Protocol – from tissues to RT-qPCR

1- Trizol extraction

Lyse samples

1. Lyse and homogenize samples in 500uL TRIzol™ Reagent (volume adjusted according to your starting material). Homogenize samples using Tissue Lyser (set up 30 Freq/sec for 2 times 2min), by adding one stainless steel bead to each epp. Beads for RNA are washed with water inside the chemical hood, rinsed with ethanol, let it dry and then autoclaved inside an epp – place the epp inside a bottle or beaker.

Separate phases

1. Incubate for 5 minutes to permit complete dissociation of the nucleoproteins complex.

2. Add 0.2 mL of chloroform per **1 mL of TRIzol™ Reagent** used for lysis, then securely cap the tube and mix vigorously by shaking or vortexing. (0.1 mL for 500uL)

3. Incubate for 2–3 minutes.

4. Centrifuge the sample for 15 minutes at 12,000 × g at 4°C.

The mixture separates into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase.

5. Transfer the aqueous phase containing the RNA to a new tube.

6. Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipetting the solution out.

IMPORTANT! Avoid transferring any of the interphase or organic layer into the pipette when removing the aqueous phase.

1. Isolate RNA

b. Add 0.5 mL of isopropanol to the aqueous phase, per 1 mL of TRIzol™ Reagent used for lysis. (0.25 mL for 500uL)

c. Incubate for 10 minutes.

d. Centrifuge for 10 minutes at 12,000 × g at 4°C.

Total RNA precipitate forms a white gel-like pellet at the bottom of the tube.

e. Discard the supernatant with a micropipettor

2. Precipitate the RNA

a. Resuspend the pellet in 1 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis. (0.5 mL for 500ul trizol)

b. Vortex the sample briefly then centrifuge for 5 minutes at 7500 × g at 4°C.

c. Discard the supernatant with a micropipettor

d. Repeat wash with 1 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis. (0.5 mL for 500ul trizol)

e. Vortex the sample briefly then centrifuge for 5 minutes at 7500 × g at 4°C.

f. Discard the supernatant with a micropipettor

g. Remove all ethanol and air dry

IMPORTANT! Do not dry the pellet by vacuum centrifuge. Do not let the RNA pellet dry, to ensure total solubilization of the RNA. Partially dissolved RNA samples have an A230/280 ratio <1.6.

3. Solubilize the RNA

a. Resuspend the pellet in 20–50 µL of **RNase-free water** (can also be 0.1 mM EDTA, or 0.5% SDS solution) by pipetting up and down and place on ice.

b. Incubate in a water bath or heat block set at 55°C for 10 minutes.

Proceed to downstream applications, or store the RNA at –70°C.

4. Determine the RNA yield

Use Nanodrop to determine RNA concentration, blank with RNase-free water, and pay special attention to **260/280 (~2 for RNA) and 260/230 (higher than 1,5) ratio.**

2- cDNA

Xpert cDNA Synthesis Kit (#GK80.0100)

1. Thaw individual reagents thoroughly and mix briefly before use.

2. Mix the following components in a RNase-free microtube kept on ice:

Component	Volume
5x Reaction Buffer (with RNase inhibitor)	4 µl
dNTP mix (10 mM each)	1 µl
primer (10 µM)	1 µl hexaprimer (N6) and/or 1 µl oligo(dT)$_{20}$
template RNA	1ng - 2 µg total RNA or 1pg – 2ng poly(A)+ RNA
Xpert RTase (200U/µl)	1 µl
RNase free water	up to 20 µl

3. Gently mix the reaction tube and then centrifuge briefly.

4. If using random primers, incubate at 25°C for 10min

5. Using a thermocycler or thermoblock, heat the microtube for 50 min at 55ºC

6. Inactivate RTase by heating for 5 min at 85ºC and chill on ice for 2 minutes.

7. Either use cDNA immediately as template in qPCR/PCR or store at -20ºC.

3- RT-qPCR

SsoAdvanced Universal SYBR Green Supermix

Reaction Mix Preparation and Thermal Cycling Protocol

1.Thaw SsoAdvanced Universal SYBR Green Supermix and other frozen reaction components to room temperature. Mix thoroughly, centrifuge briefly to collect solutions at the bottom of tubes, then store on ice protected from light.

2.Prepare (on ice or at room temperature) enough reaction mix for all qPCR reactions by adding all required components, except the DNA template:

Table 1. Reaction setup.*

Component	Volume per 20 µl Reaction	Volume per 10 µl Reaction	Final Concentration
SsoAdvanced™ Universal SYBR® Green Supermix (2x)	10 µl	5 µl	1x
Forward and reverse primers	Variable	0,4 uL each	250–500 nM each
DNA template **(add at step 4)**	Variable	Variable 1 uL	cDNA: 100 ng–100 fg Genomic DNA: 50 ng–5 pg
Nuclease-free H_2O	Variable	3,2 uL	—
Total reaction mix volume	**20 µl**	**10 µl**	**—**

* Scale all components proportionally according to sample number and reaction volumes.

3.Mix the reation mix thoroughly to ensure homogeneity and dispense equal aliquots into each qPCR tube or into the wells of a qPCR plate. **Good pipetting practice must be employed to ensure assay precision and accuracy.**

4.Add cDNA samples to the qPCR tubes or wells containing reaction mix (table). Seal wells with optically transparent film and vortex 30 sec or more to ensure thorough mixing of the reaction components. Spin the tubes or plate to remove any air bubbles and collect the reaction mixture in the vessel bottom.

5. Program the thermal cycling protocol on a real-time PCR instrument:

Real-Time PCR System	Setting/Mode	Polymerase Activation and DNA Denaturation	Amplification			Melt Curve Analysis
			Denaturation at 95°C/98°C	Annealing/ Extension and Plate Read at 60°C**	Cycles	
Bio-Rad® CFX96™, CFX384™, CFX Touch™, CFX96 Touch Deep Well, CFX384 Touch™, CFX Connect™ Systems	SYBR® only	30 sec at **95°C or 98°C** for cDNA or 2–3 min at **98°C** for genomic DNA*	5–15 sec	15–30 sec	35–40	65–95°C 0.5°C increments at 2–5 sec/step (or use instrument default setting)
Bio-Rad® iQ™5, MiniOpticon™, Chromo4™, MyiQ™, MyiQ2 Systems	Standard			15–30 sec		
ABI 7500, StepOne, StepOnePlus, 7900HT, and ViiA 7	Fast			15–30 sec		
	Standard			60 sec		
Roche LightCycler 480	Fast			15–30 sec		
	Standard			60 sec		
QIAGEN Rotor-Gene and Stratagene Mx series	Fast			15–30 sec		

* 98°C is highly recommended for genomic DNA template to ensure complete denaturation.
** Shorter annealing/extension times (1–10 sec) can be used for amplicons <100 bp. Longer annealing/extension times (40–60 sec) can be used for amplicons >250 bp, GC- or AT-rich targets, crude samples, or for higher input amounts (for example 100 ng of cDNA or 50 ng of genomic DNA).

6.Load the qPCR tubes or plate into the real-time PCR instrument and start the PCR run

7.Perform data analysis according to the instrument-specific instructions

4- RT-qPCR analysis

The run of RT-qPCR will generate two key analysis Tabs: Amplification curves and Melting Curves. Analise both carefully.

Data analysis can be performed using different models. Bellow it is detailed how to calculate fold difference of your gene of interest (**GOI**) always having into account the normalization with the **housekeeping** gene.

1) Fold difference relative to the housekeeping gene:

$\Delta Ct = Ct_{GOI} - Ct_{Housekeeping}$

Fold difference to housekeeping = $2^{-\Delta Ct}$

These is the same as to calculate:

$\Delta Ct = Ct_{Housekeeping} - Ct_{GOI}$

Fold difference to housekeeping = $2^{\Delta Ct}$

2) Fold difference compared between tested condition or **sample** (for example KO or timepoint T=X) and **control** condition (for example WT or timepoint T=0)

$\Delta Ct_{sample} = Ct_{GOI} - Ct_{Housekeeping}$

$\Delta Ct_{control} = Ct_{GOI} - Ct_{Housekeeping}$

$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{control}$

Fold difference to control = $2^{-\Delta\Delta Ct}$

Example

	Arbp0					Ckm						
	1	2	mean	std		1	2	mean	std		dCT	2-dCT
289	28,28	28,39	28,34	0,074259	289	26,23	26,10	26,17	0,090486	289	-2,17	4,49
295	28,56	28,26	28,41	0,211461	295	25,44	25,73	25,58	0,206034	295	-2,83	7,09

Bibliography

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https://eu.idtdna.com/pages/education/decoded/article/interpreting-melt-curves-an-indicator-not-a-diagnosis Melting curve analysis