TITLE:
Single-animal, single-tube RNA extraction for quantitative analysis of transcripts in the tardigrade *Hypsibius exemplaris*

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KEYWORDS:
Single-tardigrade RNA extraction, Single-tube, qRT-PCR, Heat-shock, Transcriptional Profiling

SUMMARY:
We present a rapid RNA extraction and transcript quantification method for analyzing gene expression in the tardigrade *Hypsibius exemplaris.* Using enzymatic and physical lysis, this high-throughput method requires a single tardigrade as the starting material and results in robust production of cDNA for RT-qPCR and subsequent quantification of transcripts.

ABSTRACT:
The tardigrade *Hypsibius exemplaris* is an emerging model organism renowned for its ability to survive environmental extremes.[1–3] To explore the molecular mechanisms and genetic basis of such extremotolerance, many studies rely on transcriptional profiling[4, 5] and RNA interference (RNAi)[6] to define molecular targets. Such studies require efficient, accurate, and robust RNA extraction methods; however, obtaining high-quality quantitative levels of RNA from *H. exemplaris* has been challenging[6, 7]. Possessing a layer of firm chitinous cuticle, tardigrade tissues are difficult to disrupt by chemical or mechanical means[8]. Here we present an efficient single-tardigrade, single-tube RNA extraction method (STST) that not only reliably isolates RNA from individual tardigrades but dramatically reduces the time required for extraction. We show that this RNA extraction method yields robust quantities of cDNA and can be used to amplify multiple transcripts by qRT-PCR. To validate the method, we use it to compare dynamic changes in expression of genes encoding two heat-shock-regulated proteins, Heat-Shock Protein 70 $\beta 2$ (HSP70$\beta 2$) and Heat-Shock Protein 90α (HSP90α) by quantifying their expression levels in heat-exposed and cold-exposed individuals using qRT-PCR across long-term and short-term heat stressors. Our method effectively complements existing bulk RNA extraction methods[7], permitting rapid examination of individual tardigrade transcriptional data and quantification of phenotypic variations in expression profiles amongst individuals.

INTRODUCTION:

Tardigrades are small multicellular animals that are renowned for their ability to survive extreme conditions that are lethal to most other forms of life[9]. For example, these animals can survive nearly 1000x the dose of ionizing radiation[10, 11] that is lethal to humans, nearly total desiccation[12], freezing in the absence of added cryoprotectants[13], and even the vacuum of space[3]. Owing to their unique capacity for survival in the extremes, they have become a foundational model organism for understanding extremotolerance in complex, multicellular organisms[2, 14].

Stable genetic manipulation of these remarkable animals, including transgenesis and germline gene modification, has remained elusive until very recently[15, 16]. As such, most experimentation to reveal molecular mechanisms of extremotolerance is performed through transcriptional profiling[4, 5, 17, 18]. To test the functionality of identified genes, such studies are often followed by RNAi-mediated knockdown of molecular targets[6] and quantification of extremotolerant capacity after knockdown[5]. To facilitate these transcriptional profiling studies, bulk RNA extraction and purification methods have been developed[7], and have illuminated our molecular understanding of cryptobiosis, specifically anhydrobiosis. However, because of the extremely small body size, low cell numbers, and difficulty of disrupting the cuticle of tardigrades, these preparations typically require 100 animals to yield sufficient RNA for the quantification of transcripts. Such bulk extraction of RNA transcripts from many animals prevents analysis of variation in gene expression between individuals, thus overlooking the potential richness of more refined data sets. Importantly, these studies often analyze heterogeneous populations of animals that include both animals that survive environmental stressors and those that do not. As such, the studies are confounded by averaging expression data from multiple and potentially dramatically different states of response[4, 5, 18]. Although previous methods have been developed to extract RNA from single tardigrades, these protocols as reported in the literature do not yield concentrations of RNA that are quantifiable by Real-Time PCR; rather they rely on optical density-based methods to monitor transcriptional changes[6, 19]. This limitation is likely attributable to the lack of a chemical lysis step before the physical lysis process **(Figure 2)** or the subsequent extensive purification steps that can result in decreased yields of RNA[19]. Motivated by these challenges, we sought to develop a protocol that yields RNA in high quantities that can be used for qRT-PCR from single animals.

We adapted a single-animal RNA extraction protocol developed for *Caenorhabditis elegans*[20] by optimizing it for *H. exemplaris*. The extraction method consists of a chemical-enzymatic lysis step, followed by rapid freeze-thaw cycling to physically disrupt the cuticle, allowing for a thorough extraction of RNA and subsequent cDNA synthesis. We found that our method decreases extraction time by more than 5-fold, shortening the extraction process required in previous bulk preparations[7] from 4 hours to 45 minutes. Further, the number of sample-experimenter interactions is decreased from 7 to 1, reducing the risk of contamination by exogenous ribonucleases and thereby increasing preparation success rates. The method produces sufficient quantities of RNA and cDNA to generate 25 quantitative RT-PCR reactions per single tardigrade.

We evaluated the efficacy of our method for analyzing dynamic changes in gene expression by investigating the differential expression of the genes encoding heat-shock protein-90α (HSP90 α) and heat-shock protein 70β2 (HSP70β2) in response to short-term (20 minutes) and long-term (24 hours) heat treatment as normalized to actin

transcript levels. Both HSP70β2 and HSP90α in most eukaryotic organisms are rapidly upregulated following short-term heat-shock exposure (10-20 minutes)[20–22] In tardigrade *Ramazzottius varieornatus,* HSP90α had originally been reported to be downregulated following prolonged periods (24 hours) at elevated temperatures while HSP70 had been reported to be either upregulated or downregulated in an isoform dependent manner; however, a recent corrigendum revealed that HSP90α is actually upregulated in response to prolonged heat exposure[18]. Our analysis revealed that both the HSP70β2- *and* the HSP90α-encoding RNAs extracted from single heat-treated tardigrades showed robust increases in expression following short-term (20 min) heat exposure; however, while the RNA for HSP90 in *H. exemplaris* maintained upregulation following long-term heat exposure this was not seen for HSP70, unlike what has been reported for *R. varieornatus*[18]. These findings demonstrate that our protocol can be used to reliably analyze dynamic changes in gene expression in individual animals over time.

Our extraction method effectively complements current experimental methods available to the tardigrade research community by facilitating rapid extraction of RNA for quantification from individual tardigrades. Further, the analysis of single animals makes it possible to investigate natural variation in expression among individuals in response to imposed stresses or changes in other extrinsic conditions. This method will also be valuable for assessing the efficiency and penetrance of RNAi in individuals, in a more quantitative manner than optical density alone. Finally, owing to their similar cuticular structures and physical characteristics, it is likely that this method will also be effective for analyzing gene expression in other tardigrade species[8].

PROTOCOL:

Detailed tardigrade and algal culturing procedures were performed exactly as described in McNuff et al. 2018[23, 24].

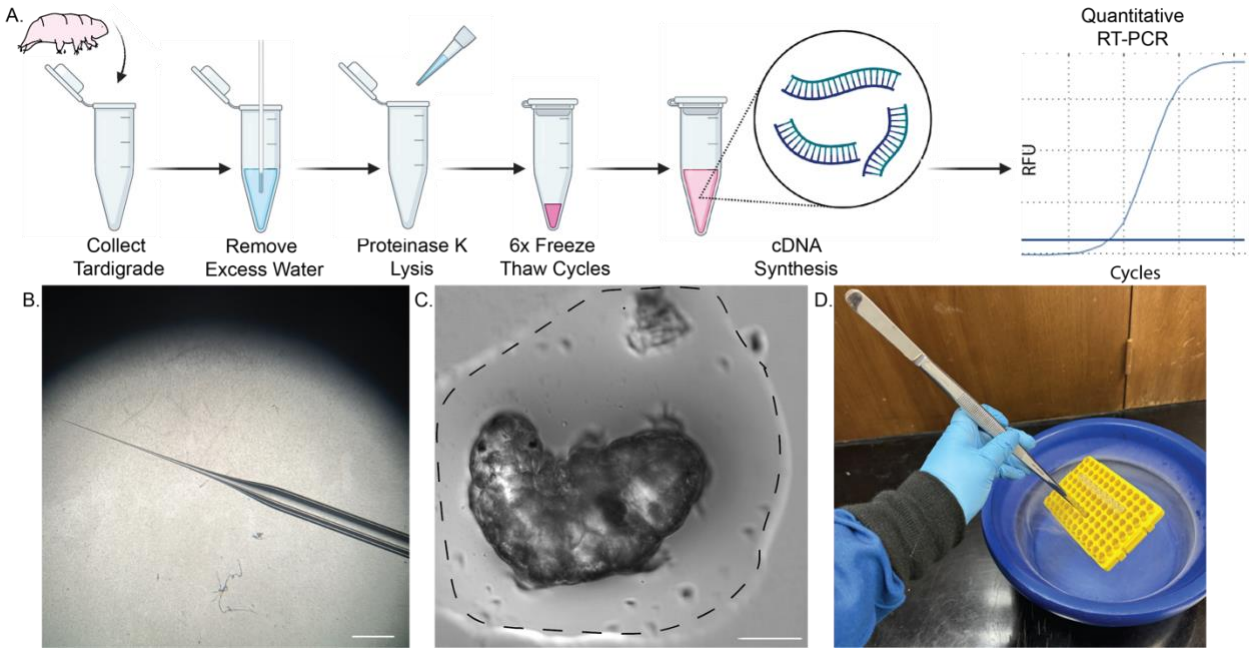

Figure 1. Single-tube pipeline for RNA extraction from a single tardigrade. a) Scheme showing the protocol for RNA extraction from a single tardigrade including

proteinase K lysis, six freeze-thaw cycles, and reverse transcription and cDNA synthesis. Samples may subsequently be used for RT-PCR and qRT-PCR. **b)** Image of micropipette taper used for removal of water. Scale, 2 mm. **c)** Bright field image of a tardigrade in a small volume of water (dotted line). Removal of most water to the extent shown is required for successful extraction and prevents dilution of lysis buffer (Scale is 50 μm). **d)** Image showing immersion of samples in liquid nitrogen using long forceps to rapidly freeze-thaw the samples safely.

Glass micropipette pulling

1. Secure a glass micropipette (O.D. 1 mm ID 0.58 mm, Length 10 cm) on a micropipette puller. Be careful to avoid contact with the heating filament as this will alter the pipette shape and damage the filament. The pulling of the pipette will need to be determined empirically for each filament and pipette puller. However, to serve as a starting point for optimization, we use 78°C and a single pull step of pull weight of 182.2 grams.

2. Allow the filament to heat and gravity to separate the glass micropipette into two sharp points **(Figure 1b)**.

3. Store these pipettes in a closed 10 mm petri dish with wax or clay to hold the pipette in place and prevent the breaking of the sharp tips.

RNA extraction

1. Prepare Tardigrade Lysis Buffer (5 mM Tris (pH=8), 0.5% (v/v) Triton X-100, 0.5% (v/v) Tween 20, 0.25mM EDTA in MilliQ Water). This solution can be stored for up to 6 months on the bench top but be careful to maintain sterility and avoid potential RNAse-contaminating sources.

2. Aliquot enough lysis buffer for your extractions (2 μL/ tardigrade).

3. Add fresh Proteinase K to the Tardigrade Lysis Buffer for a final concentration of 1 mg/ml. Vortex and spin down the solution on a bench-top centrifuge before storing the solution on ice.

   NOTE: *The lysis buffer containing Proteinase K should be made fresh before use and should always be stored on ice. Do not freeze-thaw before use.*

4. Add RNAse inhibitor to the Proteinase K/Tardigrade Lysis Buffer solution to a final concentration of 1 unit/μL. Gently vortex this solution and spin it down on a bench-top centrifuge before placing the lysis solution on ice.

5. Remove tardigrades from the culture using a sterile pipette and place them in a 35 mm Petri dish. Wash the tardigrades three times using 1 mL sterile of spring water to remove algal contaminants.

6. Transfer a single tardigrade from this washed culture to a glass 9-well plate using a sterile pipette. Use a pipette to wash the single tardigrade in 100 μL of nuclease-free water. This wash step is used to further remove contaminants, including ribonucleases.

7. Transfer the washed tardigrade to the bottom of a clean/sterile PCR tube in 1-2 μl of nuclease-free water.

8. Remove as much water as possible using a glass capillary micropipette. You may break the tip of the needle lightly outside of the tube to facilitate water removal.

   NOTE: *This is a critical step. The tardigrade should be surrounded by a small bubble of water to prevent it from drying out but as much excess water as possible should be removed to prevent dilution of the lysis buffer.* ***(Figure 1c.)***

9. Immediately after removing the water, add 2 μL of Tardigrade Lysis Buffer with Proteinase K to the bottom of the tube and briefly vortex and centrifuge the tube on a tabletop centrifuge to ensure that the sample is completely suspended in the buffer and spun to the bottom of the tube.

10. Incubate the sample for 15 minutes at 65°C in a thermocycler, then for 1 minute at 85°C to terminate the Proteinase K reaction.

11. During the Proteinase K digestion, obtain 0.5 L of liquid nitrogen in a cryo-safe container.

    CAUTION: *Liquid nitrogen is cryogenic and may cause burns if exposed to skin or eyes. Use protective clothing, splash goggles, nitrile gloves, cryo-gloves, a lab coat, and closed-toed shoes when handling. Ascertain that your container is liquid nitrogen safe before transporting the liquid.*

12. After the proteinase treatment and deactivation, immediately return the samples to ice to prevent degradation.

13. Place the samples containing heat-treated tardigrades into a PCR Tube rack and ensure that they are held tight by the rack. **(Figure 1d)**.

14. Grip the rack using a pair of long coarse forceps and gently dip the rack containing your samples into the liquid nitrogen until fully frozen.

15. Remove the sample from the liquid nitrogen and briefly dry the sample by allowing the liquid nitrogen to evaporate. Then vortex the sample at max speed (approximately 3200 RPM) until the sample is completely thawed for at least 15 seconds.

16. Repeat steps 14-15 five more times. We have found that six freeze-thaw cycles are required for maximal lysis and extraction. (**Figure 2**)

17. Once the freeze-thaw is complete, place samples on ice and *immediately* progress to the next step. Samples may not be frozen at this point for storage as this will diminish available RNA for cDNA preparation.

cDNA Synthesis

1. Make cDNA synthesis master mix. We use Maxima H Minus cDNA Synthesis Kit: a 10 µL solution containing 1 µL of random hexamer primer, 2 µL of DNase, 4 µL of 5x RT Buffer, 1 µL Enzyme Mix, 1 µL of H_2O, and 1 µL of 10 mM dNTPs.

2. Add 2 µL of cDNA synthesis master to the PCR tube containing tardigrade lysate. Briefly flick the tube and spin it down with a tabletop centrifuge, replacing the samples on ice.

3. Place the samples in a thermocycler and incubate at 25°C for 10 minutes to elongate primers, at 55°C for 30 minutes to perform reverse transcription, and finally heat inactivate enzymes at 85°C for 5 minutes.

4. After the incubation, immediately place the tube on ice and dilute the sample to a total volume of 25 µL by adding 21 µL of nuclease-free water. For extremely low copy number transcripts this dilution step can be altered as determined empirically.

qPCR

The annealing temperature of your primer set should be determined using a temperature gradient on a PCR reaction before running RT-qPCR (for all PCR settings used in this protocol refer to **Table 1 and 2.)**

Table 1. Thermocycling conditions for the Actin and HSP90 PCR reaction

Step	Temp. (°C)	Duration	No. of cycles
Initial denaturation	95	60 s	1
Denaturation	95	15 s	35
Annealing	TM	30 s	
Extension	72	1 min	
Final Extension	72	5 min	1
Hold	4	∞	-

Table 1. PCR thermocycling protocol for non-quantitative PCR. The table describes the exact thermocycling procedure used for this study. TM is denoted as the melting temperature for a reaction, the specific melting temperatures for each reaction can be found in **Table 2.**

Table 2. Melting temperature or TM for each primer pair used in this study.

Primer Set Name	Fwd Sequence	Rev Sequence	TM
Actin	CCTCAGAACAGTCGCAATGG	CCAGAGTCCAGCACGATAC	62
HSP90	CTTCACCATCCAGACGGACA	CCCAATCGTTAGTGAGGC	62
HSP70	ACGTGGTGAAGACTTGC	TGAAGCCAGCATTGAGA	65*
* 72°C (-2°C/cycle) for six cycles before a TM at 65 °C for non quantitative PCR			

Table 2. Melting temperature and sequences for primers used in this study. The table shows the forward and reverse sequences of primers used for this study and their optimized melting temperature. It is important to note that HSP70 required a brief touch-down protocol consisting of 6 cycles before the amplification to ensure specificity.

1. Thaw one tube of indicator dye supermix on ice and isolate from light. Place a 96-well PCR plate on ice and place 5 μL super mix, 2μL of water, 1 μL of each primer (100μM), and 1μl of cDNA product in the number of desired wells.

2. Seal the PCR plate with plate seal and run the Real-Time quantitative PCR using an annealing temperature appropriate for your primer set. (For all qPCR settings used in this paper refer to **Table 3.)**

Table 3. Thermocycling conditions for the qPCR

Step	Temp. (°C)	Duration	No. of cycles
Initial denaturation	95	60 s	1
Denaturation	95	15 s	30
Annealing Extension	TM	30 s	
Denaturation	95	10 s	1
Melt Curve Analysis	65 (+0.5°C)	10 s	60

Table 3. Thermocycling conditions for qPCR. The table depicts the thermocycling settings for qPCR reactions used in this study. The melting temperatures or TMs we identical to those shown in **Table 2.**

Quantification and Results Interpretation

1. Results are compared quantitatively to one or more control housekeeping genes, whose expression is expected to be constant over the imposed conditions. For our

study, we used the actin gene as our normalization control.

2. The Ct-values or cycle threshold for each well are obtained and compared to the Ct values of the control housekeeping gene reactions. The fold-change in gene expression is calculated using the following equation:

$$\Delta Ct = Ct(gene\ of\ interest) - Ct(housekeeping\ gene)$$
$$\Delta\Delta Ct = \Delta Ct(Sample) - \Delta Ct(Control\ average)$$
$$fold\ gene\ expression = 2^{-(\Delta\Delta ct)}$$

3. Fold gene expression is then plotted for each transcript and tardigrade as a $2^{-(\Delta\Delta Ct)}$.

4. In order to obtain a rough estimate of the transcript number from the Ct-value, we used the following equation:

$$N = 10x2^{(35-Ct)}$$

Where N is the number of transcripts and 2 is the assumed PCR efficiency or the fold increase in fluorescence per cycle of PCR[25].

RESULTS:

Development and optimization of single-tardigrade RNA extraction.

Adapting our protocol from Ly et. al, 2015[20] for RNA extraction in tardigrades, we optimized our system (**Figure 1a**) to maximize the quantity and quality of the preparation. As a readout for the quality and quantity, we performed RT-PCR of tardigrade actin transcripts by amplifying a 527 bp region spanning exons 1 and 2. We subsequently quantified the optical density of the expected actin band as fluorescence intensity using ImageJ/FIJI. All regions of interest quantified were of equal areal size across each gel.

We first assessed the methods of mechanical lysis required for breaking the cuticle of the tardigrade to extract RNA. In contrast to a recently published single-animal, single-tube RNA extraction protocol for *C. elegans*, which found that Proteinase K lysis and a heat-shock were sufficient to lyse the animals[20], we found that tardigrade extraction required a minimum of six freeze-thaw cycles to achieve consistent and robust RNA extraction (**Figure 2a, Figure S1**). To minimize freeze-dependent shearing and degradation, we kept the freeze-thaw cycle number at this minimal value throughout the remainder of our preparations. At lower numbers of freeze-thaw cycles (1-5), we found a higher concentration of genomic DNA (gDNA) products (higher molecular weight bands than expected 527 bp for the actin molecule) suggesting that the freeze-thaw process prior to dsDNA removal by DNase is required for complete removal of gDNA (**Figure 2a, Figure S1**). Ultimately, both the consistency of extraction success and the yield, as measured by optical density, increased with higher numbers of freeze-thaw cycles, with 6 cycles showing a 3-fold increase in cDNA yield as compared to no freeze-thaw cycling (**Figure 2b**).

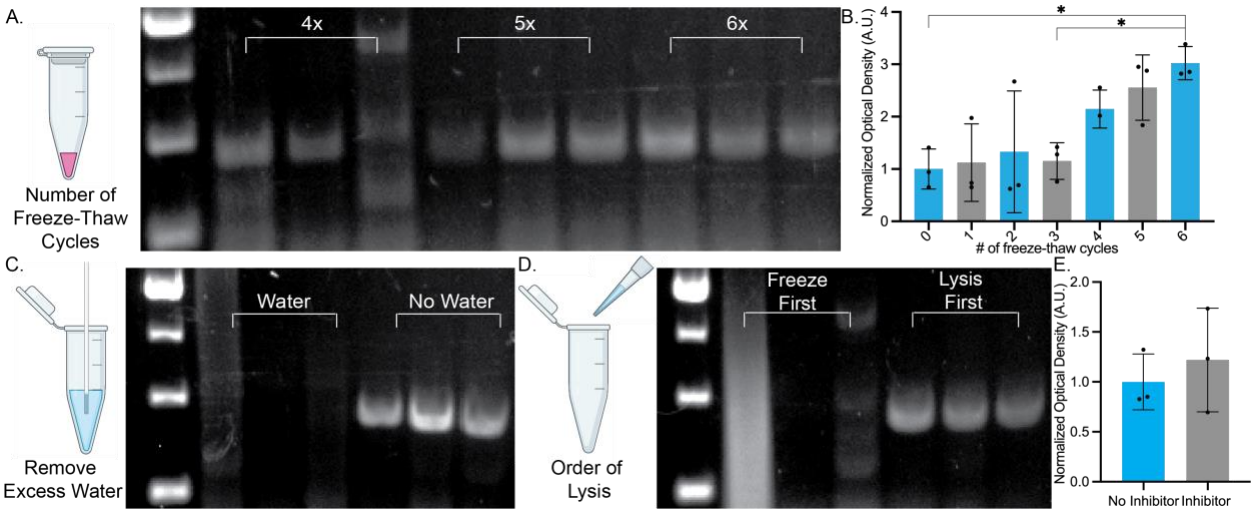

Figure 2: Optimization of single-tardigrade RT-PCR using actin cDNA as a marker for extraction quality. a) Representative gel depicting results from single-tardigrade RT-PCR extracted using 4 (lane 2-4), 5 (lane 5-7), and 6 (lane 8-10) freeze-thaw cycles to enhance lysis after proteinase K treatment and heat-shock. Full gel-containing samples from 1, 2, and 3 freeze-thaw cycles are shown in **Figure S1**. **b)** optical density quantification of ethidium bromide staining of actin RT-PCR across various freeze-thaw cycle numbers. Data represent optical density values of bands from three individual trials of actin RT-PCR per condition with statistical analysis applying Brown-Forsythe and Welch ANOVA, with Dunnett's T3 multiple comparisons test 0 vs 6, p= 0.0202 and 3 vs 6 p=0.0222, error bars represent SD. **c)** Representative gel showing the effect of removal of residual spring water from isolated tardigrades prior to chemical lysis. Samples in which water was not (lanes 1-3) and was (lanes 4-6) removed. **d)** Representative gel showing the effect of lysis order, with freeze-thaw lysis performed prior to chemical lysis (lanes 1-3) or chemical lysis with proteinase K prior to freeze-thaw lysis (lanes 4-6). **e)** Optical density quantification of ethidium bromide staining of actin RT-PCR performed in the presence or absence of RNAse inhibitor. Data represent optical density values of bands from three individual trials of actin RT-PCR per condition. Unpaired T-Test, p=0.5524 error bars represent SD. 5 μL of PCR products were loaded per lane unless otherwise noted.

We next assessed whether the near complete removal of excess water carried over from the transfer of the sample to the tube was required for consistent lysis of single tardigrades. Our rationale was, given the minute volume of lysis buffer which was minimized to prevent dilution of RNA for each sample, any residual water would significantly dilute the lysis buffer and prevent accurate concentrations of proteinase K, detergents (Triton X-100 and Tween), and EDTA, all of which are required for proper lysis. **Figure 2c and Figure S2** show results in triplicate of individual tardigrade extracts in the presence and absence of residual water from the tardigrade transfer. These data suggest that the removal of excess water is imperative, as RNA extraction and subsequent reverse transcription do not occur in the presence of excess water.

Further optimizing our method, we sought to determine whether the order of lysis is important for achieving robust extraction of RNA. We found that if the freeze-thaw lysis step was performed before the Proteinase K and enzymatic lysis, little to no RNA was

extracted and subsequently amplified by RT-PCR, as shown by the absence of bands in lanes 1-3 of **Figure 2d.** When chemical lysis is performed before freeze-thaw lysis, however, PCR products from RT-PCR were readily obtained as shown in lanes 4-6 of **Figure 2d**. This suggests that Proteinase K digestion of the cuticle is required prior to mechanical lysis to promote robust extraction of RNA. It is also possible that the proteinase K inactivates RNAse present during lysis, thereby preventing degradation of RNA after extraction from the animal.

Our final step in optimization was to assess the requirement for RNAse inhibitor prior to chemical lysis. We found that yields from reactions containing inhibitors were not significantly different from those without (**Figure 2e**). We nonetheless continued to use inhibitors throughout the remainder of the study as a precautionary measure.

Comparison to existing tardigrade RNA extraction protocols

In developing our RNA extraction protocol, we aimed to limit the time required to perform the technique, the number of sample-experimenter interactions, and the number of animals required for robust RNA extraction. In **Figures 3a and b,** we schematize a commonly used bulk RNA extraction protocol (**Figure 3a**) and our novel single-tube, single-tardigrade (STST) RNA extraction method (**Figure 3b**), highlighting each method in terms of these three aspects. Previously used RNA extraction protocols required between 4 hours to 2 days and contained seven sample-experimenter interactions prior to cDNA synthesis. Our method dramatically decreases this time to approximately 45 minutes with only one sample-experimenter interaction. The removal of sample-experimenter interactions reduces the potential for RNAse contamination, thus increasing the potential for high-quality extractions. Currently, all published RNA extraction protocols that allow for qRT-PCR assessment of transcriptional changes use approximately 100 tardigrades to generate quantitative amounts of cDNA **(Figure 3a)**, while our STST method permits the extraction of RNA from individual tardigrades at quantifiable levels.

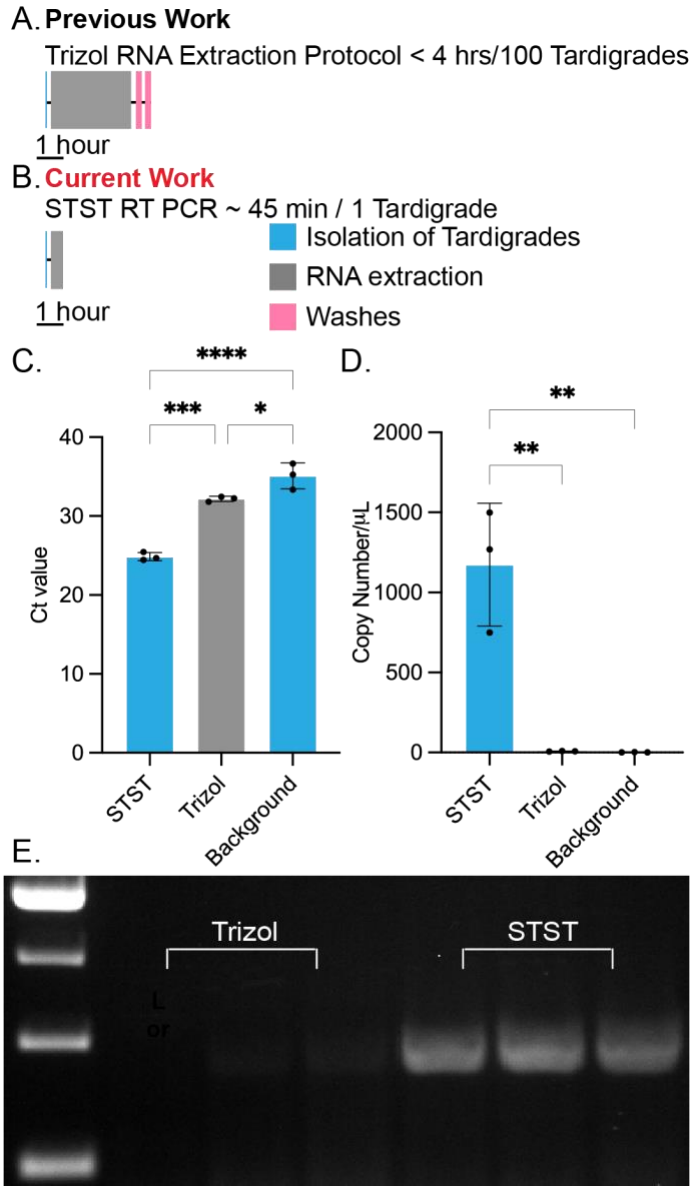

Figure 3. **Comparison of RNA extraction protocols.** Schematized time-course for **a)** an existing TRIzol®-based RNA extraction method, requiring 4 hours to 2 days and 100 tardigrades for extraction. Scale bar is 1 hour. **b)** Our single-tube, single-tardigrade RT-PCR protocol, which permits extraction in 45 minutes and requires only one tardigrade. The time courses are drawn to scale. Scale bar is 1 hour. **c)** Graph showing C_t values of actin qRT-PCR reactions run in triplicate using single-tardigrade extracts from STST RNA extraction, single-tardigrade-based extraction and the background control revealing background fluorescence from samples run on qRT-PCR in the absence of template. One Way ANOVA, Holm-Sidak's multiple comparisons test *, p= 0.0129 ***, p=0.0002 and ****, p<0.0001 error bars reflect SD. **d)** Estimated values of transcripts per µL of sample derived from the C_t values depicted in **(c).** One-way ANOVA, Tukey's multiple comparisons test **, p=0.0016 error bars reflect SD. **e)** Representative gel showing actin RT-PCR from single-tardigrade cDNA samples extracted using TRIzol®-based methods (lanes 2-4) and STST lanes (5-7).

To directly compare the efficacy of STST extractions with existing TRIzol®-based methods, we performed single-tardigrade extractions using both methods and quantitatively assessed transcript numbers using qPCR. Amplifying a 527 bp region of the housekeeping gene encoding actin, we used C_t value, i.e. the cycle number required for the fluorescence signal to be detected above the background, as an indicator of cDNA quantity. We found that on average the C_t value of single tardigrades extracted using STST was 23.49 +/- 0.52 **(Figure 3c)** while those extracted with traditional TRIzol® methods were 32.18 +/- 0.19 **(Figure 3c). Figure 3c** depicts these C_t values, showing that STST yields significantly more cDNA as compared to TRIzol®-based methods and that both extracted samples gave a signal that was above the background fluorescence obtained from sample qPCRs run in the absence of template cDNA. Using these C_t values, we were able to roughly estimate the transcript number per µL of cDNA. STST yielded ~1173+/-221 actin transcripts per µL while TRIzol®-based methods yielded ~ 7.181 +/- 1 transcripts of actin per µL **(Figure 3d)** suggesting that as a single-tardigrade extraction method, our system is >150-fold more efficient. When quantified, STST produced significantly more transcripts than TRIzol® extraction methods, which were statistically indistinguishable from background measurements **(Figure 3e).** This suggests that TRIzol®-based methods for single tardigrades do not sufficiently extract and protect the minuscule amount of RNA, resulting in undetectable quantities of cDNA. To visualize this effect, RT-PCR products from these samples were run on an analytical gel, confirming minimal amplification of TRIzol®-extracted products (**Figure 3f,** lanes 2-4) and robust amplification in the STST-extracted samples (**Figure 3f,** lanes 5-7).

Application of the single-tube single-tardigrade extraction protocol for quantifying heat-shock responses in H. exemplaris*.*

Previous work by Neves et al.[18] showed large-scale transcriptional changes in *R. varieornatus* tardigrades when exposed to heat (35°C) and cold (4°C) over 24 hours. Two key proteins highlighted in this work were HSP90 and HSP70 (**Figure 4a**) which are both molecular chaperones involved in stress response and protein folding. We replicated the previous experiment with *H. exemplaris* by treating tardigrades with prolonged heat (30 °C) or cold stress (4°C) **(Figure 4b**). Tardigrades in their active state are highly sensitive to increases in temperature[18]; thus, we selected 30°C as the high temperature for *H. exemplaris*, as this resulted in minimal lethality (**Figure S7**). We then extracted RNA from single tardigrades, generated cDNA, and analyzed the expression levels of HSP70β2 and HSP90α normalized to actin by qRT-PCR. The Ct values of actin amplicons for these samples are shown in **Figure 4c.** We subsequently quantified HSP70β2 (**Figure 4d**) and HSP90α (**Figure 4e**) transcripts, which revealed no significant difference in HSP70β2 expression between heat- and cold-treated tardigrades, but significantly elevated levels of HSP90α transcripts in heat-treated tardigrades. This lack of upregulation of H HSP70β2 transcripts suggests that different species of tardigrades may have differing roles for HSP70β2 under extreme and prolonged stressors.

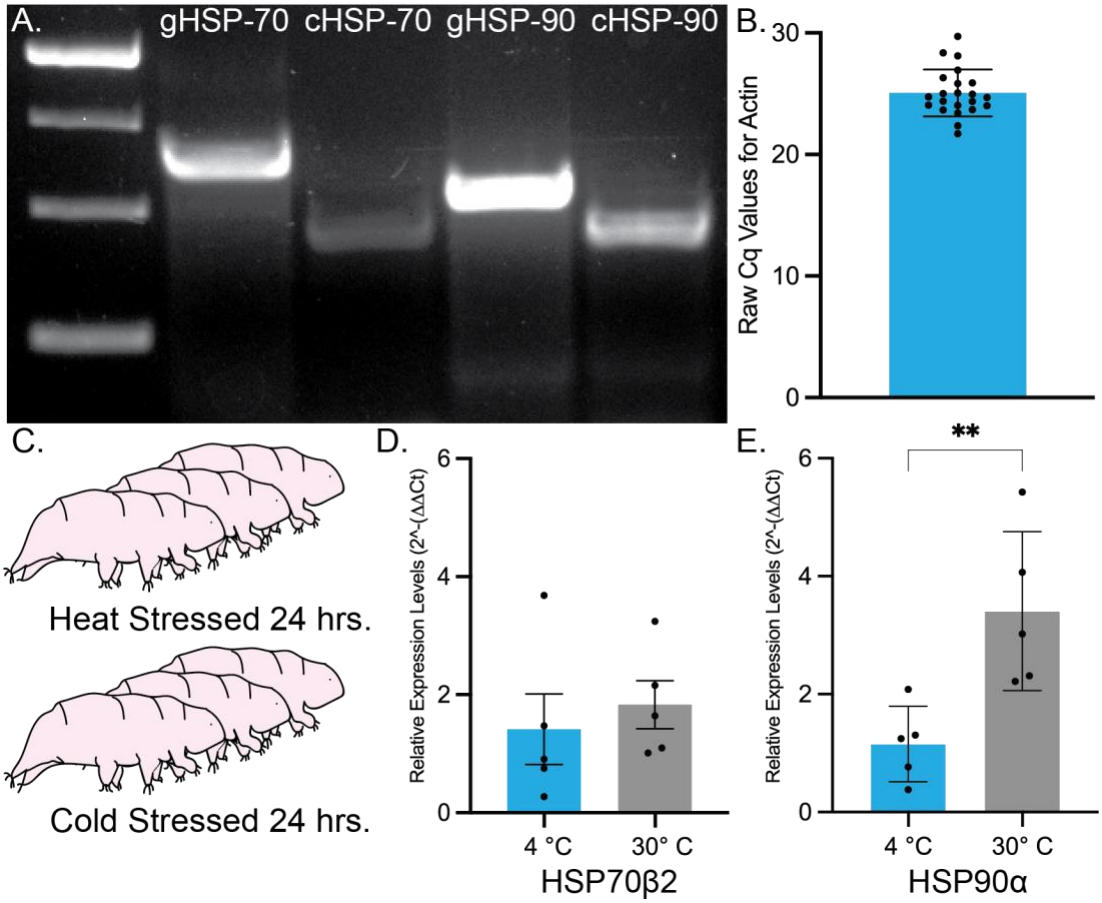

Figure 4. Expression of HSP-70 and HSP-90 transcripts. a) Representative gel showing amplification of HSP70 from genomic DNA (gDNA; lane 1) and cDNA (lane 2) and HSP90 from gDNA (lane 3) and cDNA (lane 4). Variance in gDNA and cDNA lengths reflects the primer design, which spans an intron. **b)** Raw C_t values for actin qRT-PCR from across all samples. **c)** Conditions analyzed. Relative expression level of **d)** HSP70 and **e)** HSP90 in heat- and cold-stressed animals represented as $2^{-(\Delta\Delta Ct)}$ T-Test **, p=0.0097 error bars reflect SD. Each data point represents quantification of expression levels in one independent animal.

We next sought to explore the canonical heat-shock response pathway, which results in the upregulation of both HSP70β2 and HSP90α under brief exposure to high temperatures[20]. We found that this response follows a similar pattern to that seen in other organisms[20]. We first exposed tardigrades to either rearing temperature (23°C) or high heat (35°C) for 20 minutes extracted RNA from individual animals and generated cDNA using our STST method by harvesting immediately, 1 hour, 2 hours, 4 hours, and 6 hours after exposure to the heat-shock. We subsequently quantified HSP70β2 (**Figure 5a**) and HSP90α (**Figure 5b**) using real-time PCR. Significant upregulation of HSP70β2 (~35-fold) and HSP90α (~8-fold) was observed after 1 hour, and expression slowly returned to baseline over the next several hours. These results follow the

expected patterns of the heat-shock responses seen in *C. elegans*[20].

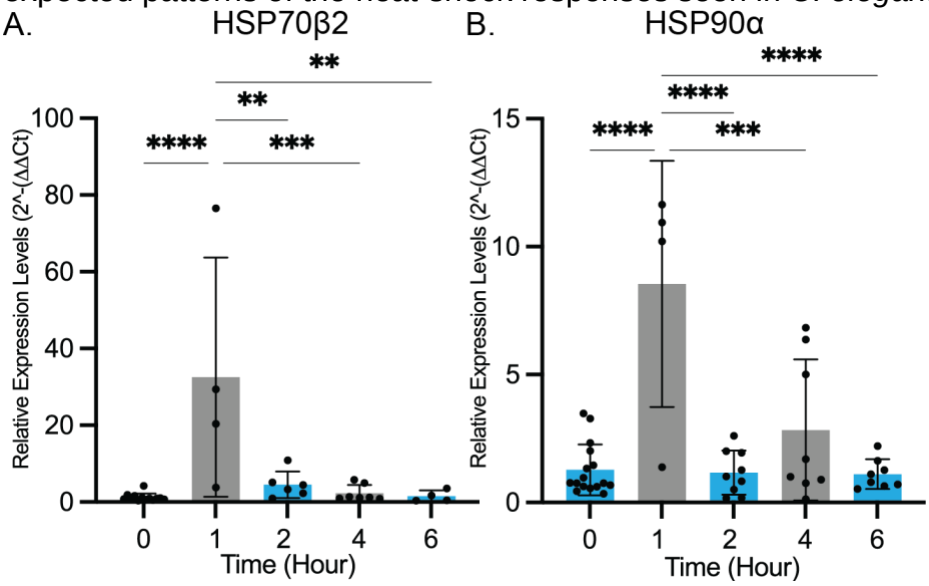

Figure 5. Tardigrade HSP70 and HSP90 short-term heat-shock response time course. a) Relative expression levels of HSP70 at the indicated time post-20-minute heat-shock at 35 °C. **b)** Relative expression levels of HSP90 at the indicated time post-20-minute 35°C heat-shock. One-way ANOVA with Tukey's multiple comparisons analysis **, p=0.0016 (1 vs 6) p=0.0017 (1 vs 2), ***, p=0.0005 and ****, p<0.0001 error bars reflect SD. Each data point represents expression levels from an individual tardigrade RNA extract.

DISCUSSION:

In this study, we present an efficient method for extraction of RNA in sufficient quantities to perform quantitatively robust single-tardigrade qRT-PCR. Directly comparing our methodology to existing TRIzol® extraction methods revealed that STST RNA extraction yields significantly higher RNA transcripts than TRIzol® extraction on single tardigrades. To apply STST to a relevant biological question, we replicated a previous study performed by Neves et al in *R. varieonatus*. As expected, we found that HSP90α was significantly upregulated after exposure to 24 hours of 30°C; however, unexpectedly we found that HSP70β2 remained unchanged after prolonged heat exposure. Finally, we assessed the short-term heat-shock response expression profile by treating animals at 35°C for 20 minutes. We found that both HSP70β2and HSP90α were strongly upregulated 1 hour after exposure, consistent with their roles as molecular chaperones that promote the refolding of heat-shocked proteins.

Although our protocol is a substantial improvement over previous methods, there remain several limitations associated with our current method that represent opportunities for improvement and further assessment. First, we have not evaluated the ability of STST to detect transcripts that are expressed at very low levels. To identify and quantify such transcripts, a linear amplification step may prove essential. Secondly, while our system showed a >90% success rate, there were occasions in which our method failed to obtain usable quantities of cDNA, perhaps as a result of RNAse activity; optimizing inhibition of RNAse activity might help to increase the success rate. Finally, owing to the high number of freeze-thaw cycles and subsequent fragmentation of the RNA, we have found that

while we can amplify targets of ~ 500 bp, the quality and quantity of RNA extracted from a single tardigrade is insufficient to obtain non-fragmented, high-quantity RNA that can be used for single-tardigrade RNAseq, as confirmed by quantification on an Agilent TapeStation. Different methods of mechanical lysis and/or linear amplification may likely be necessary to obtain long, high-quality RNAseq reads.

Overall, our findings represent an important step in the growing repertoire of molecular techniques available for use in tardigrade research. This method complements existing RNA extraction methods, which have provided for bulk preparation and quantification of transcriptional changes via RNA seq. STST allows for the quantification of transcripts isolated from single tardigrades. As most tardigrade extremotolerant responses, such as those triggered by desiccation, result in ~75-80% survival rates in *H. exemplaris*, most bulk samples contain mixed populations of tardigrades, including both survivors and non-survivors. Such a mixed population has the potential to confound analysis of transcriptional changes that occur during response and recovery from extreme stresses. In bypassing the need for bulk preparation, STST avoids the confounding problems inherent in a mixed population and allows for the full dynamic range of a response to be quantified. We envision that STST can be used not only to quantify the range of transcriptional responses and assess the efficacy of RNAi knockdowns, but will be used in many other applications in the future, including genome-wide transcriptomics of the animals.

DATA AVAILABILITY:

All raw data analytical gel data has been incorporated into the supplemental data of this manuscript. Transcript sequences were identified via Ensembl search at: https://metazoa.ensembl.org/Hypsibius_exemplaris_gca002082055v1/Info/Index?db=core HSP70β2(BV898_04401), HSP90α(BV898_50798) and Actin(BV898_02877).

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DISCLOSURES:

Authors declare no conflicts of interest to disclose.

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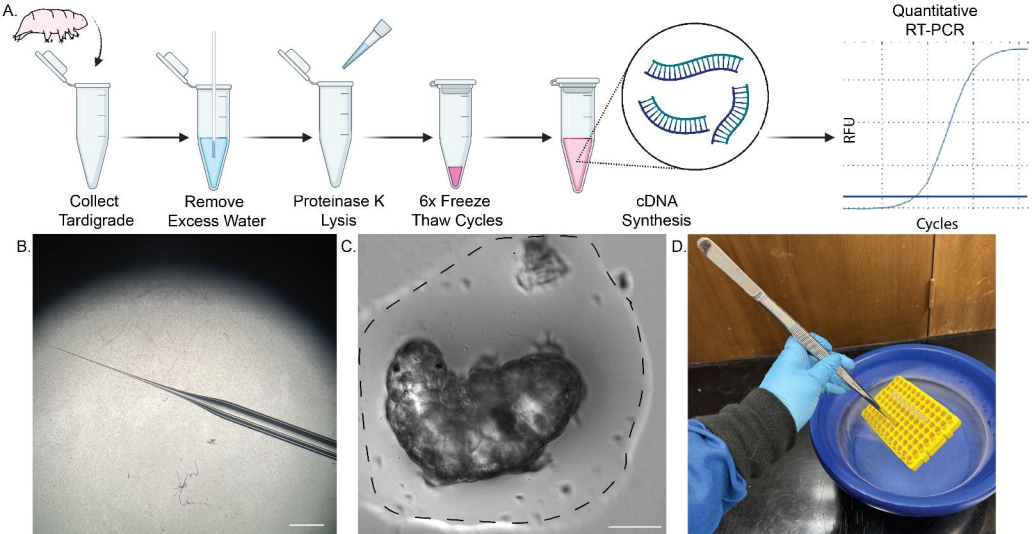
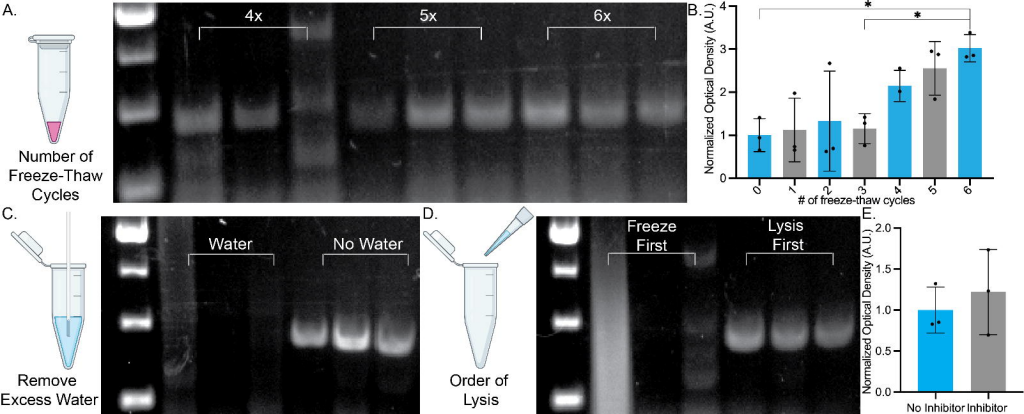

A.
Collect Tardigrade
Remove Excess Water
Proteinase K Lysis
6x Freeze Thaw Cycles
cDNA Synthesis
Quantitative RT-PCR
RFU
Cycles
B.
C.
D.


A.
Number of Freeze-Thaw Cycles
4x
5x
6x
B.
Normalized Optical Density (A.U.)
# of freeze-thaw cycles
C.
Remove Excess Water
Water
No Water
D.
Order of Lysis
Freeze First
Lysis First
E.
Normalized Optical Density (A.U.)
No Inhibitor
Inhibitor

A. **Previous Work**

Trizol RNA Extraction Protocol < 4 hrs/100 Tardigrades

1 hour

B. **Current Work**

STST RT PCR ~ 45 min / 1 Tardigrade

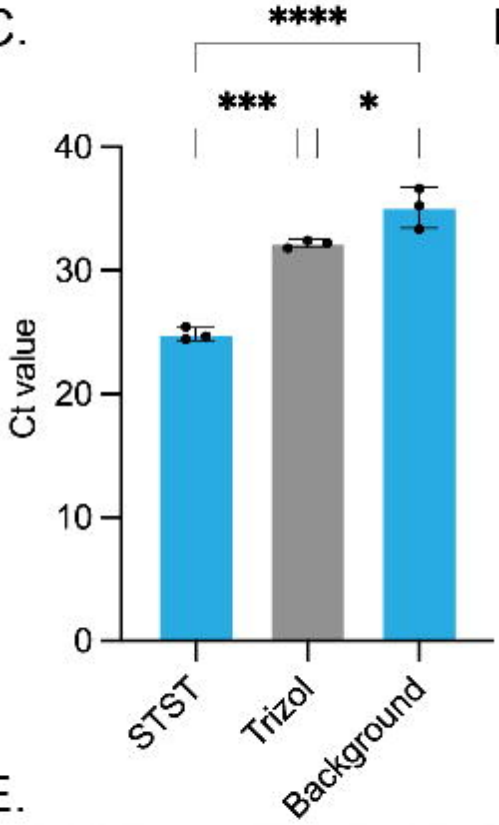
1 hour

Isolation of Tardigrades

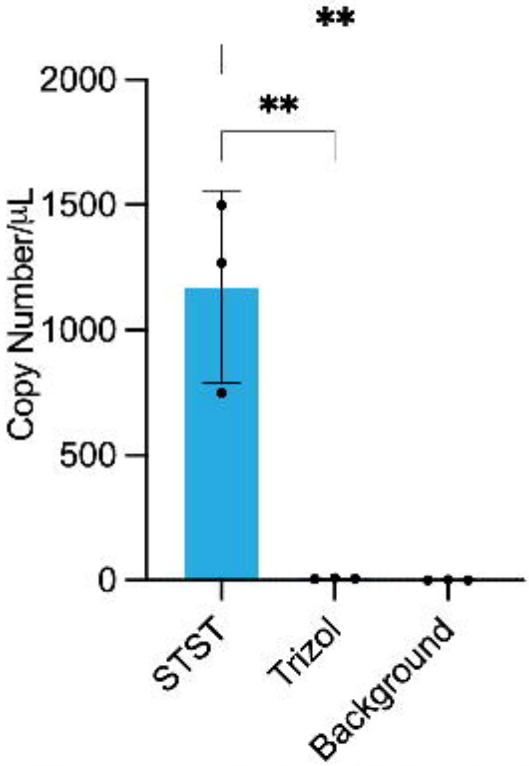
RNA extraction

Washes

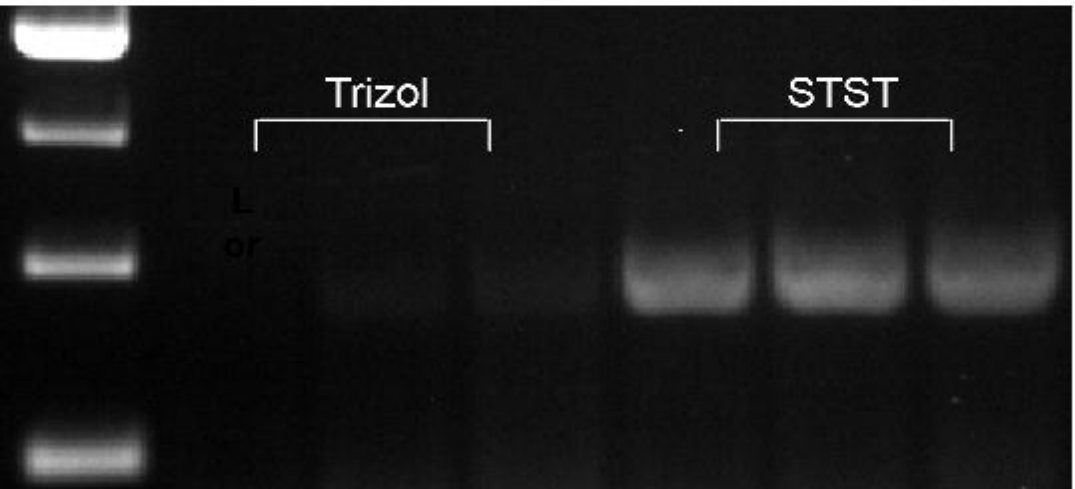
C.

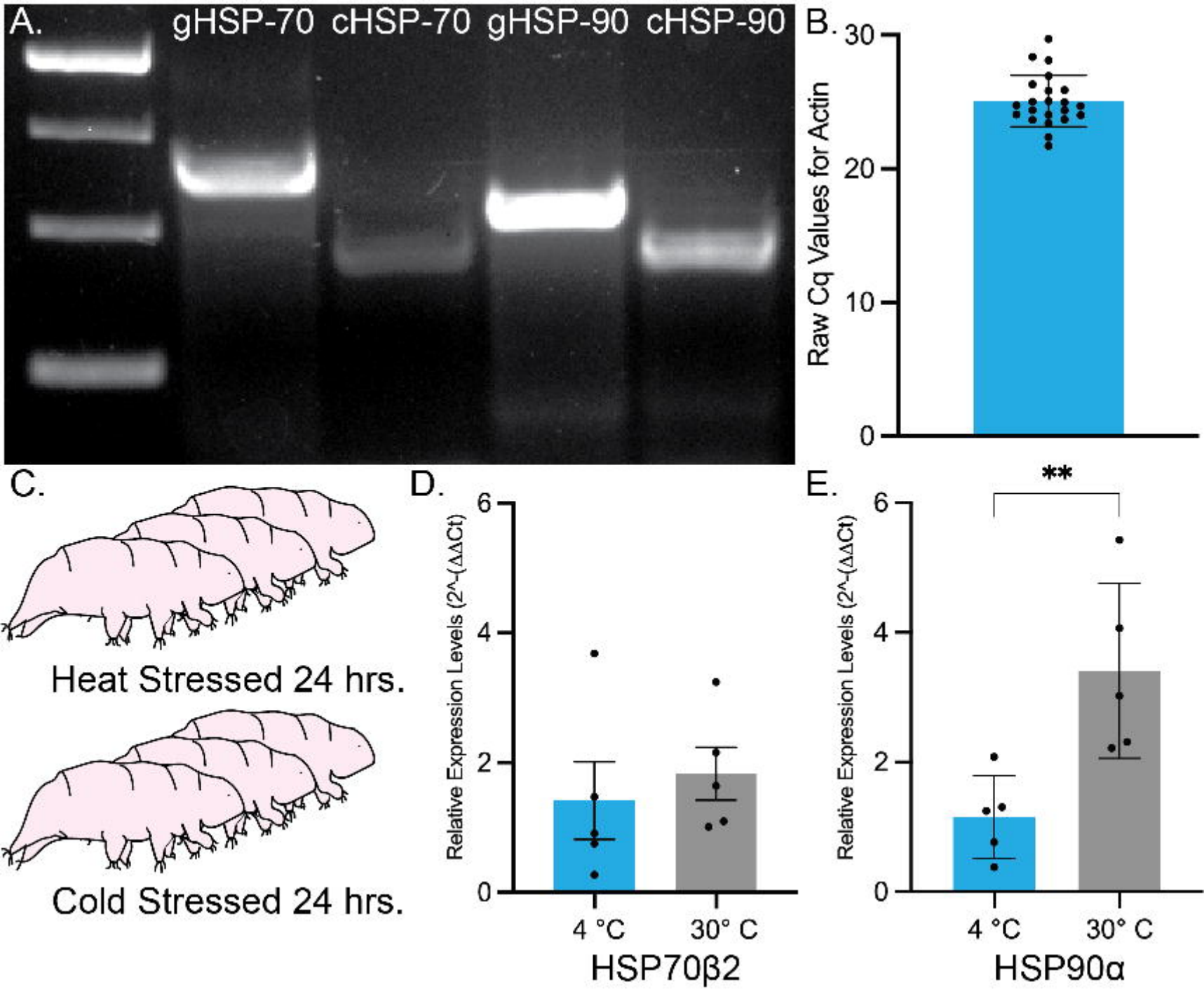


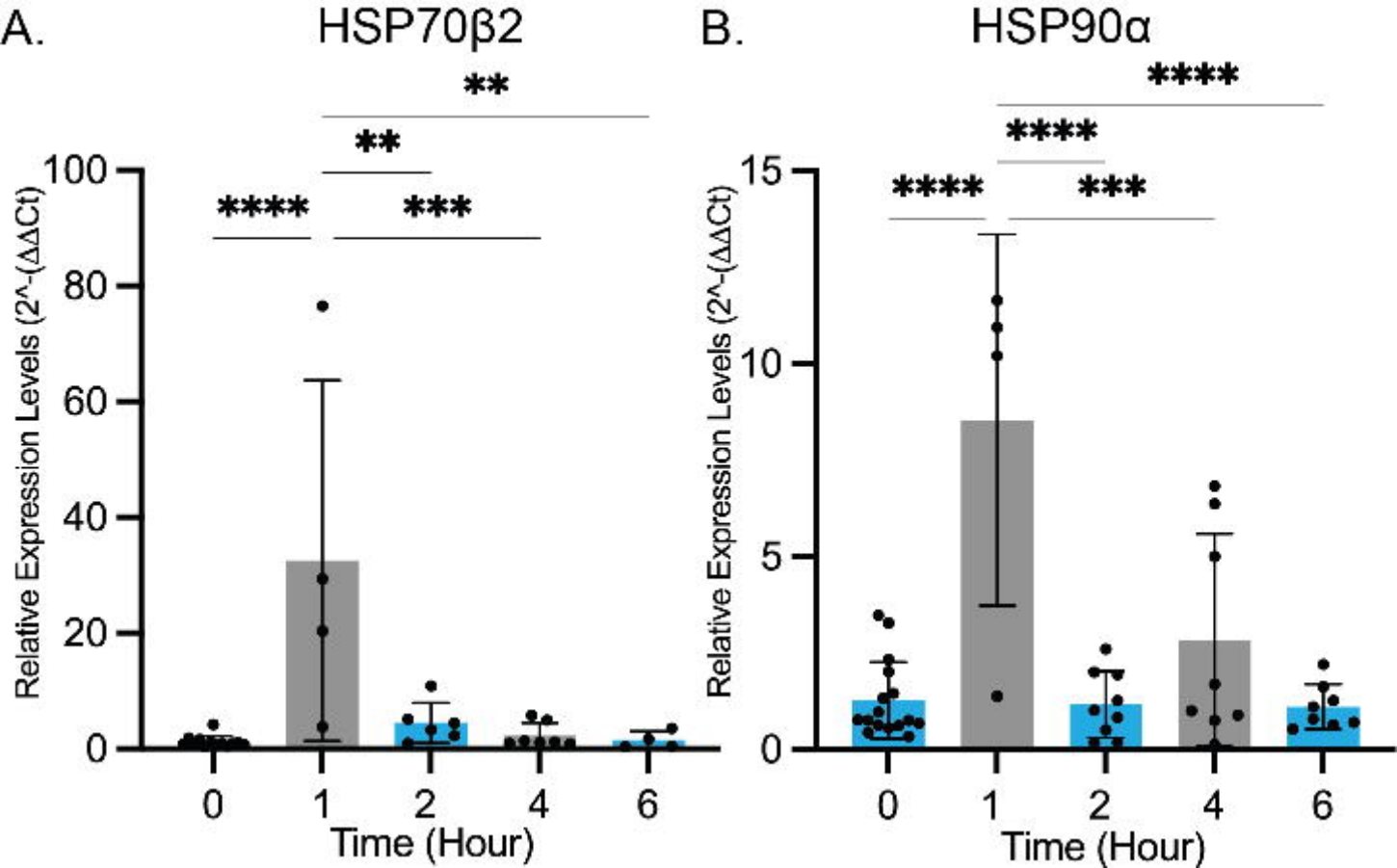
D.



E.




A.
gHSP-70
cHSP-70
gHSP-90
cHSP-90
B.
Raw Cq Values for Actin
30
20
10
0
C.
Heat Stressed 24 hrs.
Cold Stressed 24 hrs.
D.
Relative Expression Levels (2^-(ΔΔCt))
6
4
2
0
4 °C
30° C
HSP70β2
E.
**
Relative Expression Levels (2^-(ΔΔCt))
6
4
2
0
4 °C
30° C
HSP90α


A.
HSP70β2
**
**
****
***
100
80
60
40
20
0
Relative Expression Levels (2^-(ΔΔCt))
0
1
2
4
6
Time (Hour)
B.
HSP90α
****
****
****
***
15
10
5
0
Relative Expression Levels (2^-(ΔΔCt))
0
1
2
4
6
Time (Hour)