REVERSE TRANSCRIPTION – PCR OF MOUSE TISSUE RNA

REAGENTS REQUIRED

TRIzol (Invitrogen, Cat.15596-026)
Chloroform
Isopropanol
75% Ethanol (in DEPC-treated water)
DEPC-treated water or RNase free water
DNase I Amplification grade (Invitrogen, Cat. 18068-015)
Promega GoScript Reverse Transcription System (Fischer Cat. PRA5000)
Regular PCR Reagents (5x GoTaq buffer, dNTPs, spermadine, primers, Taq, ddH2O)

1 Homogenize mouse tissue

Grind up one whole ear or 1cm^2 piece of shaved flank under liquid nitrogen using a mortar and pestle previously chilled with liquid nitrogen. Transfer these skin grounds or 0.5cm^3 sized other tissue into a 14mL disposable falcon tube and add 1mL TRIzol. Homogenize tissue using the handheld homogenizer at approximately half speed until tissue has been fully homogenized. Clean/rinse homogenizer between samples by placing end of homogenizer at full speed into three different solutions sequentially: 2 flasks with ddH2O and one flask with 70% EtOH. Expel EtOH on outside of homogenizer by running homogenizer at full speed in the air/within the hood.

2 Extract RNA using TRIzol reagent following manufacturers instructions.

Resuspend RNA pellet in 30-50µL depending on pellet size. Final RNA concentration should ideally be between 150-1000ng/µL. If the concentration exceeds 1000ng/µL, dilute the sample to below this in order to effectively treat the sample with DNase.

3 Treat RNA samples with DNase

Treat 1µg RNA in a 10µL volume. Treat RNA following manufacturers instructions paying close attention to the length of each incubation step.

4 Reverse transcribe the RNA (make cDNA) using all reagents from Promega GoScript Reverse Transcription System

*NOTE – The following protocol is the one I used. Refer to the manual for troubleshooting options. Only complete +/- RT for important samples (there is not enough buffer to complete this for all samples)

Part 1: RNA/primer pair

Add 4µL of DNase treated RNA to 1µL (0.5µg) random primers in 8 well PCR strip tubes
-random primers work better than the polydT primers
-use maximal amount of RNA (ie. 4µL) because it is at a lower concentration than can be used
-same volume of all samples because concentration already normalized by the amount of RNA treated with the DNase

→in a PCR machine, incubate RNA/primer pair at 70°C for 5min, drop temp down to 4°C, then chill on ice

Part 2: RT mix

-use all solutions that come in the kit
-make a master mix using the following volumes of each reagent per RNA sample:

7.3µL nuclease-free H2O
4µL GoScript 5X buffer
1.2µL MgCl2 (25mM)
1µL dNTP
0.5µL rRNasin RNase inhibitor
1µL RT
15µL

→add 15µL RT mix to each well (containing primed RNA), spin down, and start the RT cycle

25°C – 5min
42°C – 60min
70°C – 15min
4°C – 10sec

5 PCR of target mRNA

Use 1µL of cDNA per PCR reaction in a 25µL volume.
Use 0.75µL of Taq made in house or 0.2µL (1 unit) of Promega GoTaq

17.4µL ddH2O
5µL 5x GoTaq buffer
0.3µL spermadine
0.25µL dNTP (each dNTP at a concentration of 10µM)
0.3µL primers (each primer at a concentration of 10µM)
0.75µL Taq (lab-prepared)
24µL

To increase cDNA detection level, a nested-PCR can be employed. In this case, take into account the amount of buffer already contained within the PCR product being added as a template for the second PCR. Normally, I add 2µL of product from the first PCR as a template for the second PCR. In this case, the PCR mix per reaction would resemble the following:

16.8µL ddH2O
4.6µL 5x GoTaq buffer
0.3µL spermadine
0.25µL dNTP (each dNTP at a concentration of 10µM)
0.3µL primers (each primer at a concentration of 10µM)
0.75µL Taq (lab-prepared)
23µL

6 Visualize PCR products by gel electrophoresis

Load PCR products onto a 1.8-2% agarose gel. The 20 well comb will provide sufficient sample separation.