PROTOCOL for qPCR

Materials:
Primary rat aortic VSMC (Lonza) or A-10 (LGC Standards)
6 cm dishes
Growth medium for VSMC:
Serum-reduced medium for VSMC:
Growth medium for A10: DMEM (Lonza), 200 mM L-glutamine, 10% FBS (Gibco)
peqGold total RNA kit (Peqlab, Germany)
High Capacity cDNA Reverse Transcriptase Kit (Applied Biosciences)
Light Cycler TM 480 (Roche Diagnostics, Switzerland)
Luna Universal qPCR Master Mix (New England Biolabs)
Forward and reverse primer (10 µM) for rat TRPV1
FW and Rv primer for rat GAPDH
FW and Rv primer for rat 18S
cDNA from rat DRG - positive control for TRPV1 expression
PCR plate with a sealing tape

Procedure:
1. Seed 400K cells in 5 ml growth medium on a 6 cm dish and let incubate for 24 h (or 48 h)
2. For one set of VSMC: set them on the 24 h serum starvation (switch to 5 ml/dish of 0.1% FBS medium)
3. Aspirate the medium and lyse cells using 400 µl/6 cm dish of Rna TK Lysis Buffer from the RNA extraction kit
4. Incubate with lysis buffer for 5 min at RT
5. Scrape the lysates carefully using sterile cell scrapers into one part of the dish and transfer the lysate into a new, sterile 1.5 ml concial Eppendorf tube
6. Either freeze the lysate at -80°C till further work, or procede with the RNA extraction immediately
7. Put samples over the homogenizer columns; centrifuging at 12000 g for 1 min
8. Filtrate was mixed with 400 µl 70% EtOH
9. The mixture was put over the RNA mini columns and centrifuged at 10000 g for 1 min
10. Filtrate was discarded
11. RNA mini columns were washed once with 500 µl RNA Washing Buffer, centrifugation at 10000 g for 30 sec
12. RNA mini columns were washed twice with 80% EtOH, each time centrifugation at 13000 g for 1 min
13. Drying the columns at maximum speed (15000 g) for 2 min
14. Eluting the RNA from the RNA mini columns with 50 µl of nuclease free water, centrifuging at max speed for 2 min

Determining the RNA conc using NanoDrop, making sure the A260/A280 ratio is around 2

cDNA synthesis

High Capacity cDNA Reverse Transcriptase Kit was used from Applied Biosciences

We diluted 1 µg of RNA with RNAse-free water up to 10 µl in a cooling block

2x RT master mix was prepared on ice:

for 15 samples +RT (because	µl				**for one sample -RT**		µl
10x RT Buffer	30						2
25x dNTP Mix (100 mM)	12						0.8
10x RT Random Primers	30						2
MulitScribeTM Reverse Tran	15						0
RNAse Inhibitor	15						1
Nuclease-free water	48						4.2
TOTAL	150						10

Added 10 µl of the 2x RT master mix into 10µl RNA samples (for the blind RT+ sample, it was added to 10 µl RNAse-free water)

Flicked to mix and centrifuged the samples shortly to collect all the solution

Reverse transcription program:

	Step 1	Step 2	Step 3	Step 4
Temp	25	37	85	4
Time	10'	120'	5'	indef.

PCR

Luna Universal qPCR Master Mix was used

Reaction volume 15 µl
cDNA dilution in reaction volume 1:10
for 34
reactions
(+10%),
prepared on
ice:

		for one reaction/well:
Luna Universal qPCR Master Mix	255 µl	7.5 µl
Primers (TRPV1(P1) 10 µM or for 18S and G	25.5 µl	0.75 µl
Nuclease-free water	178.5 µl	5.25 µl
cDNA (for TRPV1 undiluted, for GADPH and for 18S 1:20 dilution)		1.5 µl

Samples measured in triplicates

Cycle step	Temperature (°C)	Time	Cycles
Initial denaturation	95	60 s	1
Denaturation	95	15 s	1
Extension	60	30 s	45
Melt curve	55	30 s	1