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Title: High-Throughput Quantitative RT-PCR in Single and Bulk *C. Elegans* Samples Using Nanofluidic Technology

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y, Olympus SZX10**

2. Software: Does the part of your protocol being filmed demonstrate software usage?

N

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Script Length
Number of steps: **50**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Francesca Hodge**: Our protocol improves both the throughput and time efficiency for obtaining RT-qPCR data directly from single worm or small bulk samples without the need for RNA isolation **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Laetitia Chauve**: Using this protocol, over 9000 RT-qPCR results can be obtained in just two days of bench work, a process that would typically take approximately 5 weeks using standard 96-well qPCR **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Francesca Hodge**: This protocol provides a fast, robust, and highly sensitive RT-qPCR assay in *C. elegans* and is ideal for monitoring inter-individual variability in gene expression in isogenic worms **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Worm-to-CT Worm Lysis

2.1. Begin by picking the worms from their bacterial lawn onto a fresh, unseeded nematode growth medium plate **[1]** and allowing the worms to move around the plate for 5 minutes **[2]**.

2.1.1. WIDE: Talent picking/transferring worms

2.1.2. Worms moving on plate Author NOTE: This was taken as a SCOPE SHOT with the camera of our Nikon macrozoom dissection microscope. This should have been uploaded by SHIPHI as 2.1.2

2.2. While the worms are acclimating, in an RNase-free (R-N-ayse free) hood, mix 10 microliters of lysis buffer with 1:100 DNase (D-N-ayse) one per sample **[1-TXT]** and place the lid of a PCR strip upside down on the platform of a dissecting scope **[2]**.

2.2.1. Talent mixing buffer and DNase I, with buffer and DNase I containers visible in frame **TEXT: See text for all medium and solution preparation details**

~~2.2.2. shot removed~~

2.3. Next, add 10 microliters of the lysis mix to domed PCR tube caps **[1]** and "scoop" the worms from the plate to avoid transferring bacteria **[2-TXT]** into each slot of the lid **[3]**.

2.3.1. Talent adding mix to cap(s)

2.3.2. Worm(s) being scooped *Videographer: Important step* **TEXT: Bulk experiments: 15-30 worms/cap; Single-worm measurements: 1 worm/cap** Author NOTE: This was taken as a SCOPE SHOT with the camera of our Nikon macrozoom dissection microscope. This should have been uploaded by SHIPHI as 2.3.2

2.3.3. SCOPE: Worm(s) being added to lid(s) *Videographer: Important step* Author NOTE: This was taken as a SCOPE SHOT with the camera of our Nikon macrozoom dissection microscope. This should have been uploaded by SHIPHI as 2.3.3 and 2.3.3.2. Between those two takes, the best is 2.3.3.2, where the worm is still moving after being added to the cap

2.4. When all of the worms have been transferred, close the caps **[1]** and spin the tubes for 5 seconds **[2]** before placing the tubes in a Dewar flask of liquid nitrogen **[3]**.

2.4.1. Talent closing cap(s)
2.4.2. Talent placing tube(s) into centrifuge
2.4.3. Talent placing tube(s) into flask

2.5. After a least 5 seconds, thaw the tubes in a 40-degree Celsius water bath **[1]** before repeating the freeze-thaw procedure 9 more times **[2]**.

2.5.1. Talent placing tube(s) into water bath *Videographer: Important step*
2.5.2. NOTE: shot combined with 2.5.1

2.6. After the last freeze-thaw cycle, mix the lysed samples on a thermal mixer for 20-30 minutes at 4 degrees Celsius and approximately 1,800 revolutions per minute **[1]**.

2.6.1. Talent loading samples onto mixer/samples being mixed

2.7. At the end of the mixing incubation, spin down the samples as demonstrated **[1]** and add 1 microliter of freshly thawed stop solution to each tube **[2-TXT]**.

2.7.1. Talent placing tube(s) into centrifuge
2.7.2. Talent adding stop solution to tube(s), with solution container visible in frame **TEXT: Optional: Store samples at -80 °C ≤1 wk**

3. Reverse Transcription (RT) for Bulk Samples

3.1. For the reverse transcription of bulk worm samples, in an RNase-free hood, prepare an enzyme buffer master mix **[1-TXT]** and add 14 microliters of master mix to 11 microliters of each sample **[2]**.

3.1.1. WIDE: Talent adding buffer to tube, with buffer and enzyme buffer containers visible in frame **TEXT: See text for single-worm RT details**
3.1.2. Talent adding master mix to sample

3.2. Run the samples through a thermocycler using the indicated reverse transcription program **[1-TXT]** and dilute the resulting cDNA product at a 1:4 ratio in nuclease-free water **[2-TXT]**.

3.2.1. Talent loading sample onto thermocycler **TEXT: 37 °C for 60 min, 95 °C for 5 min, and 4 °C for infinity**
3.2.2. Talent adding water to tube(s) **TEXT: Do not dilute for single worms**

4. Target Specific Preamplification

4.1. For target specific preamplification, add 3.75 microliters of preamplification master mix

into one well per sample in a 96-well plate **[1-TXT]** and add 1.25 microliters of each cDNA solution to each well of master mix **[2]**.

4.1.1. WIDE: Talent adding master mix to well(s), with master mix container visible in frame **TEXT: See text for master mix primer preparation details**
4.1.2. Talent adding cDNA to well(s), with cDNA container(s) visible in frame

4.2. When all of the samples have been loaded, cover the plate with sealing tape **[1]** and briefly vortex the samples **[2]** before spinning them down by centrifugation **[3]**.

4.2.1. Talent sealing plate
4.2.2. Talent vortexing plate
4.2.3. Talent placing plate into centrifuge

4.3. Then load the plate onto a thermocycler **[1]** and run the program as indicated **[2-TXT]**.

4.3.1. Talent loading plate
4.3.2. NOTE: Shot combined with 4.3.1 **TEXT: 95 °C for 2 min, 10 or 15 denaturation cycles at 95 °C for 15 s, annealing/extension at 60 °C for 4 min, and 4 °C for infinity**

5. Exonuclease I Treatment

5.1. To remove unincorporated primers from the preamplification, at the end of the thermocycle, centrifuge the sample plate **[1]** and carefully remove the seal **[2]**.

5.1.1. WIDE: Talent placing plate into centrifuge
5.1.2. Talent removing seal

5.2. Add 2 microliters of exonuclease one solution to each preamplification reaction **[1]** and reseal, centrifuge, and load the plate back into the thermocycler **[2-TXT]**.

5.2.1. Talent adding mix to well(s), with mix container visible in frame
5.2.2. Talent loading plate onto thermocycler **TEXT: 37 °C for 30 min, 80 °C for 15 min, and 4 °C for infinity**

5.3. At the end of the cycle, dilute the samples with 18 microliters of Tris EDTA (triss E-D-T-A) buffer **[1]**.

5.3.1. Talent adding Tris EDTA to well(s), with Tris EDTA container visible in frame

6. Sample Mix Preparation

6.1. To set up a multi-array chip, add 3.6 microliters of assay loading master mix **[1]** and 0.4 microliters of 50-micromolar forward-reverse primer into one well per sample in a 384-well plate **[2]**.

6.1.1. WIDE: Talent loading master mix to well(s), with master mix container visible in frame
6.1.2. Talent loading primer into well(s), with primer container visible in frame

6.2. Then add 2.2 microliters of sample master mix **[1]** and 1.8 microliters of each preamplified, exonuclease one-treated sample to the appropriate wells of the plate **[2]**.

6.2.1. Talent adding master mix, with master mix container visible in frame
6.2.2. Talent adding sample to well(s), with sample container visible in frame

6.3. To set up a single-array chip, add 5.4 microliters of the assay loading master mix **[1]** and 0.6 microliters of the forward-reverse primer to one well per sample of a second 384-well plate **[2]**.

6.3.1. Talent loading master mix to well(s), with master mix container visible in frame
6.3.2. Talent loading primer into well(s), with primer container visible in frame

6.4. Then add 3.3 microliters of sample master mix **[1]** and 2.7 microliters of each preamplified, exonuclease one-treated sample the appropriate wells of the prepared single-array plate **[2]**.

6.4.1. Talent adding master mix, with master mix container visible in frame
6.4.2. Talent adding sample to well(s), with sample container visible in frame

7. Nanofluidic Chip Priming and Loading

7.1. To prime the nanofluidic chip for the first run, holding the chip at a 45-degree angle **[1]**, slowly and carefully inject 150 microliters of control line fluid into the accumulators of the chip **[2]**.

7.1.1. WIDE: Talent holding chip at 45° angle *Videographer: Important step*
7.1.2. Talent injecting fluid, with fluid container visible in frame *Videographer: Important step*

7.2. When all of the fluid has been loaded, remove the blue protective film from the bottom of the chip **[1]** and place the chip into the nanofluidics PCR priming machine with the barcode facing outwards **[2]**.

7.2.1. Talent removing film

7.2.2. Talent placing chip into machine

7.3. Then run the **Prime 153x** script **[1]**.

7.3.1. Talent running script

7.4. After priming, place the chip onto a dark surface **[1]** and, as each assay or sample mix is loaded, remove the corresponding barrier plugs if necessary **[2]** and add the appropriate volume of assay or sample mix to the corresponding inlets **[3-TXT]** of the nanofluidic chip according to the experimental plan **[4]**.

7.4.1. Talent placing chip onto dark surface *Videographer: Important step*
7.4.2. NOTE: Shot combined with 7.4.1*Videographer: Important step*
7.4.3. Talent loading assay or sample mix, with assay and sample mix containers visible in frame *Videographer: Important step* **TEXT: 3 microliters for multi-array chip; 5 microliters for single-array chip**
7.4.4. Added shot: CU of loading chip action in 7.4.3

7.5. After loading, place the chip into the nanofluidic thermocycler **[1]** and run the appropriate protocol in the data collection software **[2-TXT]**.

7.5.1. Talent loading chip into thermocycler NOTE: Authors added shots but no VO, please put together in a way that looks good and fits current VO

Added SHOT: placing chip in nanofluidics PCR priming machine for loading run. This shot is labelled as 7.4.5 in the video log

Added SHOT: CU of placing Chip in thermocycler. Shot labelled as 7.5.1B in video log

Added SHOT: CU running loading script. This shot is labelled as 7.4.6 in the video log.

7.5.2. Talent running protocol, with monitor visible in frame **TEXT: See text for protocol suggestion details**

7.6. If the whole multi-array chip is not used, perform a postscript run to allow downstream use of remaining chip arrays **[1]**.

7.6.1. reuse 7.4.5
7.6.2. Added shot: CU of running post script program on nanofluidics PCR priming machine

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.3., 2.5., 7.1., 7.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?

4.1., 5.2., 5.3., 6.1., 6.2., 6.3., 6.4., 7.4. – loading so many wells can be difficult in keeping track of which wells have been used as sometimes the liquid is difficult to see, use the pipette tips in the racks sequentially in the same pattern as the wells which works as a bookmark.

Results

8. Results: Representative Worm-to-CT Analysis

8.1. To test if the Worm-to-CT protocol is a valid cDNA extraction method **[1]**, the method **[2]** was compared to standard guanidium thiocyanate-phenol-chloroform extraction methods **[3]**.

8.1.1. LAB MEDIA: Figure 3
8.1.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize worm-to-Ct side of graph*
8.1.3. LAB MEDIA: Figure 3 *Video Editor: please emphasize phenol-chloroform side of graph*

8.2. Globally, hsp-70 (H-S-P-seventy) mRNA expression levels per 100 nanograms of total RNA were comparable using both the phenol-chloroform and worm-to-CT methods **[1]**.

8.2.1. LAB MEDIA: Figure 3

8.3. However, in the cases of highest hsp-70 expression **[1]**, the expression was higher with the Worm-to-CT method, indicating an improved sensitivity **[2]**.

8.3.1. LAB MEDIA: Figure 3 *Video Editor: please emphasize N2 + data bars*
8.3.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize worm-to-Ct data bar and/or bracket between N2 + data bars*

8.4. For guanidium thiocyanate-phenol-chloroform extraction of bulk samples **[1]**, hsp-70 decreased by 82.7% in *hsf-1* (H-S-F-one) mutants compared to controls **[2]** and by 92.3% for Worm-to-CT, indicating that the decrease was comparable between the two methods **[3]**.

8.4.1. LAB MEDIA: Figure 3
8.4.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize phenol-chloroform hsf-1 + data bar*
8.4.3. LAB MEDIA: Figure 3 *Video Editor: please emphasize worm-to-Ct hsf-1 + data bar*

8.5. Using cDNA obtained from either bulk samples of 25 worms **[1]** or from an average of 36 single worm samples **[2]**, the methods detected comparable expression levels for all of the chaperones tested **[3]**, indicating that the parameters obtained from single worms are reliable **[4]**.

8.5.1. LAB MEDIA: Figure 5 *Video Editor: please emphasize pools/bulk data bars*
8.5.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize av. single worms data bars*
8.5.3. LAB MEDIA: Figure 5 *Video Editor: please add/emphasize brackets over each pair of data bars*
8.5.4. LAB MEDIA: Figure 5

8.6. Here the mean expression of multiple *hsp* transcripts from single worms following a short heat shock are shown **[1]**. As observed, the variability in the expression of the transcripts differed dramatically across different genes **[2]**.

8.6.1. LAB MEDIA: Figure 6A
8.6.2. LAB MEDIA: Figure 6A *Video Editor: please sequentially emphasize gene sections of graph*

8.7. For every transcript tested using single-worm samples, the technical coefficients of variability were lower **[1]** than the biological coefficients of variability **[2]**, indicating that technical triplicates was not required for parameter estimation when qPCR was performed on single worms **[3]**.

8.7.1. LAB MEDIA: Figure 7 *Video Editor: please emphasize green data points*
8.7.2. LAB MEDIA: Figure 7 *Video Editor: please red data points*
8.7.3. LAB MEDIA: Figure 7

Conclusion

9. Conclusion Interview Statements

9.1. **Laetitia Chauve**: When performing this procedure, it is essential to pipette small volumes accurately and to not pipette air bubbles into the nanofluidic chip **[1]**.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (7.4.)

9.2. **Francesca Hodge**: This protocol can be used to obtain large sets of qRT-PCR data, which can then be exported and processed as desired using excel or R scripts **[1]**.

9.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*