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Protocol status: Working
 We use this protocol and it's working

Created: Jun 23, 2022

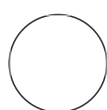
RNA extraction and quantitative PCR to assay inflammatory gene expression

OLIVIA HARDING^{1,2}, Erika L.F. Holzbaur^{1,2}

¹Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104;

²Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815

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OLIVIA HARDING

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-κB effector complex molecules, NEMO and IKKb. We developed this protocol to test levels of NF-κB 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

[470-984.pdf](#)

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:

Last Modified: Jul 31, 2023

PROTOCOL integer ID:
65140





Keywords: RNA extraction,
Reverse transcription, cDNA,
Polymerase chain reaction
(PCR), Quantitative realtime
PCR, Gene expression,
ASAPCRN

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
	

BEFORE START INSTRUCTIONS


- 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 $^{\circ}\text{C}$  00:10:00 -  00:15:00 .

25m






Note

Afterward, set heat bath or block to  65 $^{\circ}\text{C}$.







20 Measure concentration of RNA with NanoDrop or other.

Reverse Transcriptase Reaction to generate cDNA



21 Thaw 5X first-strand buffer and  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  10 millimolar (mM) dNTP Mix (equal parts each base),  1 μL of oligo(dT)20 ( 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  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  0.5 Molarity (M) EDTA and  5 µL  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 , , and discard the flow through.

30s

38



Add DNA Wash Buffer to the column.

39



Centrifuge , , and discard the flow through.

30s

40



Centrifuge max speed, , .

1m

41



Transfer the column to a new clean tube and add water to the matrix.

42



Centrifuge at , , to elute.

30s

43

Measure 260/280 for final conc. The product can be saved at .

Set up PCR Reactions

1d

44

A	B	C	D	E

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 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.


45




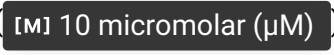
A	B	C	D	E	F
Replicate	Sample	SYBR Master Mix (V / 2)	cDNA (11 * T ug)	Nuclease free water V - (0.33*n) - (V/2) - cDNA volume	Fwd and Rev Primers (10 uM stock to 300 nM final) (0.33 uL * n) add later
N1	No template control	132	-	130	1 of each
	veh	132	5.2	124.8	1 of each
	TNF	132	3.5	126.5	1 of each
	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
	A0	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 ( 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			

A	B	C	D
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	-

Note

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

- Export all data as an .xls file.
- Analyze with $\Delta\Delta$ method.