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Confirmation of Gene Knockdown with RT-qPCR

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

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Abstract

This protocol confirms knockdown of individual genes from CRISPRi sgRNAs using RT-qPCR. Details below specifically validate gene knockdown of complex subunits within the electron transport chain in K562 cells.

Materials

- VIC-MGB human ACTB (β-actin, ThermoFisher #4326315E)

  Human ACTB (Beta Actin) Endogenous Control (VIC™/MGB probe, primer limited) **Thermo Fisher Catalog #**4326315E

FAM-MGB TaqMan Gene Expression Assays

- NDUFA8 (Thermo Fisher, assay ID: Hs00204417_m1-NDUFA8)
- NDUFA1 (Thermo Fisher, assay ID: Hs00244980_m1-NDUFA1)
- NDUFA12 (Thermo Fisher, assay ID: Hs00984333_m1-NDUFA12)
- NDUFB7 (Thermo Fisher, assay ID: Hs00958815_g1-NDUFB7)
- NDUFAB1 (Thermo Fisher, assay ID: Hs00192290_m1-NDUFAB1)
- NDUFB4 (Thermo Fisher, assay ID: Hs00853558_g1-NDUFB4)
- NDUFS8 (Thermo Fisher, assay ID: Hs00159597_m1-NDUFS8)
- NDUFS5 (Thermo Fisher, assay ID: Hs02578754_g1-NDUFS5)

- 7900HT 371 Fast Real-Time PCR System (Applied Biosystem)

Equipment

7900HT Fast Real-Time PCR System	NAME
Applied Biosystems	BRAND
4329001	SKU
https://www.thermofisher.com/order/catalog/product/4329001	LINK

- TaqMan Gene Expression Cells-to-CT kit (Thermo Fisher, # 4399002)

  TaqMan™ Gene Expression Cells-to-CT™ Kit **Thermo Fisher Scientific Catalog #**4399002

Prepare Cell Lysis

1 Collect cell pellet, wash cells in cold PBS (For K562 cells, optimized cell number is 100.000). Place cells on ice.

2 For each reaction, prepare 0.5 µL DNase I with 49.5 µL of Lysis solution.

3 Add 50 µL of Lysis solution containing DNase I to cell pellet. Mix by pipetting up and down (avoid bubble). Incubate at RT for 5min.

4 Pipette 5 µL of Stop solution into each reaction. Mix by pipetting up and down (avoid bubble). Incubate at RT for 2 min.

5 Use sample for reverse transcription reaction or store at -20^{O}C. Do not keep sample at RT for longer than 20 min.

Reverse Transcription

6 Prepare 40 µL of master mix for each reaction, place on ice
- 25 µL 2X RT Buffer
- 2.5 µL 20X RT Enzyme Mix
- 12.5 µL Nuclease-free Wate

7 Add 10 µL of cell lysis sample to each reaction. (can scale up or scale down depending on cell number, but the total volume of cell lysis sample cannot exceed 22.5 µL).

8 Spin down, mix gently by tapping.

9 Program thermal cycler for standard reverse transcription cycle (37°C for 6 minutes, followed by 95°C for 5 minutes and holding at 4°C).

10 Store samples at -20°C.

Real-time PCR

11
- Prepare 16 µL PCR cocktail master mix for each reaction, place on ice
- 10 µL TaqMan Gene Expression Master Mix (2X)
- 1 µL TaqMan Gene Expression Assay (20X)
- 5 µL Nuclease-free water

Note

Keep primers away from direct light since they are all light-sensitive. Thaw primers on ice and cover with foil.

12 Pipette PCR cocktail master mix to 386-well plate, add samples from RT reaction (4 µL each), avoid bubble. Put plate on ice if this process takes a long time.

13 Cover plate with clear plastic sticker. Spin down the plate briefly.

14 Use standard Real-time PCR program (UDG incubation of 50°C for 2 minutes, enzyme activation at 95°C for 10 minutes, and PCR cycles of 95°C for 15 seconds, 60°C for 1 minute, repeated for 40 cycles).

15 After run is complete, calculate fold changes in expression using the $2^{-\Delta\Delta CT}$method.