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TITLE:
Standardized Methods for Measuring Induction of the Heat Shock Response in *Caenorhabditis elegans*

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heat shock, heat shock response, HSF-1, *C. elegans*, HSP-16.2, HSP-70, chaperones, thermotolerance, thermorecovery

SUMMARY:
Here, standardized protocols are presented to assess induction of the heat shock response (HSR) in *Caenorhabditis elegans* using RT-qPCR at the molecular level, fluorescent reporters at the cellular level, and thermorecovery at the organismal level.

ABSTRACT:
The heat shock response (HSR) is a cellular stress response induced by cytosolic protein misfolding that functions to restore protein folding homeostasis, or proteostasis. *Caenorhabditis elegans* occupies a unique and powerful niche for HSR research because the HSR can be assessed at the molecular, cellular, and organismal levels. Therefore, changes at the molecular level can be visualized at the cellular level and their impacts on physiology can be quantitated at the organismal level. While assays for measuring the HSR are straightforward, variations in the timing, temperature, and methodology described in the literature make it challenging to compare results across studies. Furthermore, these issues act as a barrier for anyone seeking to incorporate HSR analysis into their research. Here, a series of protocols are presented for measuring induction of the HSR in a robust and reproducible manner with RT-qPCR, fluorescent reporters, and an organismal thermorecovery assay. Additionally, we show that a widely used thermotolerance assay is not dependent on the well-established master regulator of the HSR, HSF-1, and therefore should not be used for HSR research. Finally, variations in these assays found in the literature are discussed and best practices are proposed to help standardize results across the field, ultimately facilitating neurodegenerative disease, aging, and HSR research.

INTRODUCTION:

The heat shock response (HSR) is a universal cellular stress response induced by cytosolic protein misfolding caused by temperature increases and other proteotoxic stresses. Activation of the HSR in *Caenorhabditis elegans* leads to transcriptional upregulation of heat shock genes such as *hsp-70* and *hsp-16.2.* Many heat shock proteins (HSPs) function as molecular chaperones that restore protein folding homeostasis, or proteostasis, by directly interacting with misfolded or damaged proteins. The master regulator of the HSR is the transcription factor Heat Shock Factor 1 (HSF-1), whose activation is elegantly controlled via multiple mechanisms[1].

The role of HSF-1 is not restricted to stress. HSF-1 is required for normal growth and development, because deletion of *hsf-1* leads to larval arrest[2]. HSF-1 is also important during aging and age-related neurodegenerative diseases characterized by accumulation of protein aggregates and an inability to maintain proteostasis. Knockdown of *hsf-1* causes accumulation of protein aggregates and a shortened lifespan, while overexpression of *hsf-1* reduces protein aggregation and extends lifespan[3,4]. Therefore, regulation of HSF-1 at the molecular level has broad implications for organismal physiology and disease.

C. elegans is a powerful model organism for HSR research because the HSR can be measured at the molecular, cellular, and organismal levels[4–6]. Highlighting the power of this model, key advances in delineating the HSR pathway, such as tissue-specific differences in HSR regulation, have been discovered in *C. elegans*[7, 8]. Furthermore, *C. elegans* is widely used for aging research and is an emerging system for modeling diseases linked to proteostasis disruption.

Although heat shock experiments with *C. elegans* can be quick and reproducible, there are several questions to consider before beginning. For example, which temperature should be used for induction of the HSR and how long should the worms be exposed? Is it better to use a dry incubator or a water bath? Which developmental stage should be used? Unfortunately, the methodologies used to investigate the HSR vary widely from laboratory to laboratory, causing confusion when selecting the best methodologies and making it difficult to compare results across the field.

We present robust and standardized protocols for using RT-qPCR, fluorescent reporters, and thermorecovery to measure the HSR. While these three approaches are complementary, they each have unique advantages and disadvantages. For example, RT-qPCR is the most direct and quantitative measurement of the HSR, and this assay can be easily expanded to include many different heat shock-inducible genes. However, RT-qPCR is the most expensive, technically difficult, and requires the use of specialized equipment. In contrast, fluorescent reporters have the advantage of measuring tissue-specific differences in HSR induction. However, they are difficult to quantitate accurately, can only measure induction above a certain threshold, and require the use of a fluorescence microscope. Additionally, the reporter strains described here are developmentally delayed compared to the standard N2 strain. Although newer reporter strains containing single-copy transgenes are available, they have not been tested here[9]. The third assay, thermorecovery, has the advantage of providing a physiologically relevant readout at the organismal level. However, this assay is arguably the least sensitive and most indirect.

Finally, we discuss some common variations found in these assays and propose a set of best practices to facilitate research in this field.

PROTOCOL:

1. Maintenance and synchronization of *C. elegans*

1.1. Maintain worms at 20 °C on Nematode Growth Medium (NGM) plates seeded with OP50 *Escherichia coli* bacteria by transferring several adults to fresh plates approximately 2x per week[10]. Care should be taken to prevent worms from running out of food, because this can affect their physiology[11].

1.1.1. Preparation of NGM plates

1.1.1.1. Mix 3 g of NaCl, 2.5 g of Bacto-peptone, 17 g of agar, and deionized (DI) H_2O up to 1 L in a flask.

1.1.1.2. Autoclave the mixture for sterilization.

1.1.1.3. Allow mixture to cool to ~50 °C.

1.1.1.4. Add 25 mL of 1 M KH_2PO_4 (pH = 6), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 1 mL of cholesterol (5 mg/mL in 100% ethanol).

1.1.1.5. Using sterile technique, pour the mixture into 6 cm plates to yield approximately 100 plates. Pouring plates is easier if the mixture is first transferred to a 300 mL sterile beaker.

1.1.1.6. Allow 1 day to solidify at room temperature (RT) before seeding with bacteria or storing at 4 °C.

1.1.2. Seeding of OP50 bacteria onto NGM plates.

1.1.2.1. Grow a saturated overnight OP50 bacterial culture in LB at 30 °C or 37 °C.

1.1.2.2. Place approximately 300 µL of the culture onto the center of a 6 cm NGM plate.

1.1.2.3. Let plates dry at RT for 1–3 days as needed for the bacterial lawn to adhere to the plate. Plates can then be used or stored at 4 °C.

1.2. Grow the worms synchronously either by isolating freshly laid eggs or by collecting eggs after dissolving worms with bleach.

1.2.1. Transfer approximately 10 gravid adult worms to a fresh plate using a platinum wire pick. Egg-lay synchronization works best if the adults are in the first day of adulthood.

1.2.2. After approximately 1 h, remove the worms from the plate. This should result in 40–60 eggs per plate, depending on the conditions and the strain.

2. Fluorescent imaging of HSR reporters

2.1. Synchronize the worms (section 1.2) and maintain at 20 °C until the desired developmental stage. For the AM446 (*hsp-70p::gfp*) and CL2070 (*hsp-16.2p::gfp*) fluorescent reporter strains, young adult worms that have not yet reached reproductive maturity are generated 64 h after the egg-laying synchronization.

NOTE: The developmental timing varies with each strain and the temperature at which the worms are raised. Both HSR reporter strains exhibit a slight developmental delay relative to N2. Importantly, the magnitude of HSR induction declines approximately 2–4x after the onset of reproductive maturity (see Discussion).

2.2. Heat shock the worms by wrapping plates with paraffin film and submerging in a circulating water bath at 33 °C for 1 h. A thin strip of paraffin film should be wrapped 2x around the plate to seal the edges. Do not cover the bottom of the plate or it could interfere with heat transfer. Submerge the plates upside down using a test tube rack and a lead weight. Remember to include a negative control sample (no heat shock) if necessary.

NOTE: If the paraffin film is not secure, then water will enter the plate and the plate should not be used for data collection.

2.3. Recover the worms by removing the plates from the water bath and drying with a paper towel. Remove the paraffin film and incubate the worms at 20 °C for 6–24 h. This recovery period allows sufficient time for GFP synthesis and folding before imaging.

2.4. Prepare slides for imaging. Slides should be prepared fresh for each use.

2.4.1. Make a 3% agarose solution in water and heat using a microwave until the agarose is dissolved.

2.4.2. Place a microscope slide for imaging between two other microscope slides that have a strip of laboratory tape on them to create a spacer for the agarose pad.

2.4.3. Using a 1,000 µL pipette, place a drop (~150 µL) of the heated 3% agarose in the center of the microscope slide.

2.4.4. **Immediately** cover the microscope slide with a blank microscope slide perpendicular to the first slide so that the top slide rests on the laboratory tape on the adjacent slides. This spreads out the drop of agarose to create a pad of uniform width.

2.4.5. Carefully remove the top slide.

2.5. Immobilize the worms by using a 200 µL pipette to add a small drop (~5 µL) of 1 mM levamisole in M9 buffer to the center of the agarose pad. Then transfer 10 worms into the drop of levamisole using a platinum wire pick. Cover with a coverslip. Sealing the coverslip is not necessary for an upright microscope. Optionally, the worms can be aligned when they become paralyzed by spreading the levamisole off, to the outside of the agarose pad, and aligning the worms with a platinum wire pick. Alternatively, the levamisole can be soaked up using a laboratory wipe.

NOTE: Image as soon as possible, because prolonged incubation in levamisole could alter fluorescence.

2.6. Image the worms using a fluorescence microscope. The details of image capture vary by microscope and software.

NOTE: To directly compare image intensities, use identical microscope settings in one imaging session. Avoid oversaturating the image.

3. Measurement of HSR gene expression using RT-qPCR

3.1. Synchronize worms (section 1.2) and maintain at 20 °C until the desired developmental stage. For N2 worms, young adult worms that have not yet reached reproductive maturity are generated 60 h after the egg-laying synchronization.

NOTE: The developmental timing varies with each strain and the temperature at which the worms are raised. Importantly, the magnitude of HSR induction declines approximately 2–4x after the onset of reproductive maturity (see Discussion).

3.2. Heat shock worms as described in step 2.2.

3.3. Take the plates out of the water bath, remove the paraffin film, and immediately collect the worms. The worms can be collected by washing the plates gently with 1 mL of M9, collecting the liquid in a microcentrifuge tube, and then removing the M9 after centrifugation at 400 x *g* for 1 min.

3.4. Lyse the worms and purify the RNA using organic extraction**.**

3.4.1. Add 250 µL of RNA isolation reagent (see **Table of Materials**).

3.4.2. Vortex tubes by hand for 30 s.

3.4.3. Vortex tubes for 20 min at 4 °C using a microcentrifuge tube attachment (see **Table of Materials**).

3.4.4. Add 50 µL of chloroform.

3.4.5. Vortex for 30 s.

3.4.6. Incubate the samples at RT for 3 min.

3.4.7. Centrifuge at ≥14,000 x *g* for 15 min at 4 °C.

3.4.8. Transfer the aqueous layer (i.e., top layer, ~125 µL) to a new microcentrifuge tube.

NOTE: Avoid the organic layer and the material in the interface.

3.4.9. Add 50 µL of chloroform.

3.4.10. Vortex for 30 s.

3.4.11. Incubate the samples at RT for 3 min.

3.4.12. Centrifuge at ≥14,000 x *g* for 5 min at 4 °C.

3.4.13. Transfer the aqueous layer (~100 µL) to a new microcentrifuge tube.

NOTE: Avoid the organic layer and the material in the interface.

3.4.14. Precipitate RNA with an equal volume (i.e., 100 µL) of isopropanol.

3.4.15. Incubate at -20 °C for at least 30 min, but preferably overnight.

NOTE: The experiment can be paused here and the RNA can be stored at -20 °C.

3.4.16. Pellet the RNA by centrifugation at ≥14,000 x *g* for ≥30 min at 4 °C.

3.4.17. Remove as much of the supernatant as possible without disturbing the pellet.

NOTE: The pellet will be small and may not be visible. The pellet may not adhere tightly to the side of the tube, so caution is necessary to avoid dislodging it.

3.4.18. Wash the pellet with 250 µL of 70% ice-cold ethanol made with RNase-free H_2O.

3.4.19. Centrifuge at ≥14,000 x *g* for ≥5 min at 4 °C.

3.4.20. Remove as much supernatant as possible without disturbing the pellet.

3.4.21. Perform a quick spin at RT to remove any remaining 70% ethanol.

3.4.22. Dry the pellet by leaving the tubes open at RT as long as needed; typically at least 20 min. Tubes can be covered with a lint-free tissue or aluminum foil to prevent contamination.

3.4.23. Resuspend the pellet in 20 µL of RNase-free H_2O.

3.4.24. Determine the RNA concentration using a small volume spectrophotometer (2 µL).

NOTE: The experiment can be paused here and the RNA can be temporarily stored at or below -20 °C.

3.5. Remove residual DNA by incubating with DNase I. It is recommended to use a commercially available kit (see **Table of Materials**) and to follow the manufacturer's instructions.

3.5.1. With this kit, prepare a 20 µL reaction with 500 ng of RNA and 1 µL of DNase I in a 37 °C water bath for 30 min.

3.5.2. Add 2.5 µL of DNase inactivation reagent (included in the kit) to each sample and incubate at RT for 5 min with occasional flicking/vortexing.

3.5.3. Spin down at 14,000 x *g* for 2 min.

3.5.4. Without disturbing the white pellet, transfer 15 µL of supernatant to a fresh microtube for cDNA synthesis.

3.6. Conduct cDNA synthesis. It is recommended to use a commercially available kit (see **Table of Materials**) and to follow the manufacturer's instructions.

3.6.1. With the kit, prepare a 20 µL reaction with 15 µL of DNase I-treated RNA from the previous step and 1 µL of reverse transcriptase.

3.6.2. Use the following program for cDNA synthesis: 25 °C for 5 min, 46 °C for 20 min, 95 °C for 1 min, 4 °C hold.

3.6.3. Dilute cDNA by adding 80 µL of RNase-free H_2O directly to the sample.

3.6.4. Briefly vortex, then spin down and store at -20 °C until needed.

3.7. Perform qPCR. It is recommended to use a commercially available kit (see **Table of Materials**) and to follow the manufacturer's instructions.

3.7.1. With the kit, prepare a 25 µL reaction with 2 µL of cDNA, 200 nM (each) forward and reverse primers, in a 25 µL final volume in one well of a 96 well plate.

3.7.2. Primer sequences for measuring the heat shock genes, *hsp-70* and *hsp-16.2*, and 18S rRNA (for a normalization control) are listed in the **Table of Materials**. Multiple normalization controls can be used as desired.

3.7.3. Dilute cDNA samples 50x before measurement of 18S to ensure that the assay is in the linear range. Appropriate qPCR conditions vary with the kit and primers used (see Representative Results).

3.7.4. Use a real-time PCR detection system (see **Table of Materials**) for qPCR with 40 cycles of 95 °C for 5 s denaturation, 58 °C for 30 s annealing, and 72 °C for 30 s extension.

NOTE: Optimal annealing temperatures can vary by primers and conditions.

3.7.5. Quantify using either the ΔΔCt or standard curve method[12].

4. Thermorecovery assay for measuring HSR at the organismal level

4.1. Synchronize the worms (section 1.2) and maintain at 20 °C until the desired developmental stage. For N2 worms, young adult worms that have not yet reached reproductive maturity are generated 60 h after the egg-laying synchronization.

NOTE: The developmental timing varies with each strain and the temperature at which the worms are raised. Importantly, the magnitude of HSR induction declines approximately 2–4x after the onset of reproductive maturity (see Discussion).

4.2. Heat shock the worms as described in step 2.2 for 6 h.

4.3. Remove the plates from the water bath, remove the paraffin film, and allow the worms to recover by incubation at 20 °C for 48 h.

4.4. Count the number of worms that can immediately crawl away after mechanical stimulation without jerky movement or paralysis.

NOTE: The 6 h incubation is optimal for examining conditions that reduce thermorecovery, but longer exposure times may be needed to look for conditions that enhance thermorecovery.

REPRESENTATIVE RESULTS:

Using the protocols described in this manuscript, HSR induction was measured using fluorescent reporters, RT-qPCR, and thermorecovery assays. In each case, the procedure in section 1.2 was used to generate synchronized, young adult worms that had not reached reproductive maturity.

To visualize HSR induction at the cellular level, the AM446 (*hsp-70p::gfp*) and CL2070 (*hsp-16.2p::gfp*) fluorescent reporter strains were analyzed following section 2 of the protocol. In the negative control samples without heat shock, the *hsp-16.2* reporter only showed normal autofluorescence, but the *hsp-70* reporter had constitutive fluorescence in the anal depressor muscle as previously reported (**Figure 1A**)[4]. After 1 h of heat shock at 33 °C, robust fluorescence was observed in both reporters; however, the pattern of expression was distinct depending on which reporter was used (**Figure 1B**). The *hsp-70* reporter was brightest in the intestine and spermatheca, whereas the *hsp-16.2* reporter was brightest in the pharynx. Additionally, the *hsp-16.2* reporter had a high degree of worm-to-worm variability in the amount of induction as previously described, but the *hsp-70* reporter did not[13].

A commonly used variation of section 2 is to perform the heat shock in a dry incubator instead of a circulating water bath. Therefore, the difference between the two methodologies was also tested. It was found that both protocols resulted in robust induction of the two fluorescent reporters using our conditions, although a circulating water bath is recommended as a best practice (see Discussion) (**Figure 1B**).

To test the dependence of the reporters on the transcription factor HSF-1, feeding RNAi was used to knockdown *hsf-1* before reporter induction was measured. It was found that fluorescence of both strains was severely reduced upon HSF-1 knockdown, indicating that these reporters are HSF-1-dependent as described in the literature[4] (**Figure 2**). However, it was also observed that pharyngeal fluorescence persisted in both reporters upon *hsf-1* knockdown, which is consistent with previous reports that the pharyngeal muscle is resistant to RNAi by feeding[14].

To quantitate whole worm induction of the HSR at the molecular level, two endogenous HSPs were measured with RT-qPCR using section 3 of the protocol. Samples were measured in triplicate, a standard curve was generated for each of the primers, and a melt curve was analyzed for each sample for quality control. It was found that a 33 °C heat shock for 1 h resulted in more than a 2,000x increase in relative expression for two heat shock genes, *hsp-70* and *hsp-16.*2 (**Figure 3**). These results show that both endogenous genes are suitable for measuring HSR induction and that a 33 °C heat shock for 1 h is sufficient to generate a substantial response. However, caution should be used in interpreting the absolute degree of heat shock induction, because the mRNA levels in the absence of heat shock are very low.

To analyze a physiological response to heat shock, an organismal thermorecovery assay was tested using section 4 of the protocol. It was found that exposure of worms to a 6 h heat shock at 33 °C led to a 20% decrease in worms with normal movement after a 48 h recovery (**Figure 4A**). The dependence of this assay on the HSF-1 transcription factor was tested using feeding RNAi to knockdown *hsf-1* before exposing worms to the stress. It was found that knockdown of *hsf-1* caused a dramatic decrease in normal movement, with >95% of worms showing jerky movement or paralysis after being prodded with a platinum wire pick.

We compared this thermorecovery assay to a widely used alternative organismal assay commonly referred to as thermotolerance. In the thermotolerance assay, worms are exposed to a continuous 35 °C temperature using a dry incubator, and the percentage of worms alive are measured at various timepoints. Using this assay, it was found that control worms continuously exposed to 35 °C died after approximately 8 h of exposure (**Figure 4B**). However, when the dependence of this assay on HSF-1 was tested using RNAi knockdown, it was found that inhibition of *hsf-1* did not cause a decrease in thermotolerance. Similar results have been previously shown using HSF-1 mutations (see Discussion). Therefore, the use of the thermotolerance assay to measure HSR is not recommended, and thermorecovery is the preferred method for examining HSR at the organismal level.

FIGURE LEGENDS:

Figure 1: HSR induction measured with fluorescent reporters. (**A**) The basal and (**B**) heat-inducible expression of *hsp-70p::gfp* and *hsp-16.2p::gfp* reporter strains after 1 h of heat shock at 33 °C in a water bath or incubator. Worms were raised on OP50 bacteria for 64 h, heat shocked, and then recovered at 20 °C for 8 h before imaging. For reference, the no heat-shock worms in (**A**) were renormalized in (**B**) to match the range and saturation of the heat-shocked worms. Representative images of two experimental replicates are shown. Scale bar = 250 µm.

Figure 2: HSR induction measured with fluorescent reporters is dependent on HSF-1. Strains containing the *hsp-70p::gfp* and *hsp-16.2p::gfp* reporters were raised on control (L4440 empty vector) or *hsf-1* RNAi plates for 64 h, exposed to a 1 h heat shock at 33 °C in a water bath, and then recovered at 20 °C for 8 h before imaging. Representative images of two experimental replicates are shown. Scale bar = 250 µm.

Figure 3: HSR induction measured with RT-qPCR. N2 worms were raised on HT115 bacteria for 60 h and then heat shocked for 1 h in a 33 °C water bath. The relative mRNA levels of *hsp-70 (C12C8.1)* and *hsp-16.2* are shown normalized to the no heat-shock control. Values plotted are the mean of four biological replicates and error bars represent ± SEM. Statistical significance was calculated using an unpaired Student's t-test. **p < 0.01.

Figure 4: Thermorecovery, but not thermotolerance, is dependent on HSF-1. N2 worms were raised on control (L4440) or *hsf-1* RNAi plates for 60 h and then shifted to either: (**A**) A 35 °C dry incubator and removed every 2 h until dead (thermotolerance), or (**B**) A 33 °C water bath for 6 h and recovered at 20 °C for 48 h before scoring for normal movement (thermorecovery). Each assay was done with n ≥ 60 individuals on 2 independent days. The average is shown.

DISCUSSION:

In the literature a wide variety of temperatures, times, and equipment have been used to assay the HSR, which has introduced unnecessary caveats and led to difficulty in comparing results between laboratories. For example, temperatures ranging anywhere from 32–37 °C and times from 15 min to several hours have been used to induce the HSR[15]. However, it is reported that lethality occurs as early as 3 h at 37 °C for all stages and 1.5 h for day 1 adults[15]. Furthermore, we show that exposure of worms to 35 °C causes lethality that is not HSF-1 dependent, making these conditions poorly suited for analysis of the HSR. In contrast, a heat shock of 33 °C for 1 h is robust enough to elicit strong induction of heat shock genes, yet mild enough to not affect worm viability. Indeed, exposure to 33 °C for as long as 6 h only causes 20% of worms to display abnormal movement. Therefore, we propose using a temperature of 33 °C and a time of 1 h as a standardized condition for RT-qPCR and fluorescent reporter assays.

Recent experiments have revealed that developmental staging of worms for HSR experiments is particularly important. It was recently shown that in *C. elegans* the inducibility of the HSR declines (i.e., collapses) by >50% when hermaphrodites begin egg laying[5]. Staging the worms correctly is critical because there are often differences in developmental timing in strains carrying mutations. If temperature-sensitive mutants are used, this will also impact results if they are not synchronized by their reproductive age. Therefore, it is recommended to carefully measure the onset of egg laying for every strain to determine when the collapse occurs. The window of time after the L4 molt and before the initiation of reproductive maturity is narrow; therefore, care must be taken so that the HSR collapse does not inadvertently cause variability in results.

In addition to developmental timing, surprisingly small changes in temperature, as little as 1 °C, can have substantial effects on the HSR. For example, thermosensory neurons in *C. elegans* are sensitive to temperature changes as small as ±0.05 °C[16]. Thus, it is imperative to use a thermometer that can accurately measure the temperature. Therefore, we propose as best practice the use of a calibrated device for temperature measurement that is precise enough to measure temperatures within ±0.1 °C. Furthermore, a thermometer with a data-logging functionality should be used to measure temperature variations across time. Many incubators are specified to have thermal variations of more than 1 °C in different parts of the incubator and across time, which can have significant effects on HSR experiments. As a best practice, we suggest using incubators that have sufficient insulation and circulation to minimize temperature fluctuations. For conducting heat shock experiments, we propose a best practice of a circulating water bath. The time it takes for an agar plate to reach a desired temperature is approximately 6–7 min in a water bath but much longer in a dry incubator[15,17]. However, if a circulating water bath is not available, we have shown that robust HSR induction also occurs in a dry incubator using our conditions. If a dry incubator is used, opening of the incubator for the duration of the stress should be minimized.

It is well-established that induction of heat shock genes is dependent on the master regulator of the HSR, HSF-1. Here, we present evidence that the two more indirect assays, fluorescent reporters and thermorecovery, are also dependent on HSF-1. Significantly, we found that a commonly used alternative organismal assay, thermotolerance, is not HSF-1 dependent using *hsf-1* RNAi (**Figure 4**). Similar results have been previously reported using an *hsf-1* mutant or a *ttx-3* mutant, which blocks the HSR[18–20]. Together, these results indicate that the thermotolerance assay should not be used for HSR research. Furthermore, this suggests that a best practice is to test the HSF-1 dependence for any assay used to measure the HSR.

Taken together, we present a series of standardized protocols and best practices for robust and reproducible measurement of HSR induction in *C. elegans*. We hope that these methodologies will decrease variability in HSR experiments and increase reproducibility. Facilitating direct comparisons of HSR research between laboratories will serve to accelerate research in the HSR field. Furthermore, standardization will benefit research into aging and neurodegenerative diseases with which the HSR is intimately associated.

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DISCLOSURES:

The authors have nothing to disclose.

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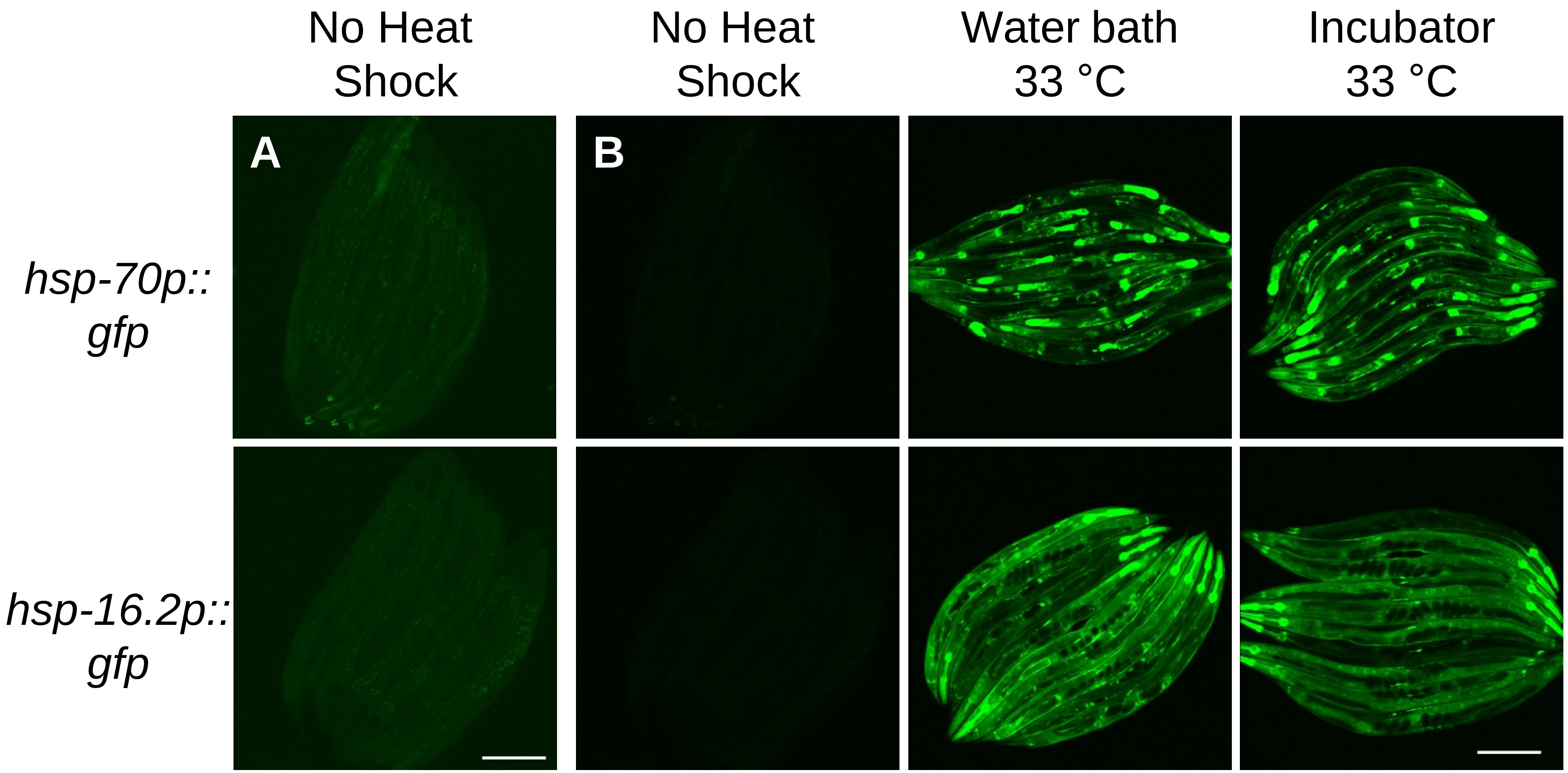
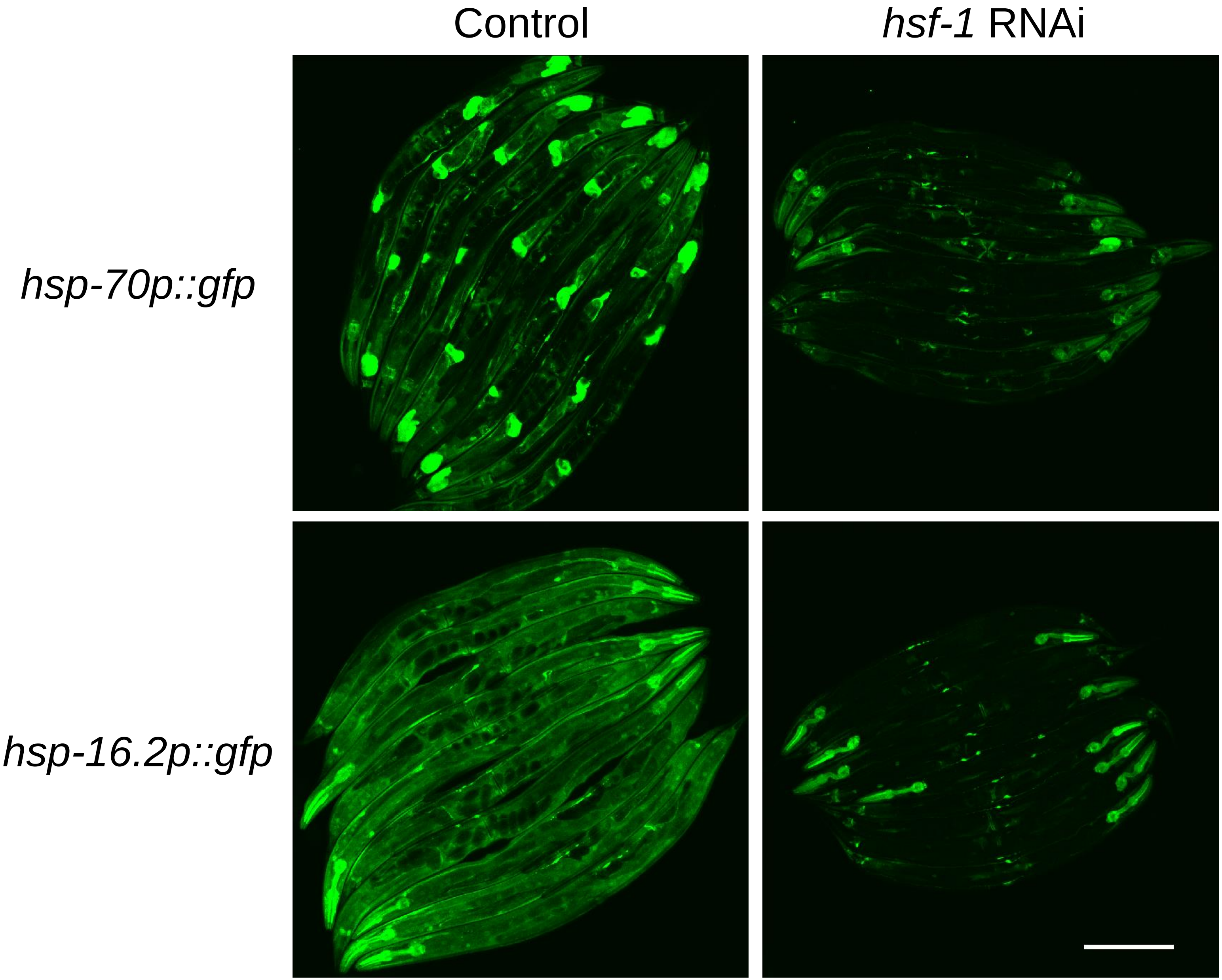
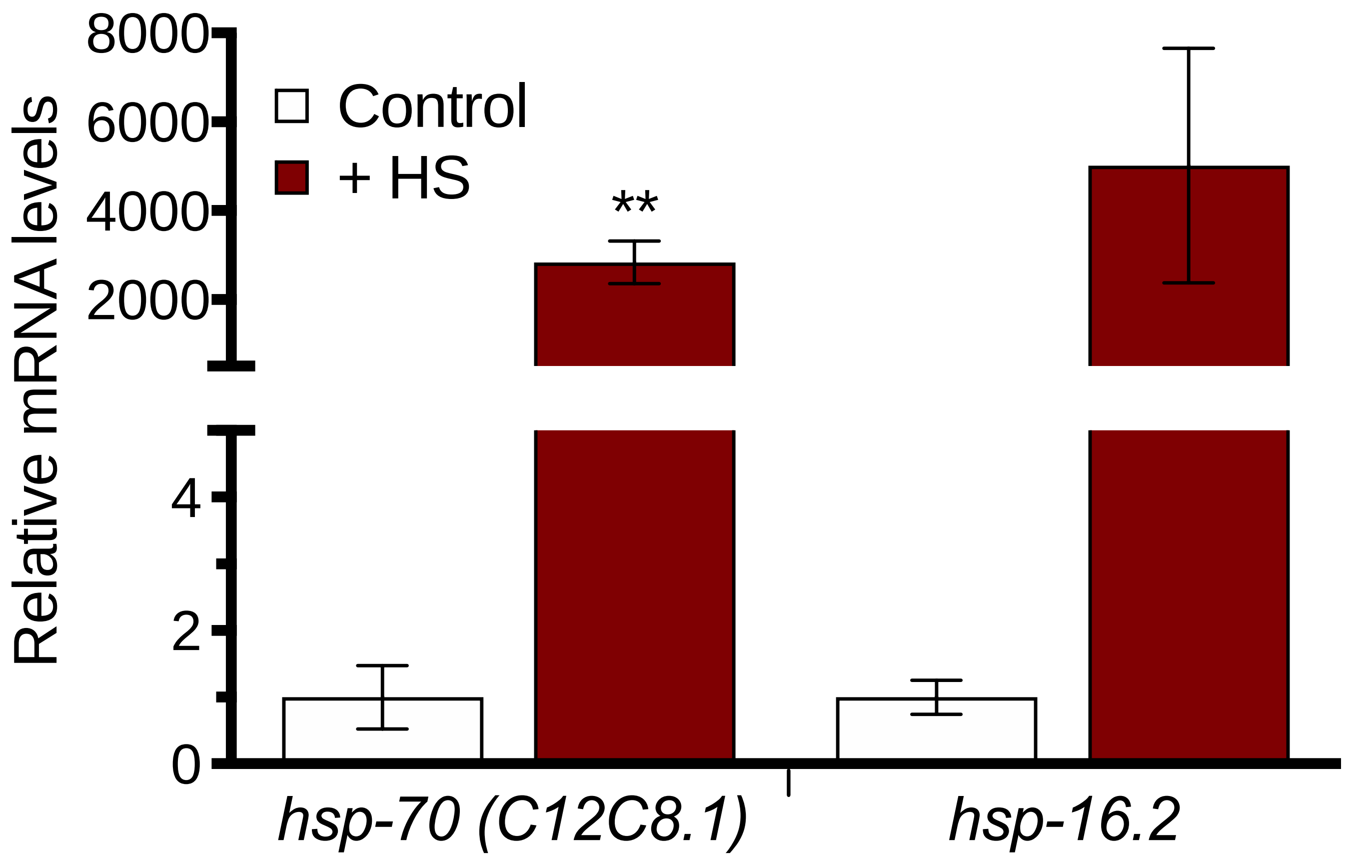

No Heat Shock
No Heat Shock
Water bath 33 °C
Incubator 33 °C
hsp-70p::gfp
hsp-16.2p::gfp
A
B


Control
hsf-1 RNAi
hsp-70p::gfp
hsp-16.2p::gfp


Relative mRNA levels
8000
6000
4000
2000
4
2
0
Control
+ HS
**
hsp-70 (C12C8.1)
hsp-16.2

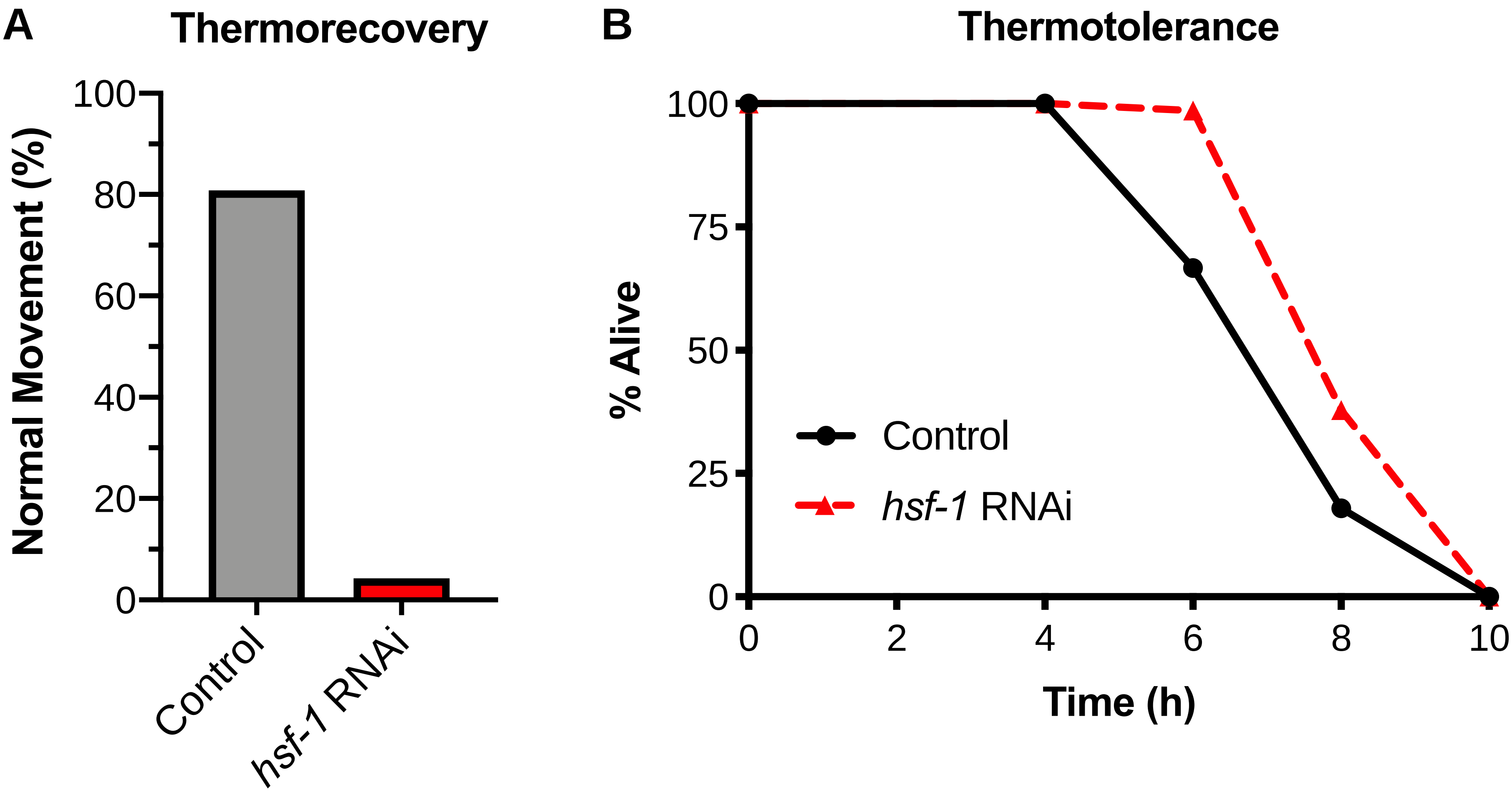

A
Thermorecovery
Normal Movement (%)
100
80
60
40
20
0
Control
hsf-1 RNAi
B
Thermotolerance
% Alive
100
75
50
25
0
0
2
4
6
8
10
Time (h)
Control
hsf-1 RNAi

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials List.xlsx

Equipment
18S- forward primer
18S- reverse primer
AM446 rmIs223[phsp70::gfp; pRF4(rol-6(su1006))]
C12C8.1- forward primer
C12C8.1- reverse primer
CFX Connect Real-Time PCR Detection System
CL2070 dvIs70 [hsp-16.2p::GFP + rol-6(su1006)]
EasyLog Thermistor Probe Data Logger with LCD
Greenough Stereo Microscope S9i Series
Hard Shell 96 Well PCR Plates
hsp-16.2- forward primer
hsp-16.2- reverse primer
iScript cDNA Synthesis Kit
iTaq Universal Sybr Green Super Mix
Laser Scanning Confocal Microscope
MultiGene OptiMax Thermo Cycler
N2 (WT)
Nanodrop Lite Spectrophotometer
Parafilm M Roll
RapidOut DNA Removal Kit
Recirculating Heated Water Bath
Traceable Platinum Ultra-Accurate Digital Thermometer
TRIzol Reagent
TurboMix Attachment
Vortex-Genie 2

Company
Morimoto lab
Bio Rad
Caenorhabditis Genetics Center (CGC)
Lascar
Leica
Bio Rad
Bio Rad
Bio Rad
Nikon
Labnet
Caenorhabditis Genetics Center (CGC)
Thermo Scientific
Bemis
Thermo Scientific
Lauda Brinkmann
Fisher Scientific
Invitrogen
Scientific Industries
Scientific Industries

Catalog Number
http://groups.molbiosci.northwestern.edu/morimoto/
1855200
https://cgc.umn.edu/
EL-USB-TP-LCD
HSS9601
1708891
1725121
Eclipse 90i
TC9610
https://cgc.umn.edu/
ND-LITE
5259-04LC
K2981
RE-206
15-081-103
15596026
SI-0564
SI-0236

Notes

TTGCGTCAACTGTGGTCGTG
CCAACAAAAAGAACCGAAGTCCTG

GTACTACGTACTCATGTGTCGGTATTT
ACGGGCTTTCCTTGTTTTCC

ACTTTACCACTATTTCCGTCCAGC
CCTTGAACCGCTTCTTTCTTTG

RNA isolation reagent

Author's Response:
We would like to thank the editors and the reviewers for their thoughtful comments. We have made all suggested changes per the Editorial Comments. Specific responses to reviewers' comments are included below in bold font.

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Parafilm, Trizol, RapidOut, iScript, iTaq.

Protocol:
1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Figures:
1. Please provide 1 file per Figure (4 in total). Please remove 'Figure 1' etc. from the figures themselves.
2. Figure 1: Please use '33 °C' instead of '33°C' (include a space).

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The manuscript describes three methods for evaluating the heat stress response in C. elegans.

Major Concerns:

Statistical methods for analysis of data not described for Figure 3 and 4.

Figure 3 has been updated with statistical significance shown and described in the figure legend. Statistics were not calculated for Figure 4 as two replicates were performed as described in the figure legend.

Line 89 - moving 2 adults each generation can lead to rapid selection of deleterious mutations. Picking at least 8 worms is better practice.

WormBook recommends 3-4 adult hermaphrodites to be transferred per plate. We have addressed this comment by replacing 'at least 2' with 'several'.

The authors do not describe whether worms move "normally" in their thermorecovery assay. However, they do not describe what "normal" means. This is very subjective. Perhaps, the authors can also score inability to move or death in their assays?

We agree. This comment highlights the utility of the JOVE video format. We will highlight the difference between normal and impaired movement in the video. Additionally, in the protocol section IV 4, we clarified that we scored "the number of worms that can immediately crawl away after mechanical stimulation without jerky movement or paralysis." In line 368-369 we changed the text from "abnormal movement" to "jerky movement or paralysis after being prodded with a platinum wire pick."

Minor Concerns:

Line 75-76: The authors state that fluorescent reporters for the HSR are not quantitative. This is not the case - imaging and then analysis by FIJI/ImageJ is commonplace.

We have changed the text from "not as quantitative" to "difficult to quantitate accurately."

Descriptions of HS treatment are very repetitive. Consider consolidating.

Consolidated after first mention to "heat shock worms as described in protocol II.2."

Best practice is to use more than one normalisation control for RT-PCR experiments.

We amended the text to state that “multiple normalization controls can be used as desired.”

Figure 3: only the gene name for hsp-16.2 is shown but for hsp-70 (C12C8.1) is also shown. Make consistent.

***C12C8.1* is listed for clarity because this is a cosmid name that was extensively used before the gene was renamed to *hsp-70*.**

Line 88: Write "OP50 Escherichia coli" instead of OP50

Done.

Reviewer #2:

Manuscript Summary:
The manuscript entitled "Standardized Methods for Measuring Induction of the Heat Shock Response in Caenorhabditis elegans" by Golden et al. aims to identify different parameters that determine the magnitude and physiological significance of the heat shock response in Caenorhabditis elegans. While the authors picked an important and relevant topic that could potentially serve as an important guide to the field, the manuscript has many drawbacks/limitations/omissions that need to be addressed for it to be useful.

Major Concerns:

1. There is the lack of discussion about how the methods detailed here relate to the question(s) that are typically studied. If transcription factor binding is being studied, then it is unlikely that the timing recommended by the authors is the most appropriate. Alternately, if mRNA levels are used to validate some aspect of C. elegans physiology, it is unclear why "heat shock of 33 °C for 1 hr" is being recommended. Similarly, statements such as "heat shock of 33 °C for 1 hr is robust enough to elicit strong induction of gene expression changes, yet mild enough so that worm viability is not affected" are unhelpful, as heat shock for 30 minutes also fits this criterion. A more rigorous discussion of how the methods relate to specific questions is warranted.

We have clarified this section of the manuscript as requested. We acknowledge that our recommended conditions are not the only conditions that induce the HSR, however we believe that the field would benefit from a single set of standardized conditions. We detailed methods for only the three assays that we describe and we agree that these protocols would not be suitable for other assays, such as transcription factor binding.

2. Many of the parameters that affect the response of C. elegans (even amongst those that the authors discussed) are ill defined. Examples are listed below.

The reviewer has correctly noted that there are many variables beyond those that we have enumerated. Unfortunately, it is not possible in the scope of this manuscript to explore all of these parameters. For example, the reviewer suggests that we include 1) a description of the optimal density of worms and 2) a discussion of whether different plate densities affect heat transfer and therefore the HSR. We have detailed the variables that we know to be relevant for these assays, for example by noting that lack of food affects the HSR assays. However, we do not routinely measure our plate weights nor have we conducted extensive examination of worm-to-food ratios in relation to HSR activation. Therefore, we have included additional information as detailed in the response to reviewer 1 where appropriate, but were unable to respond to all of the comments. Furthermore, we have referenced WormBook for basic aspects of worm husbandry, and we have referenced a publication that details RT-qPCR analysis.

89-90: "Care should be taken not to let the worms run out of food as this can affect their physiology. A description of the optimal density to be used, would be more appropriate.

102-103: "Allow one day to solidify at room temperature (RT) before seeding with bacteria or storing at 4 °C." Both solidifying for 1 day and storing at 4°C can cause changes in the densities of the agar plates and therefore their heat transfer. Would this not affect heat shock? Weight of the plates and the rates of temperature increase on the surface of plates would be more appropriate.

109: "3. Let plates dry at RT for 1-3 days as needed. Plates can then be used or stored at 4 °C. It is unclear what determined the decision to let the plates dry for 1-3 days. Again weights of plates become an issue when talking about heating the whole plate on which the animals live. What is the OD of OP50 you use to seed plates? How big a lawn? Do these aspects matter?

133-134 "2. Heat shock worms by wrapping plates with Parafilm and submerging in a circulating water bath at 33 °C for 1 hr." Why 1 hr? How quickly is the response induced? What is the difference between these different responses?

134-136: "A thin strip of Parafilm should be wrapped twice around the plate to seal the edges. Do not cover the bottom of the plate or it will interfere with heat transfer". Again, it seems like many of the manipulation described here for heat shock will affect heat transfer. Does the rate of heat transfer affect the results? If so, how?

165-166: "…levamisole can be soaked up using a Kimwipe. Note: image as soon as possible as prolonged incubation in levamisole can alter fluorescence. How long is 'prolonged"?

238- 239: "7. Perform qPCR. It is recommended to use a commercially available kit such as iTaq Universal SYBR Green Supermix and to follow the manufacturer's instructions. No mention has been made about matching the efficiency of amplification or generating standard curves.

293-295: "..tested. We found that both of the protocols resulted in robust induction of the two fluorescent reporters using our conditions, although a circulating water bath is recommended as a best practice (see discussion) (Figure 1B)." Is robust induction of the reporters a sufficient measure for all the studies the authors are expecting to persuade to use their methods? Do plates dry out in a dry incubator? How do these two studies compare?

322-323: "… tested using protocol IV. We found that exposure of worms to a 6 hr heat shock at 33 °C leads to a 20% decrease in worms with normal movement after a 48 hr recovery (Figure 4A). " What is 'normal movement'? How was this measured?

326-327: "..a dramatic decrease in normal movement, with greater than 95% of worms showing abnormal movement." What are you measuring? Speed? Body bends?

376-377: ".. heat shock of 33 °C for 1 hr is robust enough to elicit strong induction of gene expression changes, yet mild enough so that worm viability is not affected. Indeed, exposure to 33 °C for 6 hr only." It has been shown that HSF1 binds its promoter within minutes of exposure to heat, and mRNA is induced with 5 min HS. By 30 mins mRNA continues to accumulate but one cannot tell transcription from mRNA stability. It is unclear what justifies picking this time and temperature for all studies.

Minor Concerns:
Line 46: Heat shock factor 1 should be spelt out the first time it is used

This has been addressed (see lines 46-47).