Stable knockdown of attached cells with pSUPER.retro.puro vector

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Method Article

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Abstract

This protocol describes how stable knockdown is performed using the pSUPER.retro.puro (pRS) lentiviral vector (OligoEngine) to form short hairpin RNAs for RNAi. Transient co-transfection of HEK293GP packaging cells is used to generate cell-free viral supernatants, which is then used to infect target cells. Stable shRNA production is then maintained by puromycin selection.

This protocol has been optimized for attached cultured cell lines.

Introduction

Reagents

pSUPER.retro.puro (pRS) retroviral vector (OligoEngine, VEC-PRT-0002)

Plasmid encoding the glycoprotein of vesicular stomatitis virus (VSV-G) (originally provided by BD Biosciences Clontech, Addgene plasmid #14888 may be an alternative)

HEK293GP packaging cells (CVCL_E072)

Opti-MEM (Gibco, 11058021)

DMEM (Gibco, 11960044)

PBS without calcium/magnesium (e.g. Thermo Fisher Scientific, 10010023)

Trypsin-EDTA (0.05%) (Gibco, 25300054)

Fetal Bovine Serum (lot to be tested individually prior to experiment)

Polyethyleneimine (any source, e.g. Polysciences Inc. 26435-1)

Polybrene (made in-house)

Puromycin (Thermo Fisher Scientific, A1113803)

Standard tissue culture equipment (for both regular culture and lentiviral work)

Equipment

BD™ 10ml Luer-Lok™ syringe (BD, 309604)

Acrodisc® Syringe Filters with Supor® Membrane, Sterile - 0.2 µm (Pall, 4652)

15 mL tubes (Falcon, 352096)

Standard tissue culture equipment (for both regular culture and lentiviral work)

Procedure

Before proceeding: cloning of the target sequence into the pRS vector should be performed according to manufacturer's instructions.

http://www.oligoengine.com/products/psuper/documentation/protocols/pSUPER_retro_puro_protocol.pdf

Transient transfection of packaging cells

1. Grow HEK293GP cells to 50% confluency in a 10-cm dish.

2. Replace growing medium with 5ml of Opti-MEM.

3. Mix 5 µg of pRS plasmid and 1 µg plasmid encoding the VSV-G with 24 µl polyethylene imine (PEI) in 400µl. Opti-MEM. Mix and incubate for 20 minutes at room temperature.

4. Add the transfection mix to the HEK293GP cells. Mix gently and incubate for 5hrs in a regular incubator.

5. Replace medium with 10ml complete DMEM medium. Transfer cells to an incubator in a culture room appropriate for viral work and incubate for 48hrs for viral production.

Notes:

-From here on, all work related to the viral supernatant should be performed in a culture room appropriate for viral work and all steps (as well as waste management) should be performed in accordance with local safety protocols.

-During the 2 days of incubation of the viral supernatant, passage target cells to ensure an appropriate confluency for the infection. Typically, one 10cm dish per condition, at approximately 50% confluency on the day of the infection is required.

Infection and selection of target cells

6. Two days after the transfection, label 15ml tube for each condition and add 80 µg polybrene per tube.

7. Collect the viral supernatant of HEK293GP in a 10ml syringe.

8. Filter the supernatant in a tube containing the polybrene. Mix by inverting the tube 5 times.

9. Remove the culture medium of target cells and replace by the viral supernatant (with polybrene).

10. Put cells in the incubator for 24 hrs.

11. Remove and dispose of the viral supernatant. Perform 3 washes with 10ml PBS to get rid of viral particles. Cells can be transferred back to regular culture room at this point.

12. Passage target cells and perform selection with 1.5 µg/ml puromycin.

13. Validate knockdown by method of choice (e.g. RT-qPCR or western blot).

Note: although 1.5 µg/ml works with most cell lines, the concentration of puromycin may be modified depending on the cells. Cells are typically selected within 2-3 days and the selection is maintained constantly.