Protocol: RNA extraction and quantitative PCR to assay inflammatory gene expression

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Abstract

Real-time quantitative PCR (RT-qPCR) is a sensitive assay to determine the production of selected mRNA transcripts in various conditions. We required such an assay to demonstrate the effects of mitochondrial depolarization in the presence of Parkin, since we found that damaged mitochondria recruited the NF-kB effector complex molecules, NEMO and IKKb. We developed this protocol to test levels of NF-kB response genes in a cell model transiently over-expressing Parkin. With this technique we found significant upregulation of key pro-inflammatory genes normalized to a housekeeping gene, *Gapdh*.

Keywords

RNA extraction | reverse transcription | cDNA | polymerase chain reaction (PCR) | quantitative real-time PCR | gene expression

Guidelines

- When working with RNA, take caution to keep space clean to avoid sample degradation by RNases. Clear bench space and wipe with RNaseZap. Change gloves often and wear a mask.
- Use new, sterile supplies of pipet tips and tubes
- Since RNA is vulnerable to degradation, proceed through the extraction and reverse synthase procedures on the same day to avoid storing RNA samples
- Day 1, extract RNA and produce cDNA for all samples for all biological replicates. Day 2, carry out PCR reactions for all replicates.

Materials

- 1.5 mL capped tubes (Millipore Sigma, EP022364120)
- 0.2 mL 96-well PCR plates (Amplifit, 1149K06)
- RNaseZap (Sigma, R2020-250ML)

Reagents

- TRIzol (Ambion, 15596018)
- Chloroform
- Isopropanol
- Ethanol
- RNase/DNase free water (Corning, 46-000-CI)
- 10 mM dNTP mix (Invitrogen, 100004893)
- oligo (dT)20 (Life Tech Corp., 58063)
- First-Strand Buffer (Invitrogen, Y02321)
- 0.1 M DTT (Invitrogen, Y00147)
- RNaseOUT (Invitrogen, 100000840)
- SuperScript III (Invitrogen, 56575)
- 0.5 M EDTA
- 1 M NaOH

- Oligo Clean and Concentrator Kit (Zymo Research, D4060)
- Primers of interest (see Materials and Methods for the corresponding manuscript for our primer sequences)
- PowerUp SYBRGreen Master Mix (AppliedBiosystems, A25742)

Equipment

- Two user-controlled heat sources (water baths or blocks)
- Equipment to measure concentration of nucleic acids, such as NanoDrop (ThermoFisher 840274200)
- QuantStudio 3 Real-Time PCR System Machine (Applied Biosystems, A28567)

Before Start

- Set one heat source to 60 degrees
- Set one heat source to 50 degrees
- Prepare 75% ethanol with RNase/DNase free water
- The start point for this protocol is after cells grown on 6 cm dishes have been transfected with relevant constructs for 18-24 hours and treated with appropriate small molecules or vehicles. 18-24 hr before collection, transfect 1.5 ug Parkin and .2 ug EGFP-NEMO to 70-80% confluent cells on each 6 cm dish. These should yield ~1 million cells per dish
- For each replicate, one dish was treated with AntA/OligA, one dish was treated with TNFa (positive control), and one dish was treated with vehicle (control) for 5 hr.

Step-by-step

Initial RNA extraction

- Aspirate media from each dish
- Add 300 uL cold TRIzol per million cells directly onto the cells and pipet up and down to homogenize
- transfer to 1.5 mL tube
- Incubate 5 min, r.t.
- Add 200 uL chloroform per mL TRIzol
- Mix by inversion until cloudy homogenous solution
- Incubate 2-3 min at r.t.
- Centrifuge 15 min at 12 G, 4 degrees.
    - Should separate into red phenol-chloroform (bottom), an organic phase, and colorless aqueous (top)
- Transfer aqueous phase (top) containing RNA to new tube by angling at 45 degrees and carefully pipetting out. The other phases can be saved for protein or DNA isolation
- Add 500 uL isopropanol to aqueous phase per 1 mL TRIzol used
- Incubate 10 min, r.t.
- Centrifuge 10 min, 12 G at 4 degrees
    - RNA will pellet as white, gel-like
- Discard sup
- Resuspend pellet in 1 mL 75% EtOH per 1 mL Trizol used
- Vortex quickly then centrifuge 5 min 7.5 G at 4 degrees
- Discard sup
- Air dry pellet 5-10 min
    - do not totally dry it; it should start to clarify over drying
- resuspend the pellet in 50 uL RNase free water by pipetting up and down
    - it’s normal if this doesn’t go into suspension

- incubate at 60 C 10-15 min
    - Afterward, set heat bath or block to 65 C
- measure concentration of RNA with NanoDrop or other

Reverse Transcriptase Reaction to generate cDNA

- Thaw 5X first-strand buffer and 0.1 M DTT at RT immediately before use. refreeze immediately after.
- Calculate the volume of each sample needed for 5 ug
- To 5 ug RNA, add 1 uL 10 mM dNTP Mix (equal parts each base), 1 µl of oligo(dT)20 (50 µM); and sterile water to 13 uL
- Heat at 65 degrees, 5 min
    - Afterward, set heat bath or block to 70 C
- Incubate on ice 1 min
- Briefly centrifuge
- Add 4 uL First-strand buffer, 1 uL 0.1 M DTT, 1 uL RNase OUT inhibitor, 1 uL SuperScript III
- Gently pipet up and down to mix
- Incubate at 50 degrees for 45 min
    - Afterward, set heat source to 65 C
- inactivate by heating to 70 degrees for 15 min
- The result is cDNA.

Clean cDNA (EDTA/NaOH and Zymo Oligo Clean & Conc. Kit)

- Add 5 uL 0.5 M EDTA and 5 uL 1 M NaOH to each, mix by inversion
- Heat at 65 C 15 min
- Adjust volumes to 50 uL with water
- Add 100 uL Oligo Binding Buffer to each 50 uL
- Add 400 uL ethanol and mix briefly by pipetting. Transfer to Zymo-Spin Column in the kit
- Centrifuge 10 G, 30 sec, R.T. and discard the flow through
- Add 750 uL DNA Wash Buffer to the column
- Centrifuge 10 G, 30 sec, R.T. and discard the flow through
- Centrifuge max speed, 1 min, R.T.
- Transfer the column to a new clean tube and add 15 uL water to the matrix
- Centrifuge at 10 G, 30 sec, R.T. to elute
- Measure 260/280 for final conc. The product can be saved at -20.

Set up PCR Reactions

Sample	SYBR Master Mix	Fwd and Rev Primers (10 uM stock to 300 nM final)	cDNA (1:100 dilutions)	Nuclease free water (to 44 uL)
For one reaction (total 11 uL)	5.5 uL	0.33 uL	11 ng (this is the maximum mass)	varying

- We use the following worksheet to plan volumes needed for each reaction. **The following is our example**.

Number of different primer sets = _____8____(p)

Number of replicates per primer set = ___3_____(n).

___8___(p) * __3_____(n) = ___24____(T) = number of reactions per cDNA sample.

__24____(T) * 11 uL = ____264_____(V) = volume for each set of cDNA

Replicate	**Sample**	SYBR Master Mix (V / 2)	cDNA (11 * T ug)	Nuclease free water V – (0.33*n) – (V/2) – cDNA volume	Fwd and Rev Primers (10 uM stock to 300 nM final) (0.33 uL * n) *add later*
N1	**No template control**	132	-	130	1 of each
	veh	"	5.2	124.8	"
	TNF	"	3.5	126.5	"
	AO	"	4.5	125.5	"
N2	**No template control**	"	-	130	"
	veh	"	4.08	125.9	"
	TNF	"	2.1	127.9	"
	AO	"	2.07	127.9	"
N3	**No template control**	"	-	130	"
	veh	"	3.32	126.7	"
	TNF	"	4.88	125.1	"
	AO	"	2.18	127.8	"

- Mix these then centrifuge quickly
- Split into __8____(p) tubes > (____3____(n) * 10 uL = ____30_____($P_{initial}$)) in each tube
- add 0.33 uL * n = ___1_____ uL each primer (10 uM) respectively to get total

  ____32_____(~P_{final} uL)/tube
- Mix again, centrifuge, and add 10 uL each reaction to wells
- Seal the plate with an adhesive cover then centrifuge to get rid of air bubbles and ensure components are combined.
- Can store this at R.T. 24 hr.
- Run the reaction in the QuantStudio with the following procedure

Step	*Temp (C)*	*Duration*	*Cycles*
Cycling Mode			
UDG activation	50	2 min	-
Dual Lock DNA polymerase	95	2 min	-
Denature	95	15 sec	40
Anneal	*56**	15 sec	
Extend	72	1 min	
Dissociation curve			
1	1.6C/sec to 95	15 sec	-
2	1.6C/sec to 60	1 min	-
3	0.15C/sec to 95	15 sec	-

* is variable annealing temp, chosen taking into account the melt curve of all primers

- export all data as an .xls file.
- Analyze with ΔΔ method