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RNA extraction and quantitative PCR to assay inflammatory gene expression

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OLIVIA HARDING[1,2], Erika L.F. Holzbaur[1,2]

[1]Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; [2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815

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OLIVIA HARDING
Children's Hospital of Philadelphia

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We use this protocol and it's working

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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.

Attachments

PDF

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:

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

Spectrophotometer
TYPE

Thermo Scientific™
BRAND

840274200
SKU

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

Equipment

QuantStudio 3 Real-Time PCR System
NAME

Real-Time PCR
TYPE

Applied Biosystem
BRAND

A28567
SKU

4 excitation filters (450–600 nm)
4 emission filters (500–640 nm)
SPECIFICATIONS

PNG

Troubleshooting

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 1.5 µg Parkin and 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 05:00:00 .

Initial RNA extraction

1 Aspirate media from each dish.

2 Add 300 µL 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 . 5m

5 Add 200 µL chloroform per mL TRIzol.

6 Mix by inversion until cloudy homogenous solution.

7 Incubate 00:02:00 - 00:03:00 at Room temperature . 5m

8 Centrifuge 00:15:00 at 12 x g , 4 °C . 15m

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 500 µL isopropanol to aqueous phase per 1 mL TRIzol used.

11 Incubate 00:10:00 , Room temperature . 10m

12 Centrifuge 00:10:00 , 12 x g at 4 °C . 10m

Note

RNA will pellet as white, gel-like.

13 Discard supernatant.

14 Resuspend pellet in 1 mL 75% EtOH per 1 mL Trizol used.

15 Vortex quickly then centrifuge 00:05:00 7.5 x g at 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 50 µL RNase free water by pipetting up and down.

Note

It's normal if this doesn't go into suspension.

19 Incubate at 60 °C 00:10:00 - 00:15:00 . 25m

Note

Afterward, set heat bath or block to 65 °C .

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 5 µg .

23 To 5 µg RNA, add 1 µL [M] 10 millimolar (mM) dNTP Mix (equal parts each base), 1 µL of oligo(dT)20 ([M] 50 micromolar (µM)); and sterile water to 13 µL .

24 Heat at 65 °C , 00:05:00 . 5m

Note

Afterward, set heat bath or block to 70 °C .

25 Incubate On ice 00:01:00 . 1m

26 Briefly centrifuge.

27 Add 4 µL First-strand buffer, 1 µL [M] 0.1 Molarity (M) DTT, 1 µL RNase OUT inhibitor, 1 µL SuperScript III.

28 Gently pipet up and down to mix.

29 Incubate at 50 °C for 00:45:00 . 45m

Note

Afterward, set heat source to 65 °C .

30 Inactivate by heating to 70 °C for 00:15:00 . 15m

31 The result is cDNA.

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

32 Add 5 µL [M] 0.5 Molarity (M) EDTA and 5 µL [M] 1 Molarity (M) NaOH to each, mix by inversion.

33 Heat at 65 °C 00:15:00 . 15m

34 Adjust volumes to 50 µL with water.

35 Add 100 µL Oligo Binding Buffer to each 50 µL .

36 Add 400 µL ethanol and mix briefly by pipetting. Transfer to Zymo-Spin Column in the kit.

37 Centrifuge 10 x g , 00:00:30 , Room temperature and discard the flow through. 30s

38 Add 750 µL DNA Wash Buffer to the column.

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 15 µL water to the matrix.

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

43 Measure 260/280 for final conc. The product can be saved at -20 °C .

Set up PCR Reactions 1d

44

	A	B	C	D	E
	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	B	C	D	E
	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) * 11 µL = ____264_____(V) = volume for each set of cDNA.

45

	A	B	C	D	E	F
	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	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

	A	B	C	D	E	F
		AO	132	2.18	127.8	1 of each

Mix these then centrifuge quickly.

46 Split into __8____(p) tubes > (____3____(n) * 10 µL = ____30_____(Pinitial)) in each tube.

47 Add 0.33 µL * n = ___1_____ uL each primer ([M] 10 micromolar (µM)) respectively to get total ____32_____(~Pfinal uL)/tube.

48 Mix again, centrifuge, and add 10 µL 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.

	A	B	C	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	-

	A	B	C	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.