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Small-Scale Colorimetric Assays of Intracellular Lactate and Pyruvate in the Nematode *Caenorhabditis elegans* --Manuscript Draft--

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Author Comments:	<p>Response Letter (visible to both Editorial Office) May 9, 2018 Dr. Bing Wu, Review Editor (JoVE Editorial Office),</p> <p>Thank you for your e-mail and some suggestion for our revised manuscript entitled 'Small-Scale Colorimetric Assays of Intracellular Lactate and Pyruvate in the Nematode <i>C. elegans</i>.' Below are our responses to the review that number the comments, where sentences in bold font indicate your comments and those in plain font are our responses. We hope this revised manuscript will be considered for publication and look forward to receiving as a Report in JoVE in due course.</p> <p>Sincerely yours, Sumino Yanase, Daito Bunka University School of Sports & Health Science Iwadono 560, Higashi-matsuyama Saitama 355-8501, Japan +81-493-31-1558 (ext. 6922)</p> <p>1) Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Reply to this comment: Thank you for your suggestion. Our manuscript was proofread by a native English speaker twice.</p>

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TITLE:

Small-Scale Colorimetric Assays of Intracellular Lactate and Pyruvate in the Nematode *Caenorhabditis elegans*

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KEYWORDS:

Caenorhabditis elegans, energy metabolism, lactate, pyruvate, common precipitant, colorimetric assay

SHORT ABSTRACT:

We describe a modified small-scale extraction and colorimetric assays of lactate and pyruvate in the nematode *C. elegans*. When utilizing commercial assay kits, the technical development of their sensitivity and accuracy is important. Protein precipitation in extraction is the most critical step for the quantitative determination of intracellular metabolites.

LONG ABSTRACT:

Lactate and pyruvate are key intermediates of intracellular energy metabolic pathways. Monitoring the lactate/pyruvate ratio in cells helps to determine whether there is an imbalance in age-related energy metabolism between mitochondrial oxidative phosphorylation and aerobic glycolysis. Here, we show the utilization of commercial colorimetric assay kits for lactate and pyruvate in the model organism *C. elegans*. Recently, the sensitivity and accuracy of the colorimetric/fluorimetric assay kits have been improved greatly by the research and development conducted by reagent manufacturers. The improved reagents have enabled the use of small-scale assays with a 96-well plate in *C. elegans*. In general, a fluorimetric assay is superior in sensitivity to a colorimetric assay; however, the colorimetric approach is more suitable for the use in common laboratories. Another important issue in these assays for quantitative determination is protein precipitation of homogenized *C. elegans* samples. In our protein precipitation method, common precipitants (e.g., trichloroacetic acid, perchloric acid and metaphosphoric acid) are used for sample preparation. A protein-free assay sample is

prepared by directly adding cold precipitant (final concentration of 5%) during homogenization.

INTRODUCTION:

Lactate and pyruvate concentrations are widely regarded as intermediates of energy metabolism, and are related to the states of glycolysis, tricarboxylic acid (TCA) cycle, and electron transport chain in the cells of aerobic organisms. A series of reactions in glycolysis oxidize glucose to pyruvate, which lies at a metabolic crossroads and can be converted to carbohydrates through gluconeogenesis, to fatty acids and energy metabolism through acetyl-CoA, and to the amino acid alanine. The TCA cycle occurs under the presence of sufficient dissolved oxygen and is fundamental for the conversion of glucose to energy. Especially, the alteration of secondary metabolism is an interesting phenomenon in which glycolysis is used predominantly for energy production and aerobic mitochondrial respiration, which involves the TCA cycle and electron transport chain, is downregulated in mammalian cancer cells^{1,2}. We showed recently that the lactate levels and the consequent lactate/pyruvate (L/P) ratio decreased during aging in the model organism *Caenorhabditis elegans* (*C. elegans*). Likewise, we found that the mammalian tumor suppressor p53 ortholog CEP-1 in *C. elegans* has an important role in the age-related alterations of energy metabolism through the activation of its transcriptional targets³.

In biological assays, such as the measurement of lactate and pyruvate concentrations in cells, the sensitivity, accuracy, sample size, and incubation time of colorimetric/fluorimetric assay kits have been improved dramatically. Owing to technological innovations, we are now able to analyze various metabolites and intermediate metabolites without the large-scale culture of *C. elegans*, which is difficult given its small size. In general, the sensitivity of a colorimetric assay is an order of magnitude smaller than that of a fluorimetric assay; however, the colorimetric approach is more suitable in the setting of common laboratories. Furthermore, an extraction technique containing homogenization and protein precipitation is crucial for the quantitative determination of lactate and pyruvate concentrations in *C. elegans* cells because this nematode is enclosed in an exoskeleton called the cuticle, unlike mammalian cultured cell lines^{4,5}. Here, we describe a protocol to analyze lactate and pyruvate concentrations using commercial colorimetric assay kits including tips for sample extraction from *C. elegans*.

PROTOCOL:

1. Synchronized Culture of *C. elegans*

1.1. Before seeding, culture the *Escherichia coli* (*E. coli*) strain OP50 overnight at 37 °C in 300 mL of Luria-Bertani (LB) broth liquid medium. Store the cultured OP50 at -4 °C.

1.1.1. To make LB broth liquid medium, use 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl and 1.5 mL of 1 N NaOH, and add to 1 L with deionized water. Autoclave.

Note: OP50 and *C. elegans* strains are available from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN, USA).

1.2. To make nematode growth medium (NGM) agar, use 3 g of NaCl, 2.5 g of peptone, 17 g of agar, and 975 mL of deionized water. Autoclave. Cool to 55 °C, then sterilely add, in order, 1 mL of 1 M MgSO₄, 1 mL of 1 M CaCl₂, 1 mL of 5 mg/mL cholesterol in EtOH, and 25 mL of 1 M potassium phosphate pH 6.0 in 90-mm Petri dishes.

1.2.1. For 1 M potassium phosphate pH 6.0, use 108.3 g of KH₂PO₄ and 46.6 g of K₂HPO₄, and add deionized water to 1 L. Autoclave⁶.

1.3. Spread 1-2 mL of the cultured OP50 on the NGM agar plates. To make a thin layer of OP50, incubate the plates overnight at room temperature before adding any nematodes.

Note: The NGM agar plates inoculated OP50 can be stored at room temperature for 2-3 weeks.

1.4. Add at least 100 worms onto an NGM agar plate with OP50, and culture at 20 °C until the adult stage. At least three plates are required.

1.5. To collect eggs *in utero*, transfer gravid hermaphrodites from the three NGM agar plates each with 5 mL of S buffer in a 15-mL conical tube, and wash the worms 3 times with 15 mL of S buffer using centrifugation at 300 x *g* for 30 s at room temperature.

1.5.1. To make S buffer, use 5.9 g of NaCl and 50 mL of 1 M potassium phosphate pH 6.0, add to 1 L with deionized water. Autoclave⁶.

1.6. Dissolve the worms in an alkaline hypochlorite solution for axenization and bulk egg isolation (0.5 mL of fresh bleach or equivalent: 5-6% sodium hypochlorite, 0.1 mL of 10 M NaOH, approximately 4.5 mL of S buffer)⁶, and stand the solution for 10-15 min at room temperature with mixing by inverting.

Note: Within 15 min, adult worms should dissolve, leaving a hazy solution of eggs liberated from their carcasses (the liberated eggs should be confirmed using a stereoscopic microscope). Instead of 0.1 mL of 10 N NaOH, 0.2 mL of 5 N NaOH can also be used.

1.7. After hypochlorite treatment, wash the egg pellet 3 times with 15 mL of S buffer, and resuspend in 5-6 mL of S buffer. Hatch the released eggs during an overnight incubation at 20 °C in S buffer without *E. coli* for an age-synchronous culture of L1 stage larvae.

1.8. To determine the approximate number of L1 stage larvae, count the worms using a stereoscopic microscope in 10 µL of S buffer after resuspending the larvae at least 3 times, and calculate the average. Then, transfer the L1 stage larvae to five NGM agar plates with OP50 (1,500-3,000 worms per plate using a 90-mm Petri dish), and culture at 20 °C until they have

grown to the young adult stage, when the self-fertilization begins and a few eggs are laid (ordinarily after 3 days).

2. Extraction of Cellular Fraction from *C. elegans*

2.1. Collect the young adult stage (5-day-old animals) worms from the five NGM agar plates with S buffer (**Figure 1A**).

2.1.1. To select only living worms using the sucrose method⁶ for flotation on 30% (w/v) sucrose, mix the worms suspended in 3-4 mL of S buffer with an equal volume of ice-cold 60% (w/v) sucrose in a 15-mL conical tube. Spin the tube at 1,500 x *g* for 15 s at 4 °C and remove the floating worms into a fresh tube by moving them off the wall of the tube with a Pasteur pipette.

2.2. Wash the worms 3 times with S buffer by centrifugation at 1,500 x *g* for 30 s at 4 °C. Check the wet volume of washed worms after centrifugation using a 1,000 µL micropipette tip (**Figure 1B**).

2.3. Add the washed worms to an equal volume of ice-cold 10% (w/v) trichloroacetic acid (TCA; final concentration of 5%) for protein precipitation (**Figure 1C**). Instead of TCA, perchloric acid (PCA) or metaphosphoric acid can be used.

2.4. Homogenize the worms with the precipitant using 40 strokes of a pestle in a Teflon homogenizer (Potter-Elvehjem tissue grinder) with rotation at up to 1,300 rpm on ice.

2.5. Transfer the homogenate into a fresh 1.5-mL microtube with a Pasteur pipette, and sonicate using an ultrasonic homogenizer for 3 min (3 times of 1 min) with a 20% duty cycle on ice.

2.6. Clarify the homogenate by centrifugation at 8,000 x *g* for 10 min at 4 °C. Neutralize the supernatants with 4 M KOH (0.25 volume to 10% TCA) for 20 min on ice, and centrifuge at 8,000 x *g* for 10 min at 4 °C. The supernatant (as a test sample) can be stored at -80 °C until the following assays.

3. Lactate Assay Using a Colorimetric Assay Kit

3.1. Measure the concentration of lactate in the test samples using a colorimetric assay kit (**Table of Materials**). Carry out duplex examinations for the test samples. Add 5 or 10 µL of the test samples to a 96-well plate and adjust the volume to 50 µL per well with the Lactate Assay Buffer provided with the kit.

3.2. For the lactate standard curve, dilute 100 mM L(+)-Lactate Standard to 1 mM with Lactate Assay Buffer. Add 0, 2, 4, 6, 8, and 10 µL of the 1 mM L(+)-Lactate Standard, which is provided with the kit, into a series of wells.

3.3. Add 50 μ L of Reaction Mix (containing 46:2:2 of Lactate Assay Buffer, Lactate Enzyme Mix and Lactate Probe in DMSO, anhydrous; all reagents are provided with the kit) or Background Control Mix (containing 48:2 of Lactate Assay Buffer and Lactate Probe) into each well and incubate at room temperature for 30-60 min while the samples are protected from light.

3.4. Measure the absorbance of each well at 570 nm using a microplate reader and subtract the absorbance of the Background Control Mix from the absorbance of the Reaction Mix.

3.5. Plot the lactate standard curve. Calculate the lactate concentrations of the test samples from the lactate standard curve.

4. Pyruvate Assay Using a Colorimetric Assay Kit

4.1. Measure the concentration of pyruvate in the supernatants (test samples) using a colorimetric assay kit (**Table of Materials**). Carry out duplex examinations for the test samples. Add 10 μ L of the test samples and 90 μ L of Working Reagent (containing 94:1 of Enzyme Mix and Dye Reagent, which are provided with the kit) into a 96-well plate and incubate at room temperature for 30 min while the samples are protected from light.

4.2. Measure the absorbance of each well at 570 nm using a microplate reader.

4.3. Plot the pyruvate standard curve. Calculate the pyruvate concentrations of the test samples from the pyruvate standard curve.

5. Protein Assay for Normalization with Protein Content

5.1. Measure the protein concentration in the supernatants (test samples) using a colorimetric assay kit (**Table of Materials**). However, this step is not limited to an assay kit, and other approaches can be utilized to measure the protein concentration.

5.2. Normalize the values of lactate and pyruvate concentrations among the test samples using each total protein concentration.

Note: Protein concentration in the test samples is sufficiently detected even after protein precipitation and is utilized for the normalization step among samples.

REPRESENTATIVE RESULTS:

Using the colorimetric assays for the quantitative determination of lactate and pyruvate concentrations, we showed the accuracy of these assays compared with previous reports in *C. elegans*^{7,8}. Here, the process of protein precipitation during sample extraction was the most crucial step to generate accurate values. For protein precipitation, common precipitants (*e.g.*,

TCA, PCA, or metaphosphoric acid) can be used to prepare the test samples (**Figure 1**). In *C. elegans*, however, it was necessary to perform protein precipitation during homogenization of the worms (**Table 1**). In addition to accuracy, these assays were sufficiently sensitive to measure lactate and pyruvate concentrations in small-scale samples and were able to detect them in a short period of time (the reactive incubation time of both assays is at least 30 min) (**Figures 2-3**). Actually, we presented the data in a recent report³. Thus, we could detect age-related metabolic alterations indicating that cellular lactate levels and the consequent L/P ratio decreased during aging, and we also showed different energy metabolism in a *cep-1* mutant³.

FIGURE AND TABLE LEGENDS:

Figure 1: Process for the extraction of cellular metabolites. (A). 1,500-3,000 worms were placed on an NGM agar plate (90-mm Petri dish). Extraction from worms on five plates was sufficient for the colorimetric assays. (B). Worms collected from the five plates of 3,000 and 1,500 worms per plate are indicated on the left and right side of panel, respectively. Both the wet volumes in each 15-mL tube are ≤ 0.5 mL, which were sufficient for detection. (C). Protein precipitation using 10% TCA during homogenization of the worms. Adding the worms into ice-cold 10% TCA in a homogenizer has to be performed before the worms are homogenized. Otherwise, cellular lactate and pyruvate cannot be detected in test samples using a colorimetric assay kit (data are shown in **Table 1**).

Figure 2: Colorimetric pigmentation patterns of the lactate standards using a colorimetric assay kit. (A). Wells of the 96-well plate used in the colorimetric assay, and a dilution series of the L(+)-lactate standard. Increasing the lactate concentration resulted in a more intense pink color. BG indicates the background of a lactate probe against the dilution series. (B). Lactate standard curve for the colorimetric assay.

Figure 3: Colorimetric pigmentation patterns of the pyruvate standards using a colorimetric assay kit. (A). Wells of the 96-well plate using the colorimetric assay, and dilution series of pyruvate standard. Increasing of the pyruvate concentration resulted in increased light pink coloring. (B). Pyruvate standard curve for the colorimetric assay.

Table 1: Effects of protein precipitation on different timings for the detection of cellular lactate and pyruvate in 5-day-old animals of wild-type N2. Data indicate means \pm standard deviation (SD) of at least three determinations. ND indicates not detected. *The extraction process was performed preliminarily using ultracentrifugation without protein precipitation.

DISCUSSION:

When utilizing these colorimetric assay kits, the most critical step in sample extraction to detect cellular lactate and pyruvate accurately in *C. elegans* is the process of protein precipitation during homogenization (**Figure 1**). It is not strictly necessary to use a Teflon homogenizer, as other homogenizers (*e.g.*, Dounce and tapered tissue grinders, or bead mills) are also suited to the small-scale extraction of worms. We did not detect cellular lactate and pyruvate in test samples that were extracted using a homogenizer before protein precipitation. Furthermore,

both lactate and pyruvate levels decreased drastically in the cytosolic fraction separated using ultracentrifugation instead of protein precipitation (**Table 1**). These suggest that the sample extraction is crucial to detect the cellular metabolites in biochemical methodology using a nematode *C. elegans*. For protein precipitation, not only TCA but also several other precipitants (PCA or metaphosphoric acid) can be utilized to the biochemical methodology^{7,9}. According to a previous report, the use of TCA resulted in the disappearance of approximately 12% of NADH in the enzymatic measurement of blood lactate and pyruvate. Therefore, the authors concluded that 5% metaphosphoric acid should be selected as the protein precipitant for both lactate and pyruvate when using enzymatic assays⁹. It suggests that TCA partially denatures proteins including many enzymes. However, we had no problems regarding the protein precipitants when using colorimetric assays to detect cellular lactate and pyruvate.

In this study, we used commercial colorimetric assay kits for the quantitative determination of lactate and pyruvate (**Figures 2 and 3**). Their sensitivities and sample sizes were superior in comparison with previous assay kits⁷; therefore, we prepared the test samples using a smaller scale extraction and reported the differences in the cellular lactate and pyruvate concentrations in *C. elegans*³. We detected an age-related decrease in lactate and consequent L/P ratio in wild-type *C. elegans* and found that the p53/CEP-1 has an important role in the age-related alteration of energy metabolism through the activation of its transcriptional targets^{2,3}. Thus, the analysis of energy metabolism in nematode *C. elegans* advantageously proceeds due to using the improved colorimetric assay kits, at least in part.

As shown in **Figure 3B**, lactate concentration below 2 mM tend to be indeed larger in this colorimetric assay (not the fluorometric assay). Therefore, the fluorometric assay may be suitable for a more accurate measurement of lactate. The capabilities of these assay kits support both colorimetric and fluorometric assays, so that the assay is selected in response to the appliance in laboratory of the user. It is still more expensive to use the present commercial colorimetric assay kits to determine quantitatively the concentration of cellular metabolites. However, trials can be adequately performed in smaller laboratories using common instruments and a spectrophotometric plate reader.

Hitherto, there are relatively few reports using conventional biochemical approaches in a model organism *C. elegans*, which typically need the large-scale culture of worms, compared with genetic studies⁵. However, the recent remarkable progress of biological assay systems (*e.g.*, improvement of the sensitivity and stability of assay kits) makes studies using the nematode *C. elegans*, which is small and consists of fewer than 1,000 cells, more attractive and performed easily in the laboratory. Henceforth, effective metabolomic analysis using mass spectrometry (MS) and gas chromatography/mass spectrometry (GC/MS) can help to reduce certain problems, such as large-scale culture, analytical sensitivity, and specificity, and will help to elucidate the role of cellular metabolism in aging and human diseases including cancer¹⁰. Looking ahead, easier methodology, such as colorimetric assay kits, would be effective as a screening tool prior to more precise instrumental analyses.

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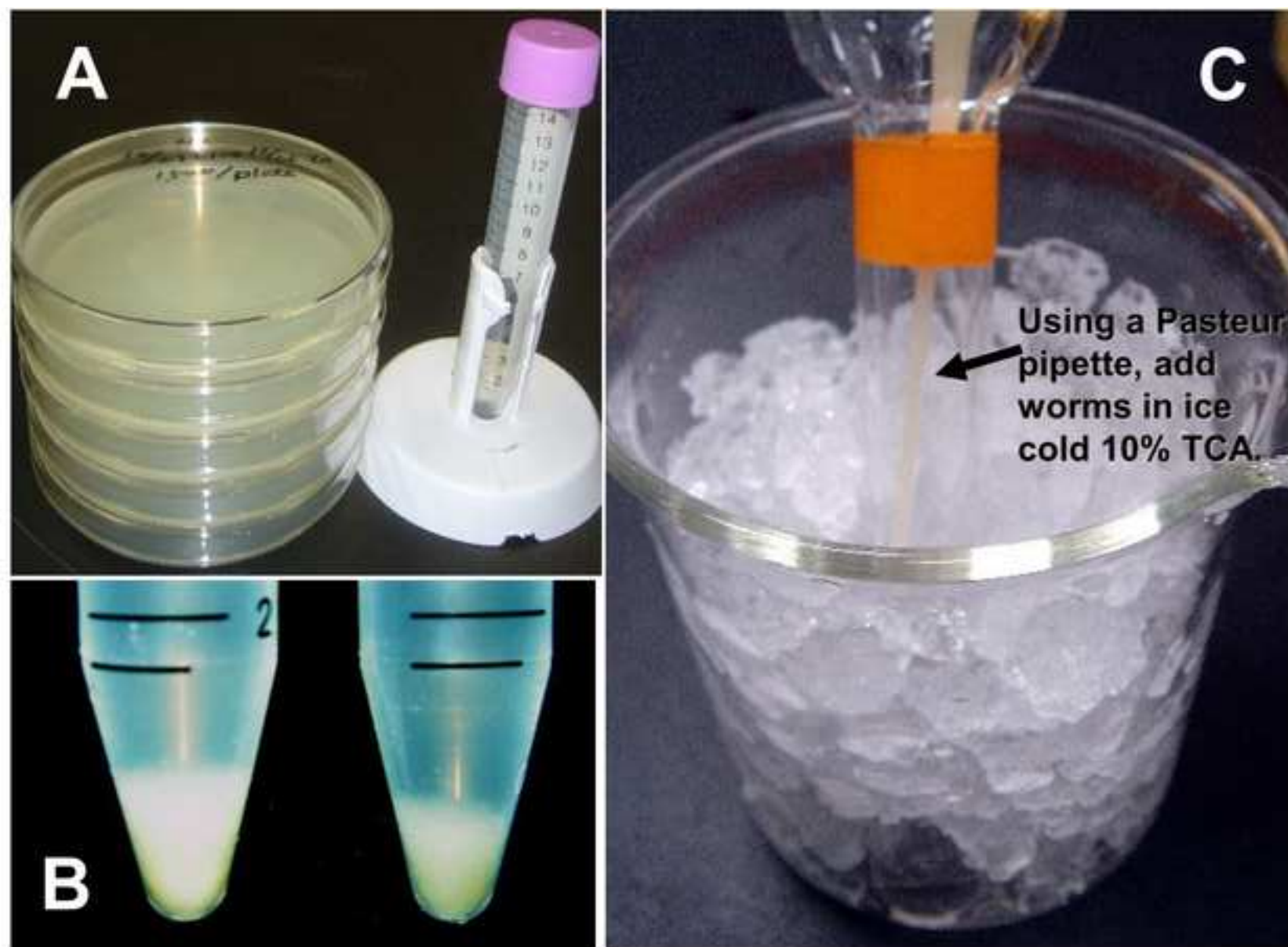
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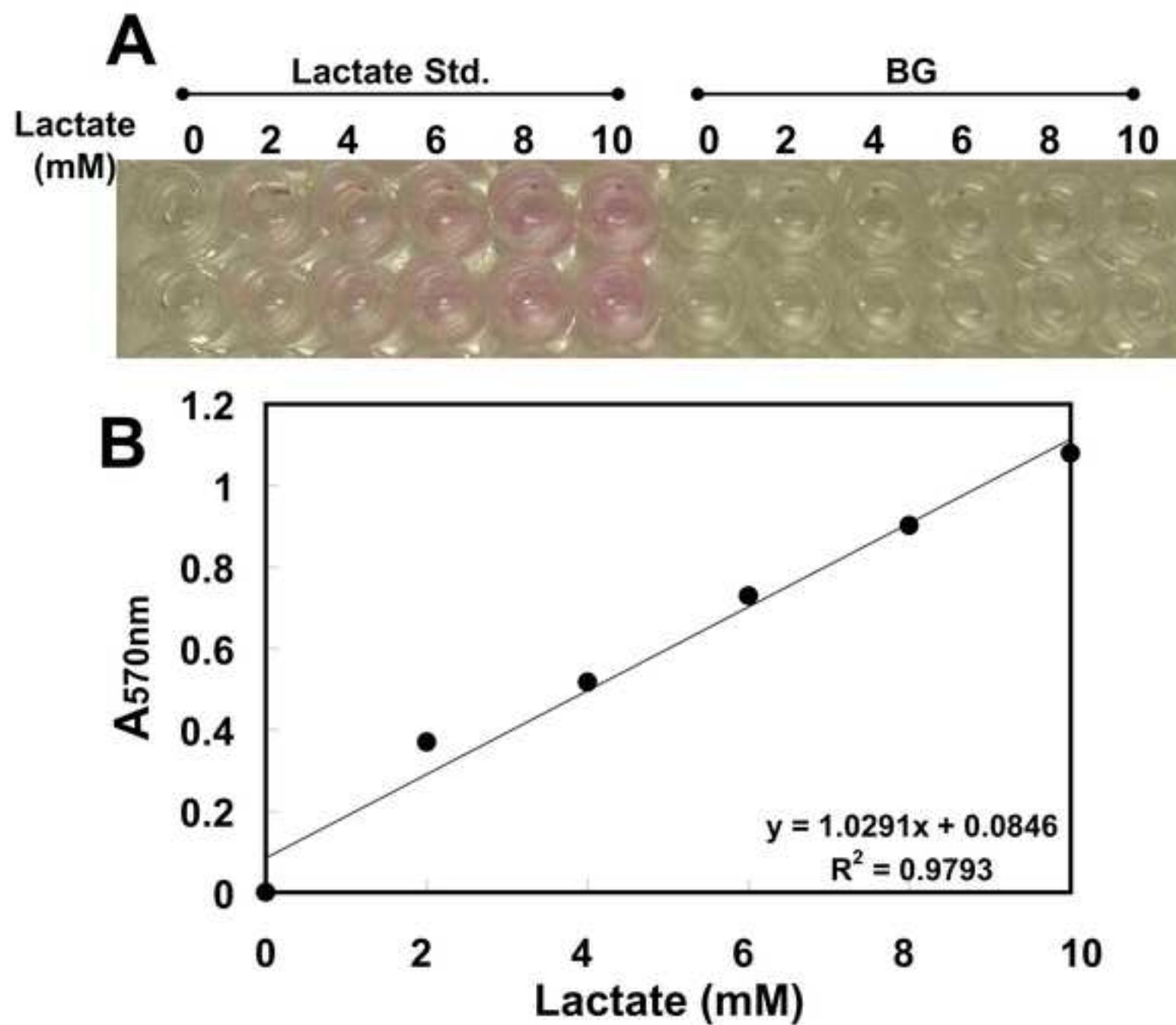
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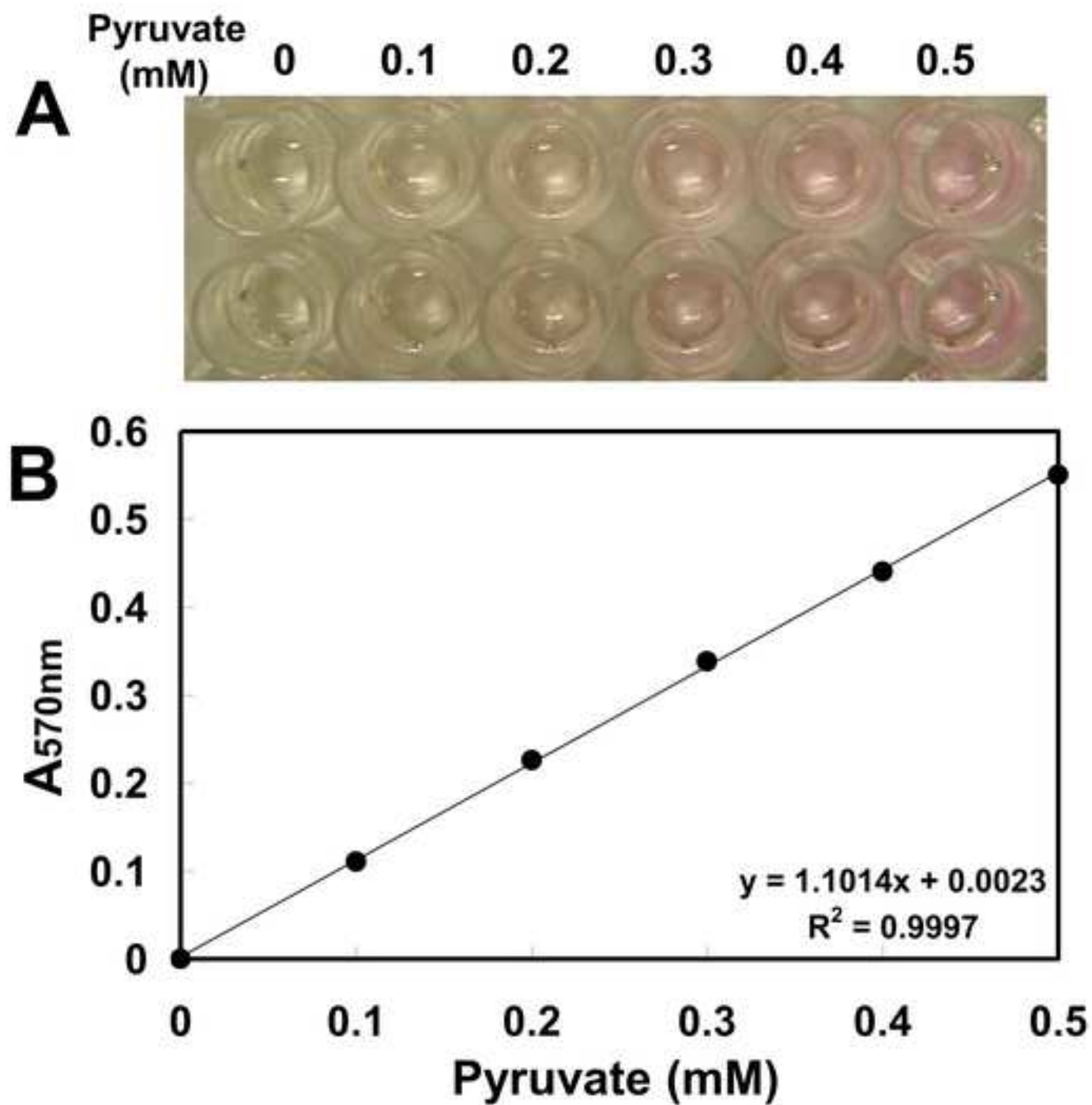
The authors declare that they have no competing financial interests.

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Test Samples	Lactate (mM)	Pyruvate (mM)
Protein-precipitated samples during homogenization	3.07 ± 0.94	0.22 ± 0.08
Protein-precipitated samples after homogenization	ND	ND
Intact cytosolic fraction after homogenization and	1.12	0.06

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Lactate Colorimetric/Fluorimetric Assay kit	BioVision	#K607-100	colorimetric/fluorimetric 100 assays; Store at -20oC
EnzyChrom Pyruvate Assay Kit	BioAssay Systems	#EPYR-100	colorimetric/ fluorometric 100 assays; Store at -20oC
BCA Protein Assay Kit	Thermo Scientific	#23225	colorimetric assay; store at room temperature
Trichloroacetic Acid	Wako Pure Chemical	#207-04955 #358034	store at room temperature Instead of Iwaki/Pyrex,
Teflon homogenizer	Iwaki/Pyrex	(Wheaton)	available by Wheaton



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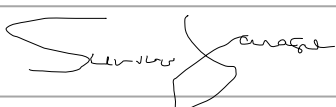
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Response Letter (visible to both Editorial Office and referees)

March 26, 2018

Dr. Bing Wu,

Review Editor (JoVE Editorial Office),

We would like to acknowledge reviewers for the valuable suggestions, constructive comments and editorial suggestions, which we believe we have adequately addressed in the revised manuscript entitled ‘**Small-Scale Colorimetric Assays of Intracellular Lactate and Pyruvate in the Nematode *C. elegans*.**’ Below are our responses to the review that number the comments, where sentences in **bold font** indicate the reviewer’s comments and those in plain font are our responses. In the revised manuscript, the changes that have been made following suggestions raised by reviewer are shown as text or PDF. We hope this revised manuscript will be considered for publication and look forward to receiving as a **Report in JoVE** in due course.

Sincerely yours,

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Reply to Referee #1,

Minor Concerns to Authors:

1) Line 67: “rule” should be role.

Reply to this comment: Thank you for your suitable suggestion. We revised “rule” to “role” in Introduction, and so on.

2) Line 87: Can the authors write how long it usually takes to dissolve the worms in the hypochlorite solution? Should the worms be completely dissolved or is it good enough to partially dissolve the worms?

Reply to this comment: We rewrote the Protocol in further detail. That is, there are new parts of 1.5) and 1.6) in Protocol 1. Synchronized Culture of *C. elegans*.

3) Line 92: Can the authors write how long it usually takes for L1 worms to become adults at 20°C?

Reply to this comment: We rewrote again the Protocol in further detail containing the condition of culture of worms. That is, there are new parts of 1.7) and 1.8) in Protocol 1. Synchronized Culture of *C. elegans*.

4) Line 101: What is the approximate number of worms usually collected per NGM plate and how much volume does that equate to? A picture of the starting density of the worms on the plate might also be useful.

Reply to this comment: On an NGM plate in 90 mm Petri dish, amounts of seeding *E. coli* as foods of worms are limited. Growing to adult worms, limit of worm numbers that we do not need to replenish *E. coli* is approximately 3,000 worms per a plate. Furthermore, counting of complete accurate numbers of 3,000 worms is technically difficult with our method using a stereoscopic microscope. We revised to explain about them in new 1.8) of Protocol.

Reply to Referee #2,

Major Concerns to Authors:

1) This almost seems like an ad for the Kits. The precipitation step implies the use of other options instead of TCA, and do not discuss method and benefit/problems of each (or even the absence of both). The grinding step in *C. elegans* is specially important to have consistent data, would be valued to explore more this step (like homogenizer, size, grinder used, speed of each stroke...).

Reply to this comment: Thank you for your reasonable indication and suggestion. As you indicated, we revised to the Protocol containing the details of culture and extraction steps. Of TCA as protein precipitant, we discussed about its denaturation with precipitation of protein in Discussion (with ref. 9).

Minor Concerns to Authors:

1) Some details are know in the community, aka, S Buffer, sucrose method...but would be good to have the details as this is a technical manuscript.

Reply to this comment: Thank you for your indication. We entirely revised Protocol 1 and 2 containing the technical details.

Reply to Referee #3,

Major Concerns to Authors:

1) In section 4.1 (3.1? as our opinion), method of generating pyruvate (lactate? as our opinion) standard curve is missing. Authors should add it to the manuscript. A list of material including reagents and buffers such as FUDR and S buffer would greatly help the readers. A few of the reagents could be moved to the list of material. In section 1.1 and 1.2, a method of defining adult stage should be included such as by duration of growth, or by identification of specific structures. It is not clear if the error bar in Fig. 4 is standard deviation or standard error of mean.

Reply to this comment: Thank you for your reasonable indication and suggestion. We mentioned the possibility of a detecting limit in the colorimetric assay kit in the second paragraph (line 5-7) of Discussion. In the revised manuscript, we think that we should explain mainly the methodology of the colorimetric assays for measurement of lactate and pyruvate in *C. elegans*. Therefore, we eliminated the data of metabolites in elder animals using an FUDR and also Figure 4 that may be conflicted to re-use the figure from our previous publication in ref. 3. Instead, we discussed and referred using the data in ref. 3. Of a method defining adult stage, we explained it (5-day-old animals as a young adult stage) in the rewriting of the Protocol 2.1) (line 1).

Minor Concerns to Authors:

A few sentences in the manuscript need to be clarified:

1) In short abstract, “we describe a modification of colorimetric assays...”. Do authors mean “we describe modified colorimetric assays”?

Reply to this comment: Thank you for your suitable indication and suggestion. We rewrote some sentences in short abstract as meaning of your suggestion.

2) In the manuscript, authors state “...common precipitants are available...”. The word “available” seems to be a wrong word to use. Maybe authors mean “used”? Such word is used many times for example, in long abstract, section 2.2, representative results, and discussion.

Reply to this comment: Thank you for your reasonable suggestion. We entirely replaced suitably “available” to “used” in your indicated points.

3) In section 2.1, specify “a quick spin” speed and time.

Reply to this comment: Thank you for your reasonable indication. We added the details (containing the speed and time) of conditions of the centrifugation (a quick spin) in the revised section 2.2).

4) In section 3 and 4, various types of solution such as Lactate Assay Buffer, Reaction Mix, and Working Reagent are used in the protocol. It is not clear to reader whether those solutions are provided by the kits or do authors make them?

Reply to this comment: Thank you for your reasonable suggestion. We entirely added about the solutions provided by the kits.

Reply to Referee #4,

Major Concerns to Authors:

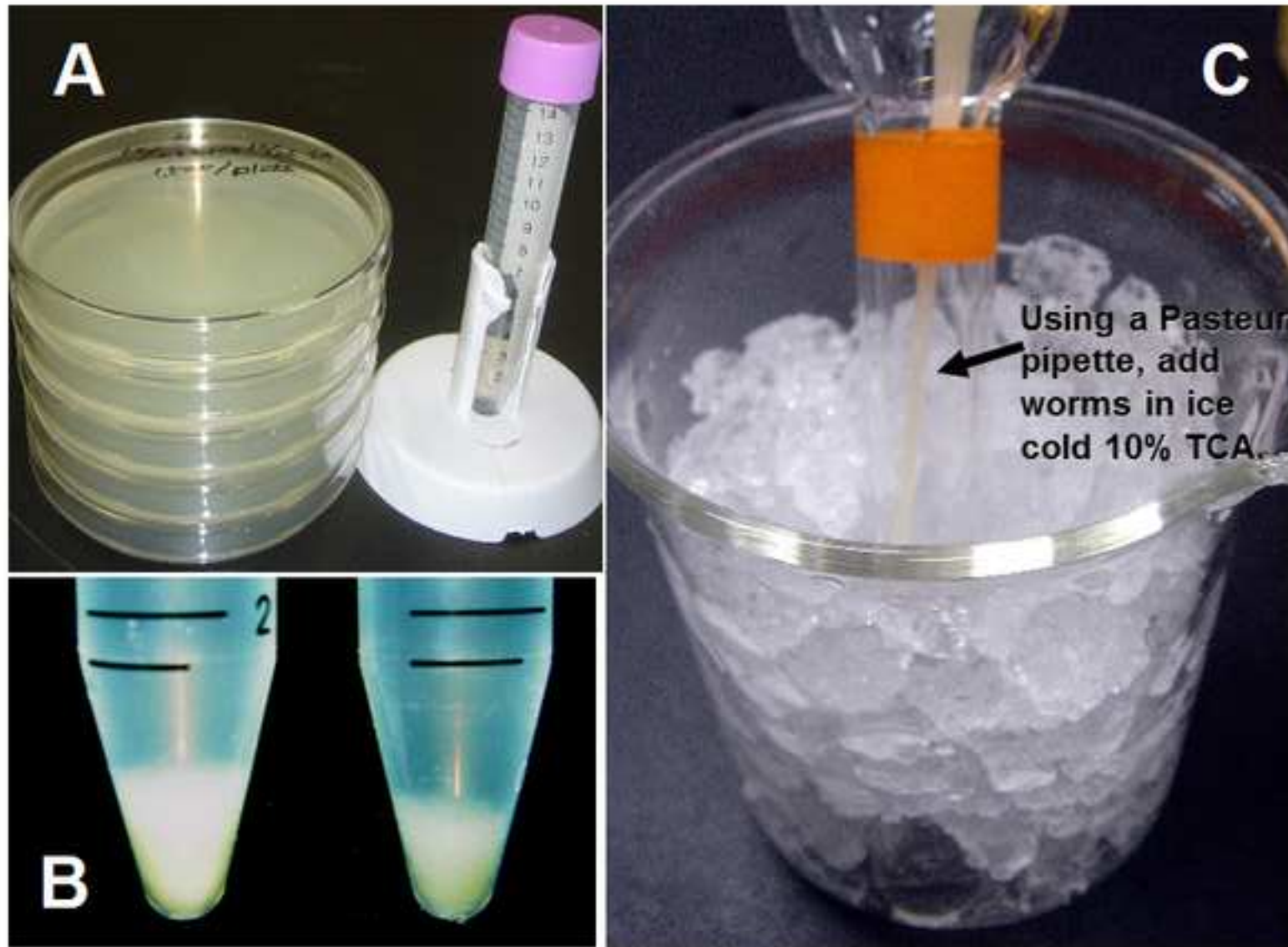
I have one major comment: it would strengthen the assay if the normalization step is based on protein content, at least not as a stand-alone. This is because (1) BCA is not a very sensitive readout of total number of worms, (2) treatments that can alter metabolism may also alter protein content, (3) the authors call for protein precipitation prior to measurement.

Reply to this comment: Thank you for your reasonable indication. We think as described below. (1) We used to utilize the BCA Protein Assay Kit in our previous reports (ref. 7, and so on), that is, there are a few past performances. (2) Normalization by protein content in worms may be unsuitable for some treatments exactly as you indicated, however, we have used to measure the lactate and pyruvate in wild-type N2 with and without hyperoxia (ref. 7). (3) We confirmed the correlation between the protein contents before and after protein precipitation. As you mention, TCA may act exactly as a protein denaturant rather than protein precipitant.

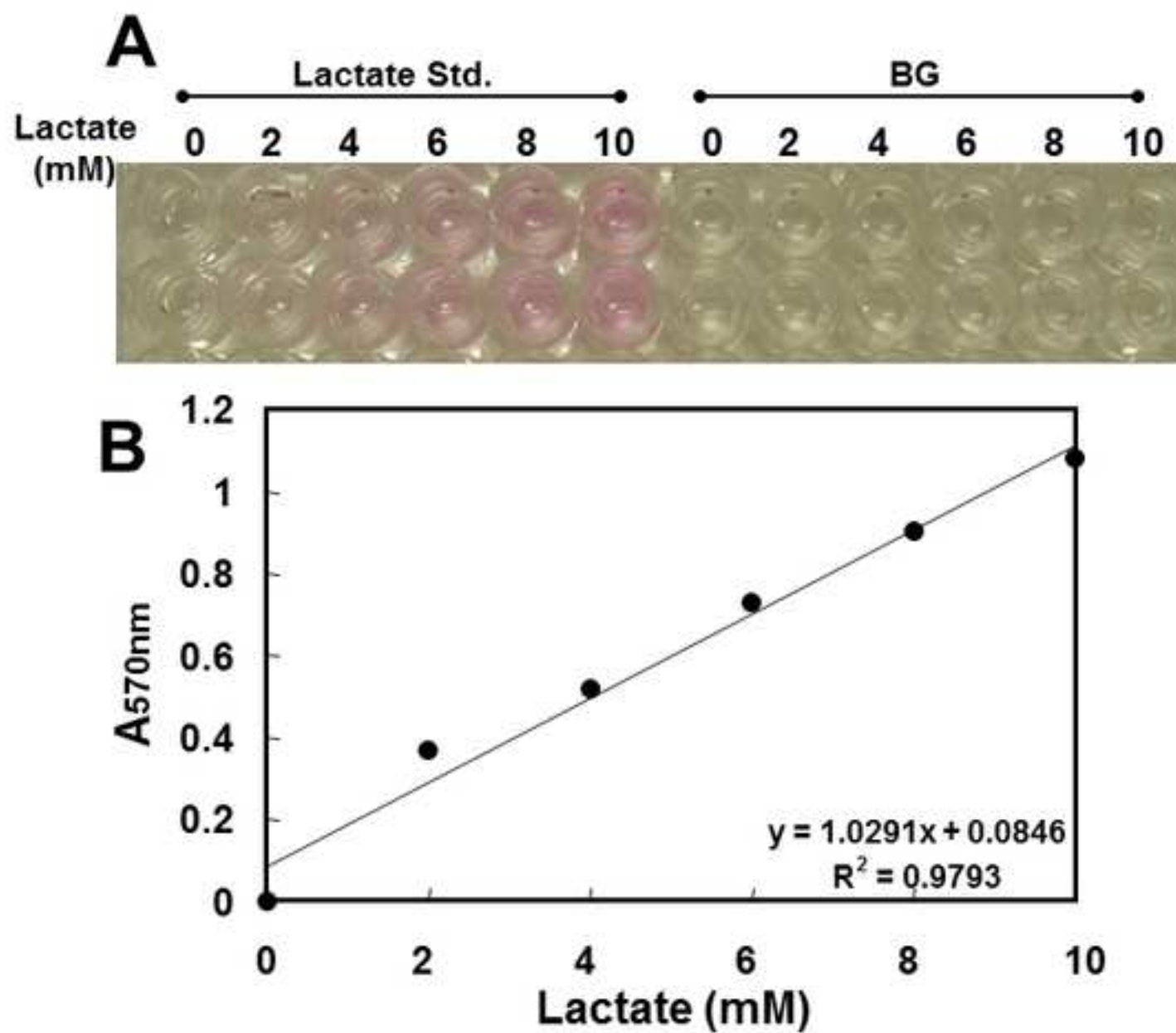
Minor concerns:

There are minor grammatical errors that can be easily fixed.

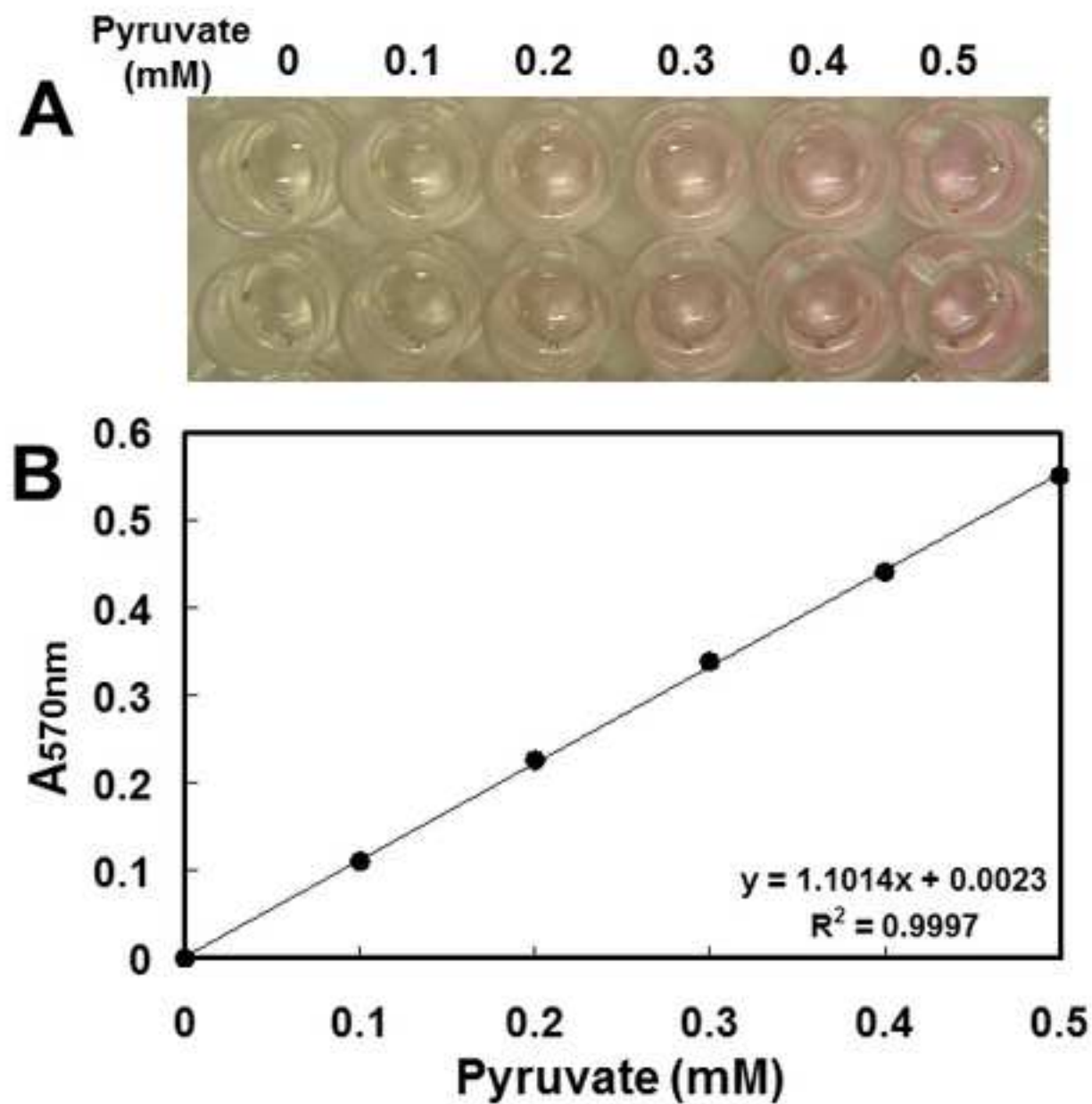
Reply to this comment: Thank you for your suggestion. We grammatically reviewed our manuscript and do English proofreading again.



(Figure 1)



(Figure 2)



(Figure 3)