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Protocol for performing PINK1 siRNA knockdown in mouse embryonic fibroblasts (MEFs)

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

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Abstract

This protocol details the siRNA knockdown in mouse embryonic fibroblasts (MEFs) for PINK1, but applicable for any other target.

Attachments

DOCX

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17KB

Guidelines

72-Hr siRNA knockdown in MEFs in a 6-well plate

- If using Immortalised MEFs – seed 100,000 cells per well.
- If using Primary MEFs – seed 200,000 cells per well.

Materials

Key Reagents

• siRNA as purchased from Horizon Discovery as ON-TARGETplus siRNA Reagents (Dharmacon™ siRNA solutions).

Note

Protocol for 5 nmol quantities of purchased siRNA

- Lipofectamine™ RNAiMAX Transfection Reagent **Thermo Fisher** **Catalog #**13778150
- Oligomycin
- Antimycin
- MLi2

Day 1 - Cell Seeding and siRNA preparation

1 For Primary MEFs seed 200,000 cells per well in a 6-well plate at a total volume of 2 mL .

2 For Immortalised MEFs seed 100,000 cells per well in a 6-well plate at a total volume of 2 mL .

3

Day 1 - Cell Seeding and siRNA preparation

4 Resuspend the dried siRNA – protocol for 5 nmol of purchased siRNA to reconstitute at [[M] 10 micromolar (µM)].

4.1 Add 400 µL of RNA-free water.

4.2 Add 100 µL of 5x siRNA Buffer.

4.3 Incubate in hood for 00:05:00 , vortex vigorously and store at -20 °C . 5m

Note

The protocol can be amended if the nmol quantity of siRNA purchased is higher/lower by adjusting the resuspension to ensure a final [[M] 10 micromolar (µM)] concentration.

Day 2 - siRNA Knock-Down 5m

5 Prepare mastermix according to the number of wells. Four tubes have to be prepared, 2 with Optimem and lipofectamine and one each with the PINK1 and scramble siRNA respectively.

Note

For PINK1 knockdown a final concentration of 25 nM of siRNA in each well is used.

5.1 Tube1 PINK1 siRNA:
Per well - 5 µL of PINK1 siRNA at [[M] 10 micromolar (µM)] diluted in 100 µL OPTI-MEMTube 3

5.2 Tube 2:
Per well - 10 µL of Lipofectamine diluted in 100 µL of siRNA-OPTI-MEM

5.3 Tube 3 scramble siRNA
Per well - 5 µL of scramble siRNA at [[M] 10 micromolar (µM)] diluted in 100 µL OPTI-MEM

5.4 Tube 4 (same as tube 2)
Per well - 10 µL of Lipofectamine diluted in 100 µL of siRNA-OPTI-MEM

6 Vortex slowly and combine the content of Tube 1 with tube 2 (PINK1 siRNA) and similarly with tube 3 and 4.

7 Vortex again slowly and incubate for 00:05:00 at Room temperature .

5m

8 Add drop by drop, 200 µL of PINK1 siRNA mix to each well for PINK1 KD and similarly of the scramble siRNA for controls.

Note

For an experiment with 4x 6 well plates (WT and mutant cells treated with scramble or PINK1
the following volumes can be used:

- Tube1 and tube 3: 60 µL siRNA + 1200 µL OPtiMEM
- Tube 2 and 4: 120 µL of lipofectamine + 1200 µL of otpimem
- This gives 2580 µL of each mix (180 µL spare)

Day 4 - Mitochondrial depolarization

5m

9 Make a 500x of Oligomycin/antimycin solution. The final concentration in the well is:

- oligo: [M] 1 micromolar (µM)
- antimycin: [M] 10 micromolar (µM)

9.1 Add 4 µL of this 500x solution will be to each well.

Note

For a 100 µL of 500x solution, we use:

	A	B
	Compound	Quantity
	Antimycin A 50 mM solution	10 ul
	Oligomycin 6.4 mM solution	7.8 ul
	DMSO	82.2 ul

10 Treat cell with Oligomycin/antimycin A for 24:00:00 . OA should be added 48:00:00 after the addition of the siRNA. Include DMSO control. 3d

Day 5 - Cell lysis 1h 50m

11 Prepare working stock of MLi2 at a concentration of [M] 100 micromolar (µM) in DMSO.

12 Treat cells with MLi2([M] 10 nanomolar (nM)) for 01:30:00 (2 ul/well). 1h 30m

13 Lyse cells using 50 µL of Lysis buffer/well

14 Lysate can be precleared by centrifugation 17000 x g for 00:15:00 at 4 °C . 15m

15 Perform protein estimation and subject lysate to immunoblotting.