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RNA extraction and quantitative PCR to assay inflammatory gene expression

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Protocol status: Working

We use this protocol and it's working

Created: June 23, 2022

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Protocol Integer ID: 65140

Keywords: RNA extraction, Reverse transcription, cDNA, Polymerase chain reaction (PCR), Quantitative realtime PCR, Gene expression, ASAPCRN, inflammatory gene expression, effects of mitochondrial depolarization, mitochondrial depolarization, kb response gene, damaged mitochondria, kb effector complex molecule, rna extraction, mrna transcripts in various condition, presence of parkin, selected mrna transcript, quantitative pcr, housekeeping gene, rna, expressing parkin

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Grant ID: Mechanisms of mitochondrial damage control by PINK1 and Parkin (ASAP-000350)

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 proinflammatory genes normalized to a housekeeping gene, Gapdh.

Attachments



470-984.pdf

226KB

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

Materials:

- 2 1.5 mL capped tubes Merck MilliporeSigma (Sigma-Aldrich) Catalog #EP022364120
- **∅** 0.2 mL 96-well PCR plates **Thomas Scientific Catalog #**1149K06
- RNaseZAP™ Merck MilliporeSigma (Sigma-Aldrich) Catalog #R2020-250ML

Reagents:

- TRIzol™ Reagent Thermo Fisher Catalog #15596018
- Chloroform
- Isopropanol
- Ethanol
- Corning® 100 mL Molecular Biology Grade Water Tested to USP Sterile Purified Water Specifications Corning Catalog #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 Catalog** #D4060
- Primers of interest (see Materials and Methods for the corresponding manuscript for our primer
- sequences)
- PowerUp™ SYBR™ Green Master Mix Thermo Fisher Catalog #A25742

Equipment:

Two user-controlled heat sources (water baths or blocks)



Equipment

Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer NAME

TYPE Spectrophotometer

BRAND Thermo Scientific™

SKU 840274200

https://www.fishersci.com/shop/products/nanodrop-onec-spectrophotometer/13400519

Equipment

NAME QuantStudio 3 Real-Time PCR System

TYPE Real-Time PCR

BRAND Applied Biosystem

SKU A28567

SPECIFICATIONS 4 excitation filters (450-600 nm)

4 emission filters (500-640 nm)



Troubleshooting

LINK



Before start

- Set one heat source to \$\ 60 °C \.
- Set one heat source to \$\ 50 °C \.
- 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 18:00:00 - 24:00:00 and treated with appropriate small molecules or vehicles. ♦ 18:00:00 - ♦ 24:00:00 before collection, transfect \bot 1.5 µg Parkin and \bot 0.2 µg 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 6005:00:00 .



Initial RNA extraction

1 Aspirate media from each dish.

- 2 Add 🗸 300 uL cold TRIzol per million cells directly onto the cells and pipet up and down to homogenize.
- 3 Transfer to 1.5 mL tube.
- 4 Incubate 00:05:00 , Room temperature .
- 5 Add 🚨 200 µL chloroform per mL TRIzol.
- 6 Mix by inversion until cloudy homogenous solution.
- 7 Incubate 000:02:00 - 000:03:00 at 8 Room temperature .
- 8 Centrifuge 6 00:15:00 at 8 12 x g , 4 °C .

Note

Should separate into red phenol-chloroform (bottom), an organic phase, and colorless aqueous (top).

- 9 Transfer aqueous phase (top) containing RNA to new tube by angling at 45 °C and carefully pipetting out. The other phases can be saved for protein or DNA isolation.
- 10 Add \perp 500 μ L isopropanol to aqueous phase per \perp 1 mL TRizol used.

5m

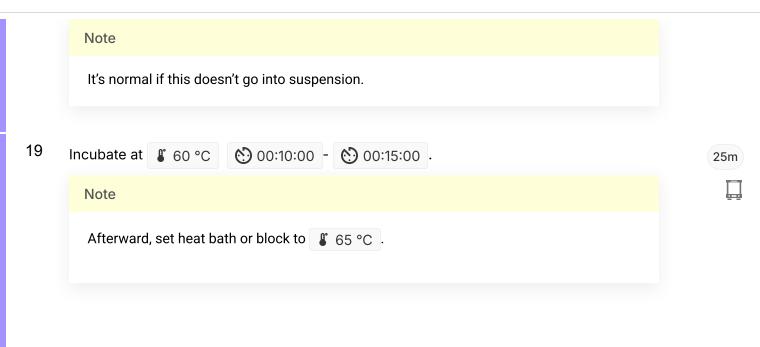
5m

15m



11 Incubate 👏 00:10:00 , 🖁 Room temperature . 10m 12 Centrifuge \bigcirc 00:10:00 , \bigcirc 12 x g at \bigcirc 4 °C . 10m Note RNA will pellet as white, gel-like. 13 Discard supernatant. 14 Resuspend pellet in 4 1 mL 75% EtOH per 4 1 mL Trizol used. 15 Vortex quickly then centrifuge \bigcirc 00:05:00 \bigcirc 7.5 x g at \bigcirc 4 °C . 5m 16 Discard supernatant. 17 Air dry pellet 00:05:00 - 00:10:00 . 15m Note Do not totally dry it; it should start to clarify over drying. 18 Resuspend the pellet in \perp 50 μ L RNase free water by pipetting up and down.





20 Measure concentration of RNA with NanoDrop or other.

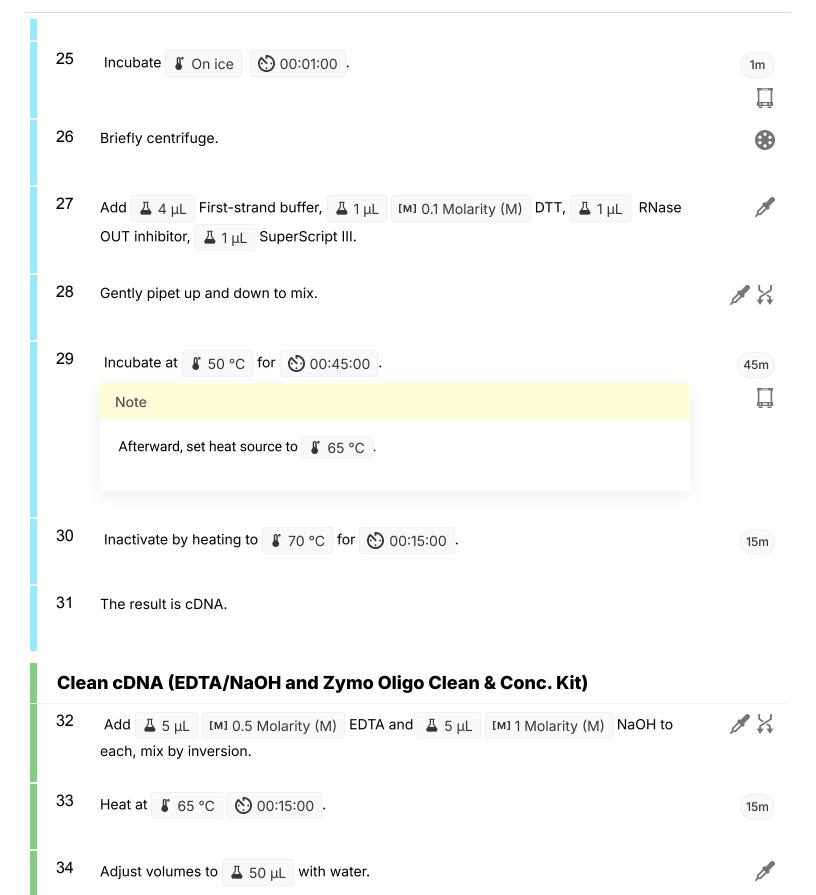
Reverse Transcriptase Reaction to generate cDNA

- 21 Thaw 5X first-strand buffer and [M] 0.1 Molarity (M) DTT at Room temperature immediately before use. Refreeze immediately after.
- 22 Calculate the volume of each sample needed for $\perp 45 \mu g$.
- 23 To $\Delta 5 \mu q$ RNA, add $\Delta 1 \mu L$ [M] 10 millimolar (mM) dNTP Mix (equal parts each base), $\perp \!\!\! \perp 1 \, \mu L$ of oligo(dT)20 ([M] 50 micromolar (μM)); and sterile water to Δ 13 μL .
- 24 Heat at \$\mathbb{8} 65 \cdot \mathbb{C} \, \bigotimes 00:05:00 \,. 5m

Afterward, set heat bath or block to \$\\ \ \ 70 \circ\$.

Note







35	Add Δ 100 μ L Oligo Binding Buffer to each Δ 50 μ L .	8
36	Add $\ \ \ \ \ \ \ \ \ \ \ \ \ $	8%
37	Centrifuge \bigcirc 10 x g , \bigcirc 00:00:30 , \bigcirc Room temperature and discard the flow through.	30s
38	Add Δ 750 μL DNA Wash Buffer to the column.	8
39	Centrifuge 10 x g , 00:00:30 , Room temperature and discard the flow through.	30s
40	Centrifuge max speed, 00:01:00 , Room temperature	1m
41	Transfer the column to a new clean tube and add $\begin{tabular}{l} \bot \ 15 \ \mu L \end{tabular}$ water to the matrix.	de

42 Centrifuge at \$ 10 x g , \$ 00:00:30 , \$ Room temperature to elute. 30s

43

Set up PCR Reactions

1d

44

А	В	С	D	Е
Sample SYBR	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	5.5 uL	0.33 uL	11 ng (this is themaximum	varying



A	В	С	D	Е
11 uL)			mass)	

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) * \bot 11 μ L = ___264_____(V) = volume for each set of cDNA.





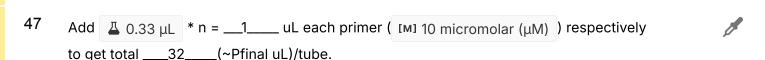
А	В	С	D	E	F
Replic ate	Sample	SYBR Master Mix (V / 2)	cDNA (11 * T ug)	Nuclease free water V – (0.33*n) – (V/2) – cDNA volume	Fwd and Rev Primers (10 uM stock to 300 nM final) (0.33 uL * n) add later
N1	No template control	132	-	130	1 of each
	veh	132	5.2	124.8	1 of each
	TNF	132	3.5	126.5	1 of each
	AO	132	4.5	125.5	1 of each
N2	No template control	132	-	130	1 of each
	veh	132	4.08	125.9	1 of each
	TNF	132	2.1	127.9	1 of each
	AO	132	2.07	127.9	1 of each
N3	No template control	132	-	130	1 of each
	veh	132	3.22	126.7	1 of each
	TNF	132	4.88	125.1	1 of each



А	В	С	D	Е	F
	AO	132	2.18	127.8	1 of each

Mix these then centrifuge quickly.

46 Split into _8__(p) tubes > (___3__(n) * \underline{A} 10 μ L = ___30___(Pinitial)) in each tube.



48 Mix again, centrifuge, and add \perp 10 μ L each reaction to wells.



49 Seal the plate with an adhesive cover then centrifuge to get rid of air bubbles and ensure components are combined.



50 Can store this at Room temperature 24:00:00.

1d

51 Run the reaction in the QuantStudio with the following procedure.



А	В	С	D
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	-



А	В	С	D
2	1.6C/sec to 60	1 min	-
3	0.15C/sec to 95	15 sec	-

Note

- * 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.