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Protocol status: Working

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

PLOS One

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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 (*Pgc1 α*) in these extracts.

MATERIALS

STEP MATERIALS

- ⊗ HALT phosphatase and protease inhibitor cocktail (100x) **Thermo Fisher Scientific Catalog #78442**
- ⊗ Pierce BCA protein assay **Thermo Fisher Scientific Catalog #23225**
- ⊗ Bovine serum albumin 2 mg/ml **Thermo Fisher Scientific Catalog #23209**
- ⊗ NUPAGE LDS sample buffer (4x) **Thermo Fisher Scientific Catalog #NP000**
- ⊗ b-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3148-25ML**
- ⊗ 10-well NUPAGE 10% Bis-Tris gel **Thermo Fisher Scientific Catalog #NP0301BOX**
- ⊗ 12-well NUPAGE 10% Tris-Bis gel **Thermo Fisher Scientific Catalog #NP0302BOX**
- ⊗ 1x NUPAGE MOPS SDS running buffer (20x) **Thermo Fisher Scientific Catalog #NP0001**

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NUPAGE antioxidant Thermo Fisher Scientific Catalog #NP0005
Pageruler Prestained Protein Ladder Thermo Fisher Scientific Catalog #26616
iBlot Gel Transfer Stacks, Nitrocellulose, Regular Thermo Fisher Scientific Catalog #IB301001
Blotting Grade Blocker Bio-Rad Laboratories Catalog #170-6404
Bovine serum albumin Fraction V Roche Catalog #10735086001
Rabbit anti-mouse pAb UCP-1 Merck MilliporeSigma (Sigma-Aldrich) Catalog #U6382
Rabbit anti-mouse pAb PGC1a Contributed by users Catalog #NBP1-04676
Horse radish peroxidase-labeled Goat anti-rabbit IgGs Contributed by users Catalog #P0448
SuperSignal™ West Femto Maximum Sensitivity Substrate Thermo Fisher Scientific Catalog #34095
Re-Blot Plus Mild antibody stripping solution Merck Millipore (EMD Millipore) Catalog #2502
rabbit monoclonal anti-mouse β -actin 13E5 antibody Cell Signaling Technology Catalog #4970L
rabbit monoclonal anti-mouse GAPDH antibody Cell Signaling Technology Catalog #2118L
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Pageruler Prestained Protein Ladder Thermo Fisher Scientific Catalog #26616
iBlot Gel Transfer Stacks, Nitrocellulose, Regular Thermo Fisher Scientific Catalog #IB301001
Blotting Grade Blocker Bio-Rad Laboratories Catalog #170-6404

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Re-Blot Plus Mild antibody stripping solution Merck Millipore (EMD Millipore) Catalog #2502
rabbit monoclonal anti-mouse β -actin 13E5 antibody Cell Signaling Technology Catalog #4970L
rabbit monoclonal anti-mouse GAPDH antibody Cell Signaling Technology Catalog #2118L

SAFETY WARNINGS

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

BEFORE START INSTRUCTIONS

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)
- 400 μ l 25% Triton-X-100 (1%)
- 100 μ l 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)

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

*Protein lysis buffer RL (10 ml):

- 1 ml 100 mM Na₂PO₄ (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 to buffer.

HALT phosphatase and protease inhibitor cocktail (100x) **Thermo Fisher Scientific Catalog #78442**

- 2 Homogenize tissues using a Fastprep Ribolyser (MP Biomedicals, Elsenne, Belgium): speed 6 time 40 sec x2, keep on ice in between.
- 3 Centrifuge at +4°C, 20 min 13000 rpm.
- 4 Collect supernatants and transfer to new labeled eppendorf tube.
- 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.
- 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).

Pierce BCA protein assay **Thermo Fisher Scientific Catalog #23225**

Bovine serum albumin 2 mg/ml **Thermo Fisher Scientific Catalog #23209**

⊗ NUPAGE LDS sample buffer (4x) **Thermo Fisher Scientific Catalog #NP0007**

⊗ b-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3148-25ML**

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

⊗ 10-well NUPAGE 10% Bis-Tris gel **Thermo Fisher Scientific Catalog #NP0301BOX**

⊗ 12-well NUPAGE 10% Tris-Bis gel **Thermo Fisher Scientific Catalog #NP0302BOX**

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

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

⊗ 1x NUPAGE MOPS SDS running buffer (20x) **Thermo Fisher Scientific Catalog #NP0001**

⊗ NUPAGE antioxidant **Thermo Fisher Scientific Catalog #NP0005**

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

- 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.

⊗ Pageruler Prestained Protein Ladder **Thermo Fisher Scientific Catalog #26616**

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

- 13 Start electrophoresis: 55min at 135V.

- 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).

iBlot Gel Transfer Stacks, Nitrocellulose, Regular **Thermo Fisher Scientific Catalog**
#IB301001

- 15 Use the Blotting roller to eliminate air bubbles.
- 16 Place the Disposable Sponge with the metal contact on the upper right corner of the lid.
- 17 Close the lid and secure the latch. The red light is on indicating a closed circuit.
- 18 Select program P1 (25V, 6 min) and press Start/Stop button. The red light changes to green.
- 19 After transfer, disassemble the stack and remove the membrane.
- 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

Blotting Grade Blocker **Bio-Rad Laboratories Catalog #170-6404**

- 21** Add primary antibody and cover:
- rabbit anti-mouse polyclonal antibody directed to uncoupling protein-1: 1/2000 in TBST supplemented with 5% BSA
 - rabbit anti-mouse polyclonal antibody directed to peroxisome proliferator activated receptor gamma cofactor 1 α : 1/4000 in TBST supplemented with 5% milk

Bovine serum albumin Fraction V **Roche Catalog #10735086001**

Rabbit anti-mouse pAb UCP-1 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #U6382**

Rabbit anti-mouse pAb PGC1 α **Contributed by users Catalog #NBP1-04676**

- 22** Incubate overnight at +4°C on shaker.

- 23** Wash membrane 3 x 5 min in TBST.

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

Horse radish peroxidase-labeled Goat anti-rabbit IgGs **Contributed by users Catalog #P0448**

- 25** Incubate 1h at room temperature on shaker.

- 26** Wash 3 x 5 min in TBST.

- 27** Detect UCP-1 and PGC-1 α 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).

- 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.

Re-Blot Plus Mild antibody stripping solution **Merck Millipore (EMD Millipore) Catalog #2502**

- 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

rabbit monoclonal anti-mouse β -actin 13E5 antibody **Cell Signaling Technology Catalog #4970L**

rabbit monoclonal anti-mouse GAPDH antibody **Cell Signaling Technology Catalog #2118L**