buffer.

Component	Volume (µL)
Forward Oligo (20 µM)	5
Reverse Oligo (20 µM)	5
NEB Buffer 2.1	5
Ultra-Pure Water	20

2.2 Heat the mixture to 95 °C for 00:05:00, then allow it to cool stepwise to 10 °C for 00:10:00 to facilitate annealing.

15m

2.3 Hold the temperature at 4 °C.

Temp (°C)	Time (Min)
95	5
70	10
64	1.5
58	1.5
52	1.5
46	1.5
40	1.5
37	5
10	10
4	Infinity

3 **Subcloning into pLKO-TRC Vector:**

3.1 Digest the pLKO-TRC vector with AgeI and EcoRI restriction enzymes.

Component	Volume (µL)
pLKO-TRC (~1 µg)	1
AgeI-HF	1
EcoRI-HF	1
CutSmart Buffer	5
Ultra-Pure Water	42

3.2 Purify the plasmids using QIAquick Gel Extraction Kit. Ligate the annealed shRNA oligonucleotides into the digested vector using T4 DNA ligase.

Component	Volume (µL)
pLKO Cut Gel Purified	1
Annealed Oligos	2
Ultra-Pure water	6
2X Quick Ligase Buffer	10
Quick Ligase	1

4 **Transformation in Competent cells:**

4.1 Transform the competent E. coli with the ligation reaction (use 5 µL - 10 µL) and plate on LB agar with appropriate antibiotics.

4.2 Pick colonies and set up 3-4 small cultures in LB media.

4.3 Isolate plasmid DNA from cultures and confirm insertion via restriction digestion or sequencing.

5 **Lentivirus Production in HEK 293FT Cells:**

5.1 Culture HEK 293FT cells in a 15 cm culture dish with DMEM containing 10% FBS and grow up to 70-80% confluence.

5.2 Transfect cells with the following plasmid mix using JetPRIME transfection reagent:

- 7.7 µg of pLKO-shRNA plasmid
- 7.7 µg of psPAX2
- 4.6 µg of pMD2

5.3 Incubate cells for 16:00:00 , then replace transfection media with 15 ml virus production media.

16h

6 **Virus Harvesting:**

6.1 Collect the supernatant containing lentivirus at 16:00:00 - 48:00:00 post-transfection.

6.2 Filter the supernatant through a 0.45 µm syringe filter to remove cellular debris.

6.3 Concentrate the lentivirus using Lenti-X Concentrator according to the manufacturer's instructions.

7 **Virus Titer Measurement:**

7.1 Use the Lenti-X GoStix Plus Assay Kit to determine the virus titer.

7.2 Store the concentrated lentivirus in small aliquots at -80 °C until use.

Note

- Use a biosafety cabinet for all steps involving lentivirus to ensure safety and sterility.
- Include appropriate controls during transfection to confirm the efficiency of plasmid delivery.
- The blue fluorescent protein (BFP) expressed from the pLKO-TRC vector can be used to confirm successful transduction in target cells.
- Optimize transfection conditions for HEK 293FT cells to maximize virus yield.