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Hutu80 and NCI h716 treatment with SCFAs and α synuclein Western Blotting

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Chaima Ezzine[1]

[1]MGP INRAE Jouy-en-Josas France

ASAP Collaborative Rese… | Team Schapira

Chaima Ezzine
MGP INRAE Jouy-en-Josas France

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We use this protocol and it's working

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Abstract

This protocol describe the treatment of human enteroendocrine cell models Hutu80 and NCI h716 parental cells with SCFAs and GCase inhibitor (CBE) in order to detect the impact of SCFAs and GCAse inhibition on alpha synuclein protein expression by immunoblotting.

Materials

Cell lysis buffer : Tris HCl pH 8.0 50 mM, EDTA 5 mM, NaCl 200 mM, Glycerol 10%, Igepal/NP40 0.5%
Protease inhibitor : Pierce™ Protease Inhibitor Tablets (Thermofisher)

Laemmeli buffer : 125 mm Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 100 mm dithiothreitol [DTT], 0.02% bromophenol blue

Migration Buffer :

Transfert Buffer :

1 Hutu80 and NCI h716 parental cells were seeded in wells at a density of 1.0 10^5 cells the day before treatments and incubated at 37 °C in a 5% CO_2 atmosphere

2 Cells were treated with differents concentrations (0.5 ; 1 ; 2 ; 4 and 6 mM) of SCFAs Acetate, propionate and butyrate (100 mM stock solutions in water (SIGMA-ALDRICH)) and with or not CBE 10 uM (Cyaman chemical) for differents time of incubation (2h ; 6h ; 24h and 48h). For CBE treaments, cells were pre-incubated with 10 uM CBE for 04:00:00 and then incubated with SCFAs. 4h

3 Cells were lysed in lysis buffer containing protease inhibitors (see materials)

3.1 -For HuTu80 cells lysis (Adherent cells): 45m

Carefully remove culture medium and wash cells 2 times with cold PBS. Add 100 uL of cold lysis buffer to the cells. Keep on ice for 00:05:00 , stirring the plate occasionally for uniform spreading.
Collect the lysate on one side using a cell scraper, collect the lysate and transfer it to a microcentrifuge tube. Centrifuge samples at 14000 x g, 4°C for 00:15:00 to collect cell debris.
Transfer the supernatant to a new tube and store at -20 °C until further use.

- For NCI h716 cells (Non-adherent cells) :

Collect cells by centrifugation at 2500 x g for 00:05:00 .
Discard supernatant and wash cells twice in cold PBS. Collect cells by centrifugation at 2500 x g for 00:05:00 . Add 100 uL lysis buffer to the cell pellet and stir up and down to suspend the pellet.
Centrifuge the mixture at 14000 x g, 4°C for 00:15:00 to sediment cell debris.
Transfer the supernatant to a new tube and store at -20 °C until further use.

Note: To increase yields, sonicate the pellet for 30 seconds with a 50% pulse before centrifuging.

Before immunoblotting, quantities of extracted protein were determined using BCA assays (Pierce™ BCA Protein Assay Kit)

4 Samples preparation : Mix samples with Laemelli buffer (see materials) containing 100 mM DTT and heat the samples 95 °C 00:10:00 — 10m

5 Samples preparation : Centrifuge samples 00:00:30 to collect condensation from tube lid and Vortex samples 00:00:10 — 40s

6 Electrophoresis and transfer : — 30m

Separate samples (~ 10 ug total protein) by polyacrylamide gel electrophoresis using precast TGX Stain-free pre-cast SDS-polyacrylamide gel (Bio-rad)and migration buffer (see materials) at 200 V 00:30:00 or until dye front reaches the bottom of the gel. Run with pre-stained size markers

7 Proteins were then transferred using liquid transfer on PVDF membranes (Bio-rad). PVDF membrane should be imbibed in EtOH 100% and equilibrate with transfer buffer (see materials) before use.

8 Soak transfer sandwich components (4 sheets of filter paper and 5 blotting pads) in transfer buffer and assemble in the transfer cassette in the following order:
Starting with the cathode plate
1 x blotting pads
2 x filter paper
gel
PVDF
2 x filter paper
1 x pad
Use a rollerin every step in order to eliminate air bubbles

9 Place cassette and a cooling unit in transfer tank placed in glace and transfer protein 100V 01:00:00 in transfer buffer — 1h

10 Immunodetection:

Incubate membrane in 4% paraformaldehyde in order to fix a synuclein protein

11 Wash membrane with TBST (see materials) 4 times 00:05:00 — 5m

12 Block non-specific binding sites in the membrane with block solution (TBST 5% skimmed milk) for 01:00:00 — 1h

13 Incubate membranes in block solution containing rabbit monoclonal antibody against α-synuclein (1:200) (ab212184) and β-actin (1:10000) Overnight 4 °C

14 Wash with TBST 3 x 00:05:00 — 5m

15 Incubate membrane with secondary antibody for 01:00:00 at Room temperature — 1h

16 Wash with TBST 3 x 00:05:00 — 5m

17 For chemiluminescent detection use an Bio-rad ECL plus kit according to the manufacturer's instructions : Add 1 mL of ECL per membrane and incubated for 00:05:00 — 5m
Visualise detected protein using iBright imaging system (Thermofisher)

18 After visualisation membranes can be washed with TBST 3x 00:05:00 and conserved at 4 °C if it will be reused. — 5m