Determination of protein levels of browning genes in murine adipose tissues by western blotting.

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Abstract

Increasing energy expenditure by stimulating thermogenesis through activation of brown adipose tissue (BAT) and/or induction of browning of white adipose tissue (WAT) is considered a promising strategy to treat/prevent obesity and related metabolic diseases. Whereas WAT is adapted to store energy as triglycerides, BAT produces heat (non-shivering thermogenesis). In brown adipocytes, the uncoupling protein-1 (*Ucp-1*) regulates conversion of energy into heat by uncoupling ATP production from mitochondrial respiration. Also in WAT adaptive *Ucp-1* positive adipocytes (brown in white: brite or beige) can arise, predominantly in subcutaneous (s) WAT. Therefore, this browning of sWAT can be visualized by making sWAT protein extracts and by analyzing protein levels of brown adipocyte-specific markers such as *Ucp-1* and peroxisome proliferator-activated receptor gamma coactivator 1 α (*Pgc1α*) in these extracts.

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Before start

A) Prepare Protein lysis buffer RL (10 ml):
-1 ml 100 mM Na_2PO_4 (10 mM) /1.5 M sodium chloride NaCl (0.15 M)
-400µl 25% Triton-X-100 (1%)
-100µl 10% sodium dodecyl sulfate (0.1%)
-1 ml 5% sodium deoxycholate (0.5%)
-1 ml 2% sodium azide (0.2%)
-6.5 ml MilliQ water

*Add fresh 1/100 EDTA and phosphatase and protease inhibitor cocktail (Halt phosphatase and protease inhibitor cocktail (100x), Thermo Fisher Scientific catalog number 78442) to buffer.

B) Prepare NUPAGE LDS sample buffer supplemented with β-mercaptoethanol (20:1)

Protocol

Step 1.

Add protein lysis buffer RL to tissues (volume of buffer (µl) = tissue weight in mg x 4) in 1.5 ml tube with glass beads.

*Protein lysis buffer RL (10 ml):

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-1 ml 5% sodium deoxycholate (0.5%)

-1 ml 2% sodium azide (0.2%)

-6.5 ml MilliQ water

*Add fresh 1/100 EDTA and phosphatase and protease inhibitor cocktail to buffer.

REAGENTS

HALT phosphatase and protease inhibitor cocktail (100x) 78442 by Thermo Fisher Scientific

Step 2.
Homogenize tissues using a Fastprep Ribolyser (MP Biomedicals, Elsene, Belgium): speed 6 time 40 sec x2, keep on ice in between.

Step 3.
Centrifuge at +4°C, 20 min 13000 rpm.

Step 4.
Collect supernatans and transfer to new labeled eppendorf tube.

Step 5.

Measure protein concentration using the Pierce BCA protein assay:

-Make standard curve with bovine serum albumin (2 mg/ml): 1 mg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.625 µg/ml, 7.8125 µg/ml. Dilutions are made with MilliQ water.

-Make dilutions of protein extracts: 1/50 and 1/100 in MilliQ water.

-Apply 50 µl of each standard and sample (in duplicate) to a 96-well plate.

-Mix reagent A (50 parts) with reagent B (1 part) of BCA protein assay and apply 200µl/well

-Incubate plate for 30 min at room temperature in the dark

-Measure absorbance at 550 nm on the microplate reader (ELx808IU, Biotek) using the Gen5 software (Biotek).

-Average protein concentrations/sample.

REAGENTS

Pierce BCA protein assay 23225 by Thermo Fisher Scientific

Bovine serum albumin 2 mg/ml 23209 by Thermo Fisher Scientific

Step 6.

Prepare protein samples for loading: combine 50 µg of protein with xµl MilliQ water (12 and 25µl in total for a 12- and 10-well gel) and xµl NUPAGE LDS sample buffer (4x) containing β-mercaptoethanol (20:1).

REAGENTS

NUPAGE LDS sample buffer (4x) NP0007 by Thermo Fisher Scientific

b-mercaptoethanol M3148-25ML by Sigma-aldrich

Step 7.

Unpack the NUPAGE 10% Bis-Tris gel and remove the white plastic strip at the bottom and comb.

REAGENTS

10-well NUPAGE 10% Bis-Tris gel NP0301BOX by Thermo Fisher Scientific

12-well NUPAGE 10% Tris-Bis gel NP0302BOX by Thermo Fisher Scientific

Step 8.

Mount the gel cassette in the running tank (X-Cell Sure Lock, Life Technologies).

Step 9.

Fill the tank with 1x NUPAGE MOPS SDS running buffer (20x), supplemented with 500 µl NUPAGE antioxidant.

REAGENTS

1x NUPAGE MOPS SDS running buffer (20x) NP0001 by Thermo Fisher Scientific

NUPAGE antioxidant NP0005 by Thermo Fisher Scientific

Step 10.

Heat protein samples for 5 min in heat block at 95°C.

Step 11.

Load the samples and 8µl of Pageruler prestained protein ladder (10 – 180 kDa). Ladder contains two reference bands orange = 70 kDa and green = 10 kDa.

REAGENTS

Pageruler Prestained Protein Ladder 26616 by Thermo Fisher Scientific

Step 12.
Close the tank carefully and connect to power supply Powerease 500 (Thermo Fisher Scientific).

Step 13.
Start electrophoresis: 55min at 135V.

Step 14.
Assemble the iBlot transfer stacks on the iBlot gel transfer device (IB1001, Thermo Fisher Scientific): iBlot NC anode stock bottom-mini (04-BLT-BM-UN002, Thermo Fisher Scientific), gel, presoaked in deionized water iBlot filter paper, iBlot cathode stock Top-mini containing nitrocellulose membrane (06-BLT-BM-UN001).

REAGENTS

iBlot Gel Transfer Stacks, Nitrocellulose, Regular IB301001 by Thermo Fisher Scientific

Step 15.
Use the Blotting roller to eliminate air bubbles.

Step 16.
Place the Disposable Sponge with the metal contact on the upper right corner of the lid.

Step 17.
Close the lid and secure the latch. The red light is on indicating a closed circuit.

Step 18.
Select program P1 (25V, 6 min) and press Start/Stop button. The red light changes to green.

Step 19.
After transfer, disassemble the stack and remove the membrane.

Step 20.

Block the membrane for 2-3hrs in TBST supplemented with 5% milk (Blotting Grade Blocker) on shaker at room temperature.

*10x TBS (1L):

-24g Tris Base

-88g NaCl

-Adjust pH to 7.6

-Add deionized water to 1L

*TBST (1L):

-100 ml 10x TBS

-0.5 ml Tween

-20 (0.05%)

-Add deionized water to 1L

REAGENTS
Blotting Grade Blocker 170-6404 by BIO-RAD

Step 21.

Add primary antibody and cover:

a. rabbit anti-mouse polyclonal antibody directed to uncoupling protein-1: 1/2000 in TBST supplemented with 5% BSA

b. rabbit anti-mouse polyclonal antibody directed to peroxisome proliferator activated receptor gamma cofactor 1α: 1/4000 in TBST supplemented with 5% milk

REAGENTS
Bovine serum albumin Fraction V 10735086001 by Roche
Rabbit anti-mouse pAb UCP-1 U6382 by Sigma-aldrich
Rabbit anti-mouse pAb PGC1a NBP1-04676 by Contributed by users

Step 22.
Incubate overnight at +4°C on shaker.

Step 23.
Wash membrane 3 x 5 min in TBST.

Step 24.

Apply secondary horse radish peroxidase (HRP)-labeled Goat anti-Rabbit antibody (1/2000) in TBST supplemented with 5% milk.

REAGENTS
Horse radish peroxidase-labeled Goat anti-rabbit IgGs P0448 by Contributed by users

Step 25.
Incubate 1h at room temperature on shaker.

Step 26.
Wash 3 x 5 min in TBST.

Step 27.

Detect UCP-1 and PGC-1α with SuperSignal™ West Femto Maximum Sensitivity Substrate.

-Mix equal volumes of reagent 1 and 2 (1 ml in total), cover tube with tinfoil.

-Place the membrane in transparent foil.

-Incubate with substrate mixture.

-Expose to chemiluminescence (lumi-imager, Chemidoc XRS+ Image Lab™ Software, 1708265 ,Bio-Rad).

REAGENTS

SuperSignal™ West Femto Maximum Sensitivity Substrate 34095 by Thermo Fisher Scientific

Step 28.

Strip membrane for re-blotting (Re-Blot Plus Mild antibody stripping solution).

-Wash away substrate for 15 min in TBST.

-Incubate the membrane in 5 ml stripping buffer (1X in MilliQ water) for 15 min at room temperature.

-Wash shortly in TBST.

REAGENTS

Re-Blot Plus Mild antibody stripping solution 2502 by Millipore

Step 29.

Re-block the membrane and continue as described above for the housekeeping proteins glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin:

a) primary rabbit monoclonal anti-mouse β-actin 13E5 antibody (1/1000):

-Incubation primary antibody: 1.5hrs at room temperature in TBST/5% milk

b) primary rabbit monoclonal anti-mouse GAPDH antibody (1/2000):

-Incubation primary antibody: overnight at +4°C in TBST/5% milk

REAGENTS

rabbit monoclonal anti-mouse β-actin 13E5 antibody 4970L by Cell Signaling Technology

rabbit monoclonal anti-mouse GAPDH antibody 2118L by Cell Signaling Technology

Warnings

-β-mercaptoethanol needs to be handled in a fume hood.