Gene expression analysis for browning in murine subcutaneous and gonadal fat tissues and for steatosis and fibrosis in liver tissues.

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Abstract

ADAMTS5, the main aggrecanase, is a protein that appears to play a functional role in development of white adipose tissue (WAT) and brown adipose tissue (BAT) and in browning of WAT. ADAMTS5 deficient or knockout (ADAMTS5$^{-/-}$ or KO) mice indeed showed lower WAT mass, higher mass of interscapular BAT and enhanced browning of WAT, as indicated by elevated uncoupling-protein-1 (UCP-1) levels. In addition, ADAMTS5$^{-/-}$ mice appeared to be protected from non-alcoholic steatohepatitis (NASH) when kept on a Western type diet. These observations were made with the ADAMTS5-/--P mice originally generated by Pfizer, in collaboration with Lexicon Genetics. To provide confirmation of these observations, we have obtained a second strain of ADAMTS5 deficient mice, generated independently by Deltagen Inc.

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Guidelines

-RNA should be as pure as possible: $OD_{260/280}$ between 1.8 – 2.0 and $OD_{260/230}$ between 1.8 – 2.0. For sWAT and GON the $OD_{260/230}$ is sometimes lower than 1.8. Still these samples are used.
-Volume of cDNA mix depends on the number genes of which the expression will be analyzed. E.g. 4 genes, 2µl/RT-PCR reaction, 2 technical replicates = 16µl; then 25µl is enough.

-Multiscribe enzyme: add enzyme last and keep it in a cold -20°C box.

Before start

All reagents (except DNAse) should be at RT; WORK QUICKLY@@

- Dry ice
- 2 ml collection tubes (1/sample)
- 1.5 ml Eppendorftubes (3/sample, 1 included in kit)
- Pure ethanol
- Ethanol (70% v/v) in RNAse free water (Molecular Biology Reagent W4502, Sigma-Aldrich, 35 ml ethanol + 15 ml water)

- Qiagen Rneasy Mini kit (Catalog number 74106)
- Qiagen RLT buffer: add 10 µl β-mercatoethanol (powder room, toxic: wear gloves and use hood) per 1 ml RLT buffer (stable for 1 month)
- Qiagen RPE buffer (check if ethanol has been added)
- RNAse-free DNAse I set (Qiagen: 79254): dissolve solid DNAse in 550 µl water provided in kit, **DO NOT VORTEX,** store at -20°C, thaw on ice. Make working solution: 10µl DNase stock solution (thaw on ice) + 70 µl RDD buffer (provided in kit) for each sample, mix by inverting (**do not vortex**). Perform on ice.
- Clean bench first with dreft and then with ethanol
- Clean eppendorf centrifuge: take out rotor, clean with dreft, rinse with hot water, rinse with ethanol and dry.
- Write Lysing matrix D tubes (MP Biomedicals).

Protocol

Step 1.

Make sure portion sizes of murine tissues are appropriate for the amount of RNA the RNAeasy column (RNAeasy kit) can hold:

-sWAT: 100 mg

-GON WAT: 100 mg

-Liver: 30 mg

REAGENTS

RNAeasy mini kit 74106 by Qiagen

Step 2.

Add 600µl RLT buffer (provided in RNAeasy kit) containing β-mercaptoethanol to each labeled 2 ml Lysing matrix D tube. Put on regular ice!

REAGENTS

b-mercaptoethanol M3148-25ML by Sigma-aldrich

Lysing matrix D tubes 6913100 by MP Biomedicals

Step 3.

Transfer tissues with an ethanol swapped forceps to the corresponding labeled 2 ml Lysing matrix D tube on ice.

Step 4.

Homogenize sample in the Fastprep-24 (speed: 6 for 40 sec; MP Biomedicals): push button (back Ribolyzer), take out first platform, put tubes on second, push 2nd platform in, put first platform on head of tubes. Fix head on rotor, turn until you hear a sound. Set speed. Press run. Take out samples by putting entire platform on ice. Then transfer tubes one by one on ice.

Step 5.
Homogenize a second time: speed 4 for 40 sec. Put samples on ice.

Step 6.
Centrifuge homogenized samples at room temperature for 3 min at 13,000 rpm (will give a nice fat layer for fat tissues so it is easy to separate)

Step 7.
Transfer the clear homogenate (45° angle, remove the supernatant even through the beads layer but avoid the upper white material) into a new eppendorftube.

Step 8.

Add 600µl ethanol (50% for liver tissues, 70% for fat tissues) to each tube containing the homogenates. Mix immediately by pipetting and transfer to the RNAeasy spin column (max. 700 µl and provided by the RNAeasy mini kit).

Step 9.
Centrifuge 30 sec at 10,000 rpm at room temperature; discard flow-through (50 ml tube).

Step 10.
Apply the rest of the sample and centrifuge 30 sec at 10,000 rpm at room temperature. Discard flow-through.

Step 11.
Add 350µl of RW1 buffer (provided in kit) to each column (take fresh tip each time).

Step 12.
Centrifuge for 30 sec at 10,000 rpm at room temperature.

Step 13.

Add 80µl DNAse I working solution directly onto the membrane (drop in middle of the membrane) and incubate for 15 min at room temperature (fresh tip each sample).

REAGENTS
DNAse I, RNAse-free 79254 by Qiagen

Step 14.
Add 350µl of RW1 buffer to each column.

Step 15.
Use a new tube to collect flow-through.

Step 16.
Centrifuge for 30 sec at 10,000 rpm at room temperature, discard flow-through.

Step 17.
Add 500µl RPE buffer (provided in kit) to each column.

Step 18.

Centrifuge for 30 sec at 10,000 rpm at room temperature, discard flow-through.

Step 19.
Add another 500µl RPE buffer to each column.

Step 20.
Centrifuge for 2 min at 10,000 rpm at room temperature, discard flow-through, use a new tube.

Step 21.
Centrifuge for 1 min at 13,200 rpm at room temperature.

Step 22.
Transfer the column in a new 1.5 ml Eppendorf tube and add xµl RNAse-free water (provided by RNAeasy Mini kit) directly onto each membrane (volume: sWAT, GON and liver: 30 µl)

Step 23.
Centrifuge for 1 min at 10,000 rpm at room temperature (fresh tip for each sample).

Step 24.
Keep DNA-free total RNA samples on ice and measure RNA concentration using the Nanodrop 2000C (Isogen Life Science)

Step 25.
Make a 10 ng/µl dilution with RNAse-free water of the DNA-free total RNA liver samples.

Step 26.

Make complementary DNA from the total RNA samples using the Taqman Reverse Transcription Reagents: per sample combine 5.725µl RNAse-free water, 2.5µl 10x RT buffer (1x), 5.5µl MgCl2 solution, 4µl dNTPs mixture (2.5 mM each), 1.25µl 50 µM random hexamers (2.5 µM), 0.4µl 20U/µl RNAse inhibitors (0.32U/µl), 0.625µl 50U/µl Multiscribe enzyme (1.25U/µl) with 5µl 10 ng/µl total RNA.

REAGENTS

Taqman reverse transcription reagents N8080234 by Thermo Fisher Scientific

Multiscribe reverse transcriptase N8080018 by Thermo Fisher Scientific

Random hexamers N8080127 by Thermo Fisher Scientific

Multiply µStrip 0.2 ml chain 72.985.002 by Contributed by users

Step 27.
Put samples in a 2720 Thermal Cycler (Applied Biosystems)

Step 28.
Cycling conditions are:-10 min 25°C-60 min 48°C-5 min 95°C-+4°C

Step 29.

cDNA samples are applied in duplicate on the 96 well fast thermal cycling plate for each gene

* Thermo Fisher Scientific ref. 4366932 Optical 96 well fast thermal cycling plates, 200 pcs

REAGENTS

Optical 96 well fast thermal cycling plates 4366932 by Thermo Fisher Scientific

Step 30.

Prepare the RT-PCR mix for each Taqman gene expression assay and ppiet samples on cycling plate: for one sample combine 5µl Taqman fast universal PCR master mix, 0.5µl Taqman gene expression assay, 2.5µl water with 2µl cDNA mix.

* Thermo Fisher Scientific ref. 4364103 Taqman fast universal PCR master mix

* VWR Filtertip 20ul max rec axygen 960 REF 732-0652

* VWR Filtertip 200ul max rec axygen 960 REF 732-0655

* VWR Filtertip 1000ul REF 732-0863* VWR ART/ REACH 10ul Pipet tips REF 732-2221

* Taqman gene expression assay A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5): Mm00478620_m1

Browning

* Taqman gene expression assay Cell death activator (Cidea): Mm00432554_m1

* Taqman gene expression assay Uncoupling protein-1 (UCP-1): Mm01244861_m1

* Taqman gene expression assay Peroxisome proliferator-activated receptor gamma cofactor-1α (PGC-1α): Mm01208835_m1

* Taqman gene expression assay PR domain containing 16 (Prdm16): Mm00712556_m1

Steatosis

* Taqman gene expression assay Cluster of differentiation 36 (CD36): Mm00432403_m1

* Taqman gene expression assay Fatty acid synthase (Fasn): Mm09253800_g1

Fibrosis

* Taqman gene expression assay Tissue inhibitor of metalloproteinases 1 (Timp1): Mm00441818_m1

* Taqman gene expression assay Collagen type 1 α1 (Col1a1): Mm00801666_g1

* Taqman gene expression assay Transforming growth factor β1 (Tgfβ1): Mm03024053_m1

Inflammation

Pan-macrophage markers

* Taqman gene expression assay F4/80 (Emr1): Mm00802529_m1

* Taqman gene expression assay Colony stimulating factor 1 receptor (Csf1r): Mm01266652_m1

M1 or pro-inflammatory macrophage markers

* Taqman gene expression assay Monocyte chemoattractant protein-1 (MCP-1): Mm00441242_m1

* Taqman gene expression assay Tumour necrosis factor-α (TNF-α): Mm00443258_m1

* Taqman gene expression assay Interleukin 1β (IL-1β): Mm01336189_m1

M2 or anti-inflammatory macrophage markers

* Taqman gene expression assay Arginase 1 (Arg1): Mm00475988_m1

* Taqman gene expression assay Mannose receptor c type 1 (Mrc1): Mm00485148_m1

Housekeeping gene

* Taqman gene expression assay β-actin: Mm01205647_g1

Step 31.

Cover plates with optical adhesive cover. Press cover firmly on plate.

REAGENTS

ABI prism optical adhesive covers 4311971 by Thermo Fisher Scientific

Step 32.
Keep plates at +4°C (same day run) or store at -20°C (run within next 5 days).

Step 33.
Allow plate to warm-up to room temperature.

Step 34.
Before running, centrifuge plates quickly at room temperature in a table-top centrifuge Sigma 4.15C (Qiagen)

Step 35.
Run plates on 7500 Fast RT-PCR System (Applied Biosystems):-95°C 20 sec-40 cycli of 95°C 3 sec and 60°C 30 sec

Step 36.
Transfer data to excel.

Step 37.
Put data together for each gene and each group.

Step 38.
Average Ct values for each gene and for the housekeeping gene β-actin.

Step 39.
Calculate ΔCt = Ct of gene of interest – Ct of housekeeping gene.

Step 40.
Calculate the average of ΔCt of the calibrator (wild-type mice) for each gene.

Step 41.

Calculate ΔΔCt = ΔCt of all experimental groups – ΔCtaverage of calibrator group for each gene.

Step 42.
Calculate fold change as 2^(-ΔΔCt) for all data

Warnings

-RNA extraction is performed in a fume hood when working with samples that contain β-mercaptoethanol.