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TBK1 knockdown and rescue in Hela-M cells

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ABSTRACT

TANK-binding kinase 1 (TBK1) is a multifunctional kinase with roles in several crucial cell processes, including innate immune response, anti-viral response, and mitochondrial clearance, the last of which is of most interest to us. In order to further understand the role of TBK1 in mitophagy, we developed a protocol to transiently deplete TBK1 from a model system, HeLa cells, and re-introduce a tagged TBK1 along with other relevant components of mitophagy. Because of the many processes that rely on TBK1, cells depleted of the kinase exhibit poor health. Our protocol accomplishes ~70% depletion of endogenous TBK1 within 24 hours without causing excessive cell death. We employ this protocol to carry out biochemistry experiments such as Western blotting and organelle fractionation, and imaging experiments such as immunofluorescence, and live cell imaging.

ATTACHMENTS

db5nbjnsp.pdf

DOI

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PROTOCOL CITATION

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tissue culture, transfection, knockdown, TBK1

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GUIDELINES

- This protocol was developed with the HeLa subtype, HeLa-M. HeLa-M cells are flatter than standard HeLa cells, making them easier to image. They also uptakes iRNA better than standard HeLa. Regardless, the protocol would be easily adaptable to standard HeLa cells or other cell culture lines.
- HeLa cells fare poorly after TBK1 depletion. For this reason, I developed a 24-hour protocol with simultaneous knockdown and rescue. Even so, there will be a number of dead cells in the dish after transfection.
- This protocol was created in order to investigate Parkin-dependent mitophagy. Parkin and several other fluorescently-tagged mitophagy components are included in the protocol.

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MATERIALS TEXT

Materials

Countess slides **Thermo Fisher Scientific Catalog #C10228**

10 ml conical tube **Corning Catalog #CLS430055**

1.5 mL capped tubes **Millipore Sigma Catalog #EP022364120**

35 mm glass-bottomed dishes **MatTek Corporation Catalog #P35G-1.5-20-C**

Reagents

Trypsin **Thermo Fisher Scientific Catalog #R001100**

Trypan blue **Thermo Fisher Scientific Catalog #T10282**

DMEM **Corning Catalog #10-017-CV**

FBS (HyClone) **Contributed by users**

GlutaMAX glucose supplement **Gibco - Thermo Fischer Catalog #35050061**

Opti-MEM **Thermo Fisher Scientific Catalog #3198507**

- Plasmid DNA
  - untagged Parkin (subcloned from YFP-Parkin, a gift from R. Youle, NIH, Bethesda, MD)
  - Mito-DsRed2 (kindly provided by. T. Schwartz, Harvard Medical School, Boston)
  - Halo-TBK1(subcloned from SNAP-TBK1, provided by T.Maniatis, see Ye et al, PNAS, 2019)

- siRNA
  - targeting 5' (UAACAAGAGGAUUGCCUGA) end of hTBK1(Horizon Discovery)
  - targeting 3' (CCACUGUUAUACUGGGAUA) end of hTBK1(Horizon Discovery)

Lipofectamine 2000 **Thermo Fisher Scientific Catalog #11668027**

Equipment
- cell incubator to maintain 37 °C and 5% CO_2 atmosphere
- Countess automated cell counter (Invitrogen, AMQAX1000)
- minicentrifuge (SouthernLabware, MLX-106)
- Compound microscope

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

- Cells are best transfected before passage 30. Higher passage number could result in lower transfection efficiency. Use cells between P5 and P25 for best results.
- siRNA is easily degraded by RNAases that exist on everyday surfaces. Follow best practices for handling siRNA by suspending desiccated reagent in RNAase-free water, aliquoting into sterilized tubes, and using barrier pipet tips to handle aliquots.

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Day 1: Plating

1 Trypsinize Hela-M cells by aspirating all media from a 10 cm dish of confluent cells, then dropping **0.75 mL Trypsin** onto cells.

2 

5m

Incubate cells at **37 °C** , **5 % CO2** for **00:05:00** .

3 Resuspend detached cells and neutralize Trypsin with **1 mL DMEM** with **10 % FBS** and **1 % GlutaMAX** for a final volume of **1.75 mL** .

4 Transfer this volume to 10 mL conical tube.

5 Combine **10 µl of suspension** with **10 µl Trypan blue** in a 1.5 mL tube.

6 Drop **10 µl of this mixture** onto a Countess slide and insert into the cell counter to calculate the concentration of cells in the resuspended solution.

7 Plate ~0.25 million HeLaM cells on 35mm imaging dish in **2 mL DMEM/ FBS/ GlutaMAX** .

The number of cells to plate should be calculated by a standard guide for confluence, in order to achieve ~80-90% confluence on Day 2.

I prefer to pipet the volume of suspension needed (usually 20-50 µL) *for each dish* directly from that 10 mL tube and add it dropwise to 2 mL in the dish. I found that the cells uptake the exogenous nucleic acids best when they are in confluent patches instead of evenly distributed on the surface.

Day 2: Transfection

8 Examine cells by compound microscope **18:00:00** - **24:00:00** after plating to confirm 80-90% confluence.

1d

If cells are not at 80-90% confluence, do not transfect. Wait until they reach 80-90%.

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9 For each dish, prepare the following two solutions in 1.5 mL tubes.

Tube 1 (nucleic acids): **200 µl Opti-mem**

- \+ **0.5 µl Halo-TBK1** (stock at 1µg/µL)
- \+ **0.25 µl mito-dsRed** (" ")
- \+ **0.5 µl Parkin** (" ")
- \+ **4.8 µl siTBK1-5'**
- \+ **4.8 µl siTBK1-3'**

Invert stock solutions of each plasmid several times in order to ensure even distribution of plasmid.

Tube 2 (Lipofectamine 2000): **200 µl Opti-mem**

- \+ **11.4 µl Lipofectamine 2000**

The Lipofectamine 2000 volume is calculated by this equation:
- (X*4)+(Y*3.2), where X = ug plasmid DNA (in this protocol, X=1.25) and Y = # of 4.8 µL aliquots of siRNA(in this protocol, Y = 2)

10 Invert tubes 8 times to distribute the contents, then:

11  10m

Incubate **00:05:00** - **00:10:00** at **Room temperature** .

12  2s

Spin **00:00:02** in a minicentrifuge.

13 

Add Tube 2 to Tube 1 and invert 8 times to mix.

14  15m

Incubate **00:05:00** - **00:10:00** at **Room temperature** .

15  2s

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Spin 00:00:02 in a minicentrifuge.

16 Add entire volume (~> 400 µl) to the cells dropwise, distributing the drops mostly in the center of the dish (where the imaging window/coverslip is).

Day 3: Collection 1d

1d

17 Cells are ready to collect for various assays 18:00:00 - 24:00:00 after transfection step.

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