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

Notes

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

Key Reagents

* siRNA as purchased from Horizon Discovery as ON-TARGETplus siRNA Reagents (Dharmacon™ siRNA solutions)
	+ Protocol for 5 nmol quantities of purchased siRNA
* Lipofectamine™ RNAiMAX Transfection Reagent (#13778150)
* Oligomycin
* Antimycin
* MLi2

Protocol

Day 1

* For Primary MEFs seed 200,000 cells per well in a 6-well plate at a total volume of 2 mL
* For Immortalised MEFs seed 100,000 cells per well in a 6-well plate at a total volume of 2 mL
* Resuspend the dried siRNA – protocol for 5 nmol to reconstitute siRNA at [10 uM]
	1. Add 400 uL of RNA-free water
	2. Add 100 uL of 5x siRNA Buffer
	3. Incubate in hood for 5 minutes, vortex vigorously and store at -20

*The protocol can be amended if the nmol quantity of siRNA purchased is higher/lower by adjusting the resuspension to ensure a final [10 uM] concentration*

Day 2

* Prepare mastermix according to number of well. Four tubes have to be prepared, 2 with Optimem and lipofectamine and one each with the PINK1 and scramble siRNA resepctively:
	1. Tube1 PINK1 siRNA:

Per well - 5 uL of PINK1 siRNA at [10 uM] diluted in 100 uL OPTI-MEMTube 3

* 1. Tube 2:

Per well - 10 uL of Lipofectamine diluted in 100 uL of siRNA-OPTI-MEM

* 1. Tube 3 scramble siRNA

Per well - 5 uL of scramble siRNA at [10 uM] diluted in 100 uL OPTI-MEM

* 1. Tube 4 (same as tube 2\_

Per well - 10 uL of Lipofectamine diluted in 100 uL of siRNA-OPTI-MEM

* Vortex slowly and combine the content of Tube 1 with tube 2 (PINK1 siRNA) and similarly with tube 3 and 4
* Vortex again slowly and incubate for 5 min at RT
* Add drop by drop, 200 ul of PINK1 siRNA mix to each well for PINK1 KD and similarly of the scramble siRNA for controls

*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 ul siRNA + 1200 ul OPtiMEM*

*Tube 2 and 4: 120 ul of lipofectamine + 1200 ul of otpimem*

*This gives 2580 ul of each mix (180ul spare)*

Day 4

* Make a 500x of Oligomycin/antimycin solution. The final concentration in the well is:
	1. oligo:1 uM
	2. antimycin:10 uM

4 ul of this 500x solution will be added to each well.

*For a 100 ul of 500x solution we use:*

*10 ul of antimycin A 50 mM solution
7.8 ul of oligomycin 6.4 mM solution
82.2 ul of DMSO*

* Treat cell with Oligomycin/antimycin A for 24h. OA should be add 48h after the addition of the siRNA. Include DMSO control

Day 5

* Prepare working stock of Mli2 at a concentration of 100 um in DMSO
* Treat cells with MLi2(100 nM) for 90 min (2 ul/well)
* Lyse cells after 72-Hour incubation
* Lysate can be precleared by centrifugation 17,000 x *g* for 15 minutes at 4°C
* Perform protein estimation and subject lysate to immunoblotting