

Real Time (RT) PCR

Before starting need to get:

- TRIzol Reagent (Invitrogen, #15596-018)
- Deoxyribonuclease I, Amplification Grade (Invitrogen, #18068-015)
- ThermoScript RT-PCR System (Invitrogen, #11146-024 for 25 reactions)
- iQ SYBR Green Supermix (BIO-RAD, #170-8882)
- DEPC-treated water; RNase free tips, tubes, seal pack; Liquid nitrogen
- Chloroform, Iso-propanol, 75% Ethanol (in DEPC-treated water)
- iCycler (Bio-Rad laboratories, München, Germany) for PCR

RNA preparation

According to manufacturer's instructions

Everything must be RNase free until cDNA is obtained

Dissect the tissue, and freeze in liquid nitrogen.

Put the tissue in a bag (seal pack, KAPAK) and mechanically powder the tissue by working on dry ice.

Isolate RNA using the TRIzol Reagent (Invitrogen, #15596-018) by adding 1 ml of TRIzol Reagent per 50-100 mg of tissue, pipette several times.

At this step samples can be stored at -80°C for at least one month.

Incubate the homogenized samples for 5 min at RT to permit complete dissociation of

nucleoprotein complex.

Add 0.2 ml of chloroform per 1 ml of TRIzol Reagent.

Shake tubes vigorously by hand for 15 sec, and incubate for 2-3 min at RT.

Centrifuge samples at $12,000 \times g$ for 15 min at 4°C .

Transfer the aqueous phase (0.4 ml) to a new tube.

Precipitate by adding 0.4 ml of isopropanol per 1 ml of TRIzol Reagent.

Incubate samples at RT for 10 minutes.

Centrifuge at $12,000 \times g$ for 10 min at 4°C . The RNA forms a gel-like pellet.

Remove the supernatant and wash the pellet once with 75% ethanol (prepared with DEPC

water) by adding 1 ml of 75% ethanol per 1 ml of TRIzol Reagent.

Briefly dry RNA pellet (air dry for 5-10 min). Do not over dry.

Re-suspend the pellet in DEPC water.

Check the RNA concentration with a spectrophotometer ($\lambda 260$, UV light). (Dilute 1 μl RNA in 99

μl DEPC water. Considering the equation $1 \text{ OD} = 40 \mu\text{g/ml}$ of RNA and the dilution

1/100, calculate the stock concentration)

RNA must be stored at -80°C .

cDNA transcription

According to manufacturer's instructions

Everything must be RNase free until cDNA is obtained

Start with 1 μg of RNA and proceed to the DNA degradation by using the Deoxyribonuclease I,

Amplification Grade (Invitrogen, #18068-015).

Prepare a master reaction mix containing (per tube):

1 μ l of 10X DNase I Reaction Buffer

1 μ l DNase I

Add DEPC-treated water to 10 μ l.

Incubate the reactions for 15 min at RT. Do not exceed the time.

Add 1 μ l of 25 mM EDTA solution and heat at 65°C for 10 min to stop the reaction (final volume = 11 μ l).

Continue the cDNA preparation using the ThermoScript RT-PCR System (Invitrogen, #11146-024 for 25 reactions).

In a 0.2-0.5 ml tube combine 9 μ l of RNA from the previous reaction with:

1 μ l of random primer (50 ng)

2 μ l of 10 mM dNTP mix (1mM final)

Denature RNA and primer by incubating at 65°C for 5 min and place on ice (optional).

Prepare a master reaction mix containing (per tube):

4 μ l of 5X cDNA Synthesis Buffer

1 μ l of 0.1M DTT

1 μ l of RNaseOUT (40U/ μ l)

1 μ l of DEPC water

1 μ l of ThermoScript RT (15 U/ μ l)

Pipette 8 μ l of master reaction mix into each reaction tube on ice.

Transfer the tubes to a thermal cycler preheated to 25°C and incubate as follow: 25°C for 10 min; 50°C for 50 min; 85°C for 5 min.

Add 1 μ l of RNase H and incubate at 37°C for 20 min.

Add 200 μ l of sterile water to make a 1/10 dilution. cDNA is ready for PCR and can be stored at

-20°C until use.

Real time PCR

The Real-Time PCR is carried out on an iCycler (Bio-Rad laboratories, München, Germany) featuring a reaction mixture with SYBR Green as fluorescent dye (BIO-RAD, #170-8882) and a 1/20 volume of the cDNA preparation as template.

You must first design specific primers that will amplify the cDNA of interest.

Primers can be designed by using the PRIMER3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/). Design primer pairs to obtain a band size between a window of 100 and 300 bp. Verify primer matching and specificity by blast (<http://www.ncbi.nlm.nih.gov/blast>). Primers can be ordered on this web site from JHU (http://www.jhsph.edu/Dept/BMB/DNA_Synthesis). Primers can be dissolved at a final concentration of 25 μ M.

It is absolutely necessary that the PCR product give one specific band since it is a fluorescent detection. Also primer mismatching must be avoided.

For the PCR reactions always prepare a master reaction mix containing (per tube):

- 12.5 µl of Supermix
- 0.5 µl of forward primer (500 nM final)
- 0.5 µl of reverse primer (500 nM final)
- 1.5 µl of sterile water

Pipette 10 µl of cDNA template (1/20 of the cDNA previously prepared), and add 15 µl of master reaction mix into each reaction tube.

The primers concentration may vary between 100 nM to 500 nM depending on the best condition to find.

Run the PCR in 0.2 µl tubes or 96-well plate.

Spin the tubes before starting the reaction.

Use the program on the computer connected to the iCycler (Bio-Rad laboratories, München, Germany).

Use a gradient temperature program to find the best annealing temperature for the first time.

This program can be applied as a starting condition:

Cycle 1 (1 time) 95°C for 3 min

Cycle 2 (50 times) 95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec

Cycle 3 (1 time) 95°C for 1 min

Cycle 4 (1 time) 60°C for 1 min

Cycle 5 (70 times) 60°C for 10 sec and increase by 0.5°C every repeat.

Melt curve

analysis

Cycle 6 (1 time) hold at 4°C

Result analysis

Adjust the threshold (beginning of the linear phase) if the automatic threshold is not correct. Check the melt curve (only one peak should be observed).

Save the report (including PCR amplification curve and melt curve).

Verify the PCR product on a 2% agarose gel for the first time.

In Microsoft Word, open the file and copy the cycle numbers.

The fold change in the target gene relative to the GAPDH endogenous control gene is

determined by:

$$\text{Fold Change} = 2^{-\Delta(\Delta C_T)}$$

where $\Delta C_T = C_{T, \text{target}} - C_{T, \text{GAPDH}}$

and $\Delta(\Delta C_T) = \Delta C_{T, \text{stimulated}} - \Delta C_{T, \text{control}}$

Example of analysis to do on excel:

Starting from the cycle C_T of GAPDH and Target, subtract $C_{T, \text{GAPDH}}$ from $C_{T, \text{target}}$. The

subtraction must match the same cDNA samples. If new cDNA need to be made from the same or new RNA sample, then PCR on GAPDH must be done again. This new number is named ΔC_T .

Then from these different ΔC_T values, calculate only the mean of the control values (i.e. WT mice).

Subtract this mean to all the different ΔC_T values (including the number from the control values and the stimulated condition, i.e. KO or treatment). This new different name $\Delta(\Delta C_T)$ should bring the mean of control values to zero.

Then calculate the fold change by applying the equation $2^{-\Delta(\Delta C_T)}$. This last calculation

should bring the mean of control values to 1.

Enter the fold change in a statistical program to test for significant difference.