Babraham Institute

21st February, 2020

Dear Dr. Cao:

We are submitting a revised version of the **JoVE61132** manuscript entitled "*A protocol for high-throughput quantitative RT-qPCR in single C. elegans using nanofluidic technology*"

We want to thank the editorial team and the reviewers for bringing up important issues related to both the manuscript and the pipeline. We have addressed all comments, highlighted changes in the manuscript and added responses to each point in this letter.

Review Editor

Editorial comments:
Changes to be made by the author(s):

- We have proofread the manuscript to ensure that there are no spelling or grammar issues.
- We have added email addresses for each author in the manuscript to lines 21-23.
- We have eliminated commercial language. This includes trademark symbols (™), registered symbols (®) throughout the manuscript, and company names before an instrument or reagent and referenced all commercial items in the Table of Materials.
    - The one exception we have made to this is in reference to the user guide referenced on line 262
- We have edited out personal pronouns (e.g., I, you, your, we, our) or colloquial phrases throughout the protocol.
- We have referred to actions in the imperative tense in complete sentences wherever possible.
- At the beginning of this protocol, on lines 120- 122, it is now specified that "Throughout this protocol, every time the word "worm" is used, this is in reference to the nematode *Caenorhabditis elegans."*

- The numbering throughout the protocol has been corrected.
- We have highlighted in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video.
- We have referenced all figures and tables in the manuscript in order of appearance.
- We have used neutral tone when discussing commercial products. And have altered the referencing to give full journal names
- We have changed the time unit "sec" to "s" and abbreviated liters to L (L, mL, µL) **Figure 1** and **Figure 2**. We have also included a space between all numbers and the corresponding unit: 0.2 µL, 37 °C, etc.
- We have defined error bars as standard error of the mean in the legend of **Figure 3**.
- We removed any ™/®/© symbols from the Table of Materials and sorted materials alphabetically.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Authors provide a detailed protocol of a high throughput Rt-qPCR method which allows for obtaining cDNA from individual worms, as well as from bulk samples. The method relies on reliable existing technology, expanding protocols applied to cell cultures, and it could be of interest to other C. elegans researchers. Authors support the suggested protocol by original data, proving the method's applicability and efficiency.

- We have added clarity to the fact that our protocol relies on existing technologies and is in fact a novel adaptation of existing methods, this has been addressed, for example, in line 42 of the abstract.

Major Concerns:
General
C. elegans needs to be italicized everywhere. Manuscripts needs language editing for typos, syntax errors, punctuation and similar issues. Moreover, especially in the Introduction, several periods are too long for no reason and without making sense. Please break them down into smaller periods with connecting sentences when necessary. Representative example: lines 88-92. A list of Materials needed would be much appreciated before or at the beginning of the Protocol.

We thank the reviewer for pointing out these issues, we have corrected everything, except that Jove requires the list of materials to be provided as a separate document.

Lines 538-539: It is a strong statement to say that technical replicates are not necessary. What authors' results reveal by comparing biological to technical replicates CV is that the method is very reliable and robust, which is great. Authors can highlight this advantage. But to show this, technical replicates were needed. And are needed in every run. Consider for example if something in future applications goes wrong (because of system failure, temperature control not working, etc), how is the user going to know without technical replicates? Technical replicates cannot be

replaced by biological replicates, because these two serve different purposes. I suggest authors remove this comment.

We thank the reviewer for bringing up this point. We agree with the reviewer that for bulk-PCR applications, technical replicates are of paramount importance to ensure reproducibility and we have added a sentence in line 527-8 to highlight this issue. However, when stepping into single-cell biology, technical replicates only add up cost to the experiments without adding value to parameter inference such as coefficient of variation and mean expression. This has been highlighted by many studies that are based on nanofluidics PCR platforms. For example, Ståhlberg *et* al, 2014 writes: "qPCR replicates are generally not performed, since they add to the cost of the experiment and do not really improve precision, since the reproducibility of qPCR generally is very high. Rather, qPCR replicates may compromise precision if the pre-amplified cDNA has to be divided into a larger number of aliquots, since this will increase sampling ambiguity. If there is space on the qPCR platform, then it is better to analyze more cells than running technical replicates". The same issue is brought up by and Livak *et* al, 2013.

We have highlighted this point in **Figure 7**, where we have used 3 technical replicates to estimate technical versus biological variance and clearly demonstrated that technical variance (blue points: variation between technical replicates) is smaller relative to biological variance (red points: variation between worms). We have performed additional experiments, which will be published elsewhere (Chauve *et* al, in preparation), where we used 30 samples each containing a total of three technical replicates were used to estimate statistical parameters such as mean expression and biological variance. The results of these experiments indicated that using technical replicates does not change parameter estimation. The number of single-worms that are required per experiment may vary depending on the biological variability of the sample, but we have estimated that 50 individual worms (biological replicates) are sufficient to provide robust parameter estimations, comparable to 90 individual samples. These results will be published elsewhere.

Minor_Concerns:
Abstract
I am not sure I would call the method novel, as the main steps are the ones suggested by the kit manufacturers and BIOMARK system guidelines. Maybe it would be more appropriate to call it a novel application or a novel expansion of an existing methodology, or something along these lines.Word "pipeline" appears a little too often; maybe the authors can find an alternative term.

Keywords
Are the authors sure that keyword "pooled worms" is useful, especially for a methods paper that on its title has "in single C. elegans"?

- The title (line: 2) has been adjusted to be inclusive of both single-worm and bulk worm samples.

Introduction
Line 61: cells in hermaphrodite C. elegans are 935, it is a number usually reported.

- The number of cells has been adjusted in line: 68

Lines 62-63: Reconsider this phrase or consider rephrasing it. It is not the hermaphroditism alone, but it is the latter in combination with the preferred self-fertilization that leads to isogenic populations. This is prominent in laboratory-maintained populations. (The term passage is more common for cell cultures, in my knowledge).

- The sentence regarding the cause of isogenicity has been adjusted to improve clarity on lines: 69-71

Lines 74-75: please rephrase, the message is unclear.

- The sentence regarding lowly transcribed transcripts falling below detectable levels in single-worm samples was rephrased for increased clarity on lines: 82-83

Line 84: replace "from" with "by".

- The word “from” was replaced with the word “by” on line: 92

Line 99: "will be published elsewhere": sure, but maybe a one-sentenced description can be spared? Or why such a specialized algorithm would be necessary?

We have collaborated with collaborators that have created a Bayesian approach that extracts technical noise from biological samples. The software estimates biological variance in a more accurate and sensitive manner. The publication should be available shortly.

Protocol
Lines 105-107: too long a period.
Mapped plans: maybe pre-made plans?

- The original sentence previously on lines 105-107 has now been split into two shorter sentences spanning lines 113 to 115.
- The phrase mapped plans have been changed to pre-made plans throughout the manuscript.

Line 136: using instead of utilizing

- On line 151 the word utilizing removed and the sentence was reworded.

Please list the manufacturers. Please add a sentence or two comparing this to the manufacturer's suggestions, so that the reader knows in what sense what you propose is different that their guidelines.

- A list of the manufacturers cannot be provided in the manuscript as there is a limit on commercial language, however, all details are provided in the Table of Materials.
- Comparisons with the manufacturer's protocol have been made on lines: 219, 224/225, 278, 297/298 and 320/321

Line 40: prepared, instead of made up.

- The phrase made up has been replaced with prepared on line: 167

Line 144: briefly centrifuge: spin down?

- The phrase "briefly centrifuge" has been replaced with "spin them down for 5 second" on line 171

No incubation in RT before step 2.3?

- There is indeed no incubation prior to step 2.3

For step 2.3, consider using rubber bands to keep the strips in place

- For step 2.3 a caution has been given suggesting the use of rubber bands to keep the strips in place on lines 180/181.

Step 2.3: why 10 times? For DNA extraction 1 time is enough, why is this different? Plus, authors need to define the time intervals: for how ling (secs) in liquid nitrogen? How long in water bath?

- 10 freeze thaws were chosen we determined that was the best number of freeze thaw cycles that break the cuticle.
- Time intervals for the freeze-thaw were defined in a note added following step 2.4 on lines 186/187 and 191/192

Step 2.4: vortex or mix? Or rock? Is it thermoblock or cold block? (see your line 159) Line 159: adjust the "vortexed" if needed

- On lines 194 and 197 the word "vortexed" was replaced with the word mixed.

Line 162: for short term storage would -20degrees be enough?

- Although the Cell-to-CT kit suggests either -20°C or -80°C can be used here, we would strongly suggest -80°C as the worm itself being dissolved releases a large volume of RNase into the solution and therefore -80°C would be safer to protect the RNA from RNase induced degradation.

Section 3.1: how is this different than manufacturer's protocol?

- Notes have been added following section 3.1 on lines 219 and 224/225 to detail the differences of our steps as opposed to the steps detailed by the manufacturer.

Step 3.1.1: Use periods. Maybe last sentence could be a NOTE.

- The period of step 3.1.1 on lines 214-215 was shortened and the last sentence was moved to a NOTE following the step on line 217.

Line 181: a negative control (instead of minus control). Please double check the volumes: maybe water should be the equivalent of the sample, not the enzyme mix.

- On line 227 the phrase ‘a minus control’ was changed to ‘a negative control’.
- On lines 227/228 we changed the solution with a volume equivalent to the sample from the enzyme mix to water

Step 3.1.3: What about hold at 4degrees as a final step?

- For step 3.1.3, on line 231, a hold at 4 was added to the end of the thermocycling programme.

Step 3.2.1: what is the volume of the sample?

- Regarding step 3.2.1, on line 240, a volume of 11 was added as the total volume of the sample up to that point.

Step 3.2.2: The / are confusing. Please replace.

- For step 3.2.2, on line 247, the symbol ’/’ was removed.

Line 204: remove "from this".

- On line 257 the words “from this” were removed and the sentence was moved to a NOTE

Line 208: When qPCR is performed on a pool of worms …

- The phrase “if you are performing qPCR on a pool of worms” on line was replaced with “when qPCR is performed on bulk worm samples” on line 249.

Line 210: a 1:4 ratio in the final solution

- The phrase “give a 1:4 ending solution” was replaced with “give a 1:4 ratio in the final solution” on lines 252/253

Step 3.4.5: this is identical to step 3.2.3

- Step 3.2.3 (line 249) has been altered for clarity not just containing the dilution step and step 3.4.5 has been changed into a note suggesting storage of the cDNA (lines 257/258).

Line 253: Add the final hold step at 4 degrees.

- A hold step at 4 degrees was added to the end of a thermocycling programme on line: 259

Line 256: Maybe authors can give here an example of the genes they have tried this on.

- Giving examples of genes we have tried on this would run the risk of divulging sensitive information to which we may publish elsewhere therefore examples were not given as they were not deemed necessary for comprehension.

The primers and identity of the genes utilized in this study are listed in the methods table.

Line 287: replace map with plan?

- On line 332, the word “map” was replaced with the word “plan”

Line 304: This step is recommended by both …

- The clarity this line was increased letting the reader know that the recommendation can be applied to both types of chip and moved to line 409 to increase relevance.

Line 348: hold the tip of the syringe away

Line 348: to avoid spillage

- On line 386, the phrase "hold the end syringe away from the center of the chip in case of any spillages" was replaced with "hold the tip of the syringe away to avoid spillage."

Line 398: What is the difference between the two?

- The note originally on line 398 was removed.

Line 421: For statistical evaluation

- On line 463. The phrase "for statistical testing" was replaced with "for statistical evaluation".

Line 430: a brief heat shock: provide time and T

- On line 569, a time of 30 min and temperature of 34 °C was added.

Figure 3, a comparison between N2+Trizol and N2+WtoCt would be appropriate. Same for the hsf1 strain. Moreover, authors should clarify whether all animals compared where subjected to the heat shock simultaneously, i.e. during the same treatment.

Shown in **Figure 3** is the comparison of bulk worm samples, where the RNA was either extracted by Trizol of using Worm to Ct. Two strains were analyzed in parallel: N2 and *hsf-1(sy441)* and those samples were exposed to heat shock simultaneously and harvested in parallel. To address the reviewer point, we have compared *hsp-70* mRNA levels in N2 +Trizol and N2 +WtoCt and similarly for *hsf-1(sy441)*. We have updated line 473 when describing the results of **Figure 3**. We noted that the yield was larger when using Worm-to-Ct method, but the differences among heat shock and non-heat shocked animals remains comparable.

Figure 5: Re 33 worms tested for each condition? Please clarify. What are the dots? Boxplots: is the horizontal line the median? Consider a scatter plot, where all data points will be shown in addition, or consider superimposing the data points. Prism 8 should allow you to do this. Fig. 5B: Horizontal axis: what does it show? Units? Explain the legend: shsps are small hsps? What are variable and flat?

We have changed the boxplot in **Figure 6A** to a scatter plot superposed to a box plot and added in the legend that 33 individual worms were tested for each condition. We have updated **Figure 6B**: vertical l axis= coefficient of variation, which is a dimensionless number (CV=standard deviation divided by the mean). The legend of figure 6 explains how the CV is calculated and that it is used to measure inter-individual variability in gene expression.

Discussion
Line 550: Have the authors also calculated the cost of purchasing the BIOMARK system?

In the discussion, we have made a note on cost-effectiveness, conditional on having access to a BIOMAK machine (line 659).

Line 554: I think unbiased is not the intended term here. Maybe the overall picture?

We think that RNA sequencing provides an unbiased picture of all transcripts present, as opposed to selecting specific transcripts to study, which is in itself a bias.

Line 567: Is this not solved by spinning down?

This problem is usually resolved by spinning down, but it occasionally fails.

Line 574: Maybe the authors can expand a little here, regarding the tissues to be used with the proposed method? What kind of tissues, i.e. fragmented samples? Old tissues? Tissue that contains a limited population of the targeted cells? This is important to help identify potential applications of the suggested protocol.

We have added the following paragraph at the end of the discussion:

Line 677 onwards. "Together, this versatile and reliable pipeline offers increased throughput and sensitivity compared to more standard techniques. This method can be very useful for validation of high-throughput screens and is an excellent choice to either monitor or validate single-worm gene expression levels. We also foresee that this method can be applied to other challenging techniques, such as the quantitation of gene expression from isolated tissues. For example, isolation of full tissues such as the intestine, gonads or of cells isolated by FACs provides enough material to perform RNA sequencing experiments. However, limited amounts of material often lead to duplicated reads which precludes quantitation of rare transcripts. In this scenario, using nanofluidics based technology should provide added sensitivity to these experiments and increase cost-efficiency if the researchers need to monitor only a subset of all transcripts in those tissues or cells"

Reviewer #2:
Manuscript Summary:
The authors described an efficient and rapid method to extract RNA from single or small number of C. elegans and to perform RT-qPCR by combining the high-throughput nanofluidic technology. This method would benefit the worm community for high-throughput gene expression analysis under single worm level.
This manuscript is qualified to publish on JoVE after some minor revisions and additional copy-editing.

Major Concerns:
1. The authors should describe how they preparing the worm sample in their protocol. Like do they wash the worms before loading to the buffer for avoiding the bacteria contamination?
We have added more details about this in the protocol:

Line 153. "2.1 Pick the worms from their bacterial lawn onto a fresh unseeded NGM plate and allow the worms to move around the plate for 10 minutes in which time most of the bacteria should be cleaned from the worm through movement. Pick the worms from the plate into the lysis mix by "scooping" them, i.e. by catching the worms underneath with the pick, to avoid bacterial contamination."

2. For the table 1 and 2, can they include the one with data they used for their representative results to replace the empty ones?
We prefer to keep the tables empty to be filled by the user.

Minor Concerns:
1. For 2.3 (line 153), what is the temperature of the warm water bath?

In line 183, point 2.4, we have added that the temperature of the water bath is 40C.

2. For 3.1.3 and 3.2.2 (line 185 and line 201), should use the same way to describe the thermocycler programs.

The programs are different for single-worms or bulk samples. We are now describing two different protocols in the same way.

3. Line 214, the 3.4.5 should be 3.2.5?

We have corrected the issue.

4. Miss label of step No. 4.
In line 270, we have corrected this issue.

5. Line 255 NOTE. Would be better to describe detailed why and in what circumstance they recommend 10 or 15 cycles.

Fluidigm recommends using pre-amplification cycles between 10 and 20. In our experience, we have used 10 cycles and 15 cycles pre-amplification. We obtained similar results in both cases. However, we encountered one case where the levels of gene expression were too high with 15 cycles pre-amplification that the measurement were "saturated" and unusable (were classified as

failed in the results). This was the case for highly expressed transcripts, such as vitellogenins in day 2 adult animals. Therefore, we would recommend using 10 cycles by default, without knowledge of the expression levels, and 15 cycles if the expression levels are low (for instance for inducible chaperones at basal levels. We have added a note about this in the protocol.

6. Line 276 NOTE. What does the Fluidigm suggestion mean? Why 5, 10, 20-fold?

The dilution has to be experimentally tested by the user, it depends on the targets of each experiment.

7. Figure 5B. not clear what is the label "shortHS.csv$family" mean.

We have eliminated this label to avoid confusion.

Reviewer #3:
Manuscript Summary:
The manuscript by L. Chauve, J. Le Pen et al. presents a hight throughput protocol for quantitative RT PCR in worms that circumvents the need for RNA extraction. The protocol is using commercial

Major Concerns:
The authors do not address the relative benefits of the presented method compared to the one published by Ly et al in 2015 (Rapid RNA analysis of individual Caenorhabditis elegans. MethodssX) It seems the Methods described in 2015requires less hands-on time for the lysis step. The question that is raised is therefore if here is a significant yield difference between the two approaches in a comparable set up.

This is a good reference to compare our method against. We have added the following paragraph to the discussion:

Lines 638-652. “When considering the choice of method to prepare cDNA from single worms, Ly *et* al have optimized a protocol that relies on proteinase-K for cuticle digestion. The cuticle is a major hurdle for the isolation of molecules from worms and proteinase K provides an effective method to break it. However, the disadvantage of using proteinase K, is that it has to be heat-inactivated to be able to use enzymes for reverse transcription. While Ly *et* al used a 10 minutes exposure to 96 C, we avoided this step because RNA is easily degradable. Instead of using proteinase K, we used repeated freeze-thaw cycles to break the cuticle. We believe that the freeze thaw is an effective method to break the cuticle because we can isolate more RNA per worm. Ly et al report that the total RNA extracted per worm is 35ng, whereas we obtain 51.75 +/- 6.74 SEM of total RNA per worm. We believe that avoidance of heat exposure coupled with pre-amplification steps, widens the Worm- to- Ct’s dynamic range of detection compared to standard protocols. Ly *et* al reports and absolute Ct values of 21.1 +/- 0.15 for *hsp-16.2* and 22.8 +/- 0.17

for *hsp-70* after heat shock. Using the same heat shock condition (1h at 30 degrees), we obtain absolute Ct values of 17.93 +/- 0.57 for *hsp-16.2* and 21.13 +/-0.33 for *hsp-70*. This indicates that our lysis method provides higher yields of RNA and is more appropriate for lowly expressed transcripts."

Minor Concerns:
Figures 1 and 2 are identical up to the cDNA synthesis step. It seems more confusing than helpful as the distinction between single worm and pooled worms protocols is harder to pin point.These two figures should be consolidated in a way that highlights the difference between the two applications of the protocol.

We have changed **Figures 1 and 2** to make the differences more clear.

Since the two protocols are provided it would also be interesting to provide a comparison of the CT obtained between bulk and single worm assays. One would expect a direct correlation in detection level depending on the amount of source material. Since the single worm protocol is so efficient it would also be interesting to have the author's opinion on when a "pool" approach may still be useful.

We have added in **Figure 7** a direct comparison of mRNA expression levels using the Worm to Ct method obtained from either the bulks protocol (from 25 pooled worms) or from an average calculated from single worms. The mRNA levels for 3 transcripts (*hsp-16.1*, F44E5.4 and *hsp-70*) are normalized per worm in each case. As depicted in the figure, the levels obtained are comparable when the data is presented as the average per worm in each case. The application of single worm versus bulk-samples really depends on the question that the researcher wants to address. For most applications, bulk-samples are adequate.

In figure 5A rather than presenting the box plot a figure presenting all the data points should be selected to allow better visualization of the sample dispersion between individuals.

We have updated the graph presented in **Figure 6A** as a scatter plot superposed with a boxplot, as requested also by reviewer #1.

In figure 5B similarly before showing the data transformed into CV it would be more straightforward to display the raw values obtained for each replicate. As the authors seem convinced that the RT PCR step is very reproducible within one sample it would be interesting to discuss how one can insure that inter individual differences observed are not caused by variation in RNA recovery or lysis efficiency rather than biological differences. Perhaps by comparing homogeneity or result between individual worms and different sizes of worm pools that should average those individual differences if they are indeed individual expression differences rather than extraction yield artifacts (that should be dampened by increasing the pool size).

We are now presenting the raw values obtained 28 individual worms in **Figure 6A**, before showing the Coefficient of variation for those transcripts in **Figure 6B**. The previous figure 5C has been updated to **Figure 7.** Gene expression is normalized by a set of highly stable genes, so technical differences in the size or preparation of the worms is eliminated using this step. If indeed, it was the case that most of the variability was technical, we would expect all genes tested to be highly variable, but as it is evident in **Figure 6A**, only transcripts from the inducible heat shock family of proteins is variable. We have used additional methods to verify this data, which will be published elsewhere. In addition, we have added **Figure 5**, where we have compared the expression values of 3 small heat shock protein transcripts measured using bulk-samples and an average of 30 individual worms. The expression levels are comparable, indicating that technical variance related to individual animals is not at the core of measured values.