Peer-Review

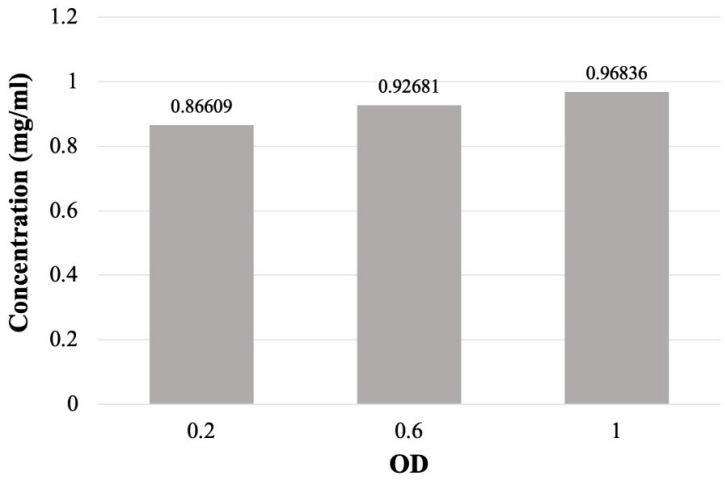
Shen, Athena. 2025. "Optimized Azidohomoalanine Labeling Protocol Enables Sensitive Detection of *de Novo* Protein Synthesis in *C. Elegans* under Heat Shock." *Journal of High School Science* 9 (4): 295–310. https://doi.org/10.64336/001c.153892

The challenge I have with your paper is that you did not present any quantitative data that
1.compared protein levels in C.elegans when e coli was incubated with the non-canonical amino acid in its (e.coli's) logarithmic growth phase versus when it (e.coli) was fed with the amino acid in its stationary (post growth) phase. There are SDS gel figures but no quantitative data.
2.I also need to see either data or references showing that when the non-canonical amino acid is directly fed (as part of the NGM growth media) to the C-elegans (along with the e.cDear Reviewer,
3.
4.Thank you for your consideration of the research paper and thought-provoking comments for revision. Your feedback has helped improve the quality of the paper, with added strength to our conclusions and clarity with procedures. We have thoroughly revised the manuscript addressing both comments. Below are detailed responses to each comment with corresponding revisions made to the manuscript.
5.
6.compared protein levels in C.elegans when e coli was incubated with the non-canonical amino acid in its (e.coli's) logarithmic growth phase versus when it (e.coli) was fed with the amino acid in its stationary (post growth) phase. There are SDS gel figures but no quantitative data.
7.
8.**Our response:** In our initial experimental design, we planned to optimize the *E. coli* labeling efficiency and then plate this culture for *C. elegans* to consume for labeling. Therefore, we did not compare the protein levels in *C. elegans*; instead, we compared the levels in *E. coli* to confirm which condition allowed for the strongest labeling efficiency. However, the comment raised a valuable consideration that led us to include important details as to why protein levels in *E. coli* are more relevant. Thus, we have added an explanation in **paragraph 1, page 8,** according to **reference 15,** to explain that *E. coli* labeling efficiency is directly correlated with *C. elegans* labeling efficiency. In other words, high labeling efficiency in *E. coli* will lead to more efficient labeling in *C. elegans.* Therefore, we can directly use the optimized experimental *E. coli* culture to label *C. elegans* in subsequent experiments, as it will ensure the highest *C. elegans* labeled protein levels.

9.
10.The comment has also inspired us to add quantitative data to compare the protein levels in *E. coli* when incubated with the non-canonical amino acid at different phases of the growth curve, strengthening our conclusion that *E. coli* labeling is optimized when induced to grow overnight in AHA M9 Medium after reaching an initial OD of 0.2. **Figure 4b** (below as well) can be found on **page 7** with a relevant description in **paragraph 1, page 7**. Additional analysis of the compared protein levels for further discussion is in **paragraph 2, page 11.**

11.**Figure 4b:**

b



12.

13.

14.I also need to see either data or references showing that when the non-canonical amino acid is directly fed (as part of the NGM growth media) to the C-elegans (along with the e.coli), the levels of labeled protein in the C.. elegans are lower than when the protocol in 1 is adopted. This will serve as confirmation that incorporation of the non-canoncial amino acid, first into e.coli is a necessary and sufficient step for its subsequent incorporation into C. elegans proteins.

15.

16.**Our response**: We have added **reference 9** (below as well) and relevant explanation in **paragraph 3, page 3** to provide clarity of the difference in labeling methods between mammalian cells (as presented in the protocol in 1) and *C. elegans* due to *C. elegans'* protective outer shell and active digestive system that would lower labeling levels if the non-canonical amino acid is directly fed to *C. elegans*.

17.Parrish AR, She X, Xiang Z, et. al. Expanding the genetic code of Caenorhabditis elegans using bacterial aminoacyl-tRNA synthetase/tRNA pairs. ACS Chem Biol. 2012 Jul 20;7(7):1292-302. https://doi.org/10.1021/cb200542j

18.

19.oli), the levels of labeled protein in the C.. elegans are lower than when the protocol in 1 is adopted. This will serve as confirmation that incorporation of the non-canoncial amino acid, first into e.coli is a necessary and sufficient step for its subsequent incorporation into C. elegans proteins.

Figure 3 shows expression after 24 hours whereas Figure 4a shows expressin after 3 hours and overnight (12 hours). Does expression peak and then fall off (say after 12 hours to 24 hours?) need reference please. Else, you are comparing expression under different conditions.

1.Please upload a word.doc of your manuscript formatted to the Journal's reguirements. We cannot work with a pdf.

2.I am confused with regard to Figure 4b. If expression level is similar across all OD's what is the specific advantage of labeling under OD 0.2 ? Unless, I am misunderstanding this and the BCA protein assay refers to the protein concentration the E.Coli was incubated with ? Please clarify in the manuscript. By the way, to justify a similarilty claim, you will need error bars and a statistical analysis for all the three concentrations.

3.Please format your references per APA format. Please do NOT use the word processing software's atuomated numbering system to number the references. Instead, do this manually.

Dear Reviewer,

Thank you for your continued consideration of the research paper and insightful comments for revision. Your feedback has helped improve the quality of the paper, adding clarity to our conclusions and strengthening explanations. We have thoroughly revised the manuscript, addressing all comments. Below are detailed responses to each comment with corresponding revisions made to the manuscript.

- Figure 3 shows expression after 24 hours whereas Figure 4a shows expressin after 3 hours and overnight (12 hours). Does expression peak and then fall off (say after 12 hours to 24 hours?) need reference please. Else, you are comparing expression under different conditions.

Our response: We have revised **Figure 4** on **page 7** to clarify that overnight refers to 24 hours. Thus, the expressions in Figures 3 and 4 are compared under the same conditions of 24-hour labeling.

- Please upload a word.doc of your manuscript formatted to the Journal's reguirements. We cannot work with a pdf.

Our response: Manuscript has been formatted accordingly and uploaded as a Word document. Thank you for the reminder!

- I am confused with regard to Figure 4b. If expression level is similar across all OD's what is the specific advantage of labeling under OD 0.2 ? Unless, I am misunderstanding this and the BCA protein assay refers to the protein concentration the E.Coli was incubated with ? Please clarify in the manuscript. By the way, to justify a similarilty claim, you will need error bars and a statistical analysis for all the three concentrations.

Our response: This thought-provoking comment prompted us to delve deeper into the analysis of compared protein levels, strengthening our conclusions. We have found reference 21 (below as well), which illustrates that the assayed samples are directly comparable, as they were processed in the same manner (using the same buffer and conditions for 24-hour incubation). Thus, we can now accurately compare the protein amounts given by the BCA protein assay, as listed in **Table 1** on **page 7.**

The data show that there is less protein in the sample with an initial OD of 0.2, the sample with the strongest fluorescence signal in Figure 4, compared to OD conditions of 0.6 and 1.0. This strengthens our conclusion in **paragraphs 1 and 2, page 11,** that incubating *E. coli* in an AHA environment after reaching OD 0.2 leads to the strongest labeling efficiency, as there is the least amount of protein, but the strongest signaling. Thus, we can confirm that the sample with an initial OD of 0.2 optimizes AHA labeling, a result of induction into the medium during the bacteria's logarithmic phase.

1. Protein Assay Data Analysis | Thermo Fisher Scientific - US. (2025). Thermofisher.com. https://www.thermofisher.com/hk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/protein-assay-data-analysis.html

- Please format your references per APA format. Please do NOT use the word processing software's atuomated numbering system to number the references. Instead, do this manually.

Our response: References have been formatted per APA style. Thank you for the reminder!

Thank you for addressing my comments except for :
part of point 3. i.e. "…… By the way, to justify a similarilty or difference claim in Table 1, you will need error bars and a statistical analysis for all the three concentrations; I am assuming each experiment (Table 1) was performed at least twice? Also, is this concentration really measurable with precision to 5 decimal places (i.e. 10 s of ng/ml?)

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Dear Reviewer,

Thank you for your continued consideration of the research paper and insightful comments for revision. Your feedback has helped improve the paper, with added strength to our conclusions. We have thoroughly revised the manuscript, addressing all comments. Below is the response to the comment with corresponding revisions made to the manuscript.

2. Thank you for addressing my comments except for : part of point 3. i.e. "…… By the way, to justify a similarilty or difference claim in Table 1, you will need error bars and a statistical analysis for all the three concentrations; I am assuming each experiment (Table 1) was performed at least twice? Also, is this concentration really measurable with precision to 5 decimal places (i.e. 10 s of ng/ml?)

Our response: The comment has helped us strengthen our conclusions in the analysis of compared protein levels in **Figure 4b**, found on **page 7**, with a relevant description in **paragraph 3, page 6**. Based on the data from two replications of the experiment, we updated the bar graph with error bars, supported by the corresponding ANOVA test, which yielded a p-value greater than 0.05. Both suggest that there is no significant difference across the three experimental groups. This conclusion has allowed us to strengthen our explanation in **paragraphs 1 and 2, page 11.** Here, we illustrate that similar protein concentrations after E. coli 24-hour incubation in the AHA environment across three initial OD conditions indicate that any difference in the fluorescence signal can be attributed to labeling efficiency, rather than a difference in protein amounts. Thus, we can confirm that the sample with an initial OD of 0.2 exhibits the most efficient AHA labeling, as indicated by the strongest fluorescence signal, which is a result of induction in the medium during the bacteria's logarithmic phase.

Additionally, the concentration is calculated through the BCA standard curve, which provides a concentration based on the absorbance of each sample. As a result, we were able to generate up to 5 decimal places. This data has been updated in the bar graph.

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Thank you for addressing my comments. Accepted.
While copyediting your manuscript (see attached), we were unable to understand the following two points. Can you please communicate via the discussion board on how we should process them?
1.You claim 16 mg enriched protein from 2 mg input ? Are you sure? This seems impossible.
2.Streptavidin yield is reported as 0.5%, but the numbers don't align with the text.
We also rewrote your title, abstract, conclusion and substantial parts of your manuscript to improve English and clarity.

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Dear Reviewer,

Below are the responses to the two final comments for publication.

1. You claim 16 mg enriched protein from 2 mg input ? Are you sure? This seems impossible.

The enriched protein is 16 **µg** from the 2 mg input. Thank you for catching this typo!

2. Streptavidin yield is reported as 0.5%, but the numbers don’t align with the text.

Figure 7 has been updated to reflect a 0.5% elution enrichment yield in the last two lanes, aligning with the description in the preceding paragraph.

Thank you for all of these valuable suggestions!

Sincerely,
Athena Shen

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Dear author,
Thank you for your review. We have also included a "Limitations" section (below) and clarified that the study was exploratory.

6. Limitations

Since this was an exploratory study, statistical analysis and controls were not as robust. For example, we did not include −AHA, +Met competition, −Cu (for CuAAC), no-alkyne tag control, unlabeled E. coli feeding, acute heat-shock regimen control (e.g., 34 °C for 1 h) vs chronic mild stress, viability/fertility controls, and total-protein loading controls. We also did not report variance, confidence intervals and effect sizes. Tests for normality and homoskedasticity were not performed. Protein loading was normalized for gels, but corrections for worm number, developmental stage, feeding rate, bacterial load, and temperature-induced changes in ingestion were not included. Sensitivity to key parameters (AHA concentration, time course, bacterial strain, worm stage, copper ligand) was not explored; no biological replicates or inter-day repeats were performed.
We will proceed with publication.
Best,
Shireesh Apte