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Peer Review

Review of: "Functional Split-tRNA: A New Perspective on the Codon Decoding Mechanism"

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This manuscript explores the novel concept of split tRNA at the anticodon loop (Fig. 1) and demonstrates the functional utility of split tRNA within the translation system (Fig. 2). The study further identifies bases 37 and 38 as critical for optimizing split tRNA activity and investigates the specific configurations that enhance its function (Fig. 3). Based on the obtained data, the authors construct a model to describe the functionality of split tRNA, which is used for analyzing chemical reaction kinetics (Fig. 4). Furthermore, they uncover a novel IRES-like translation initiation mechanism dependent on split tRNA, representing a significant advancement in synthetic biology.

From my perspective, the functional validation of split tRNA is a remarkable discovery. The proposed translational elongation mechanism, based on the use of split tRNA, is logically sound and compelling. Moreover, the development of a new translation initiation system is an original and highly valuable contribution. It is a privilege to review this excellent manuscript, which I believe is publishable in its current form. Below are my comments, which I hope the authors will find helpful to improve the manuscript further.

-- The manuscript, while comprehensive, contains plenty of data and is complex in structure. While I have reviewed it carefully, I am concerned about my potential misunderstandings. Simplifying the manuscript's structure would enhance readability and improve the reader's understanding. While I leave the specifics of such revisions to the authors and journal editors, I personally favor concise manuscripts that highlight the key points succinctly.

-- In Fig. 2, the meanings of "1xCGC" and "1xAGG" were not immediately clear. Adding explanatory notes in the legend would be beneficial.

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-- The interpretation of Fig. 2 is challenging, particularly regarding the factors influencing Vmax. Two key aspects seem relevant: (1) the competition between Ala and Arg affecting fluorescence from the product, and (2) the overall protein synthesis levels, especially given that AGG is a rare codon likely to reduce synthesis. Comparing fluorescence levels for proteins with Ala codons at position 226 under conditions with AGG versus CGC for other Arg codons could validate these interpretations. However, as this experiment may not be critical for the overall claim of this manuscript, clarifying the reasoning in the text (if correct) would suffice.

-- The results related to competitive and non-competitive conditions in Fig. 2 are difficult to interpret. For instance, intuitively, the non-competitive condition should increase the likelihood of Ala incorporation at position 226, potentially raising fluorescence levels, but this is not observed. The disparity between CGC and AGG templates is also unclear, and the trends described as "opposing" are not sufficiently explained. The experimental systems used for these comparisons may differ from those in Fig. 3, which appear more practical and informative. This raises questions about the necessity of comparing competitive and non-competitive conditions in Fig. 2. Presenting only non-competitive data might reduce confusion.

-- The two-codon assay is introduced abruptly in the manuscript. Given its limited importance to the study's central conclusions, including it here might add unnecessary complexity. Removing or recontextualizing it could improve readability.

-- The reference list for supplementary materials (79-) was not located. If present, ensuring visibility would be helpful. Apologies if I overlooked it.

-- The critical conclusion regarding the entropic and enthalpic contributions to codon-anticodon stability in split versus intact tRNAs (as stated in the main text) is significant but difficult to follow due to the manuscript's scattered data and unclear structure. Improving the manuscript's organization would dramatically enhance its readability and impact.

-- Equations such as Eq. 1 and Eq. 2 are poorly formatted in the PDF version, making them hard to read. Addressing this issue would improve the manuscript's presentation quality.

-- The experimental design in Fig. 3 addresses many of the issues I noted in Fig. 2. By using the same extract and tRNA preparation, the comparisons between competitive and non-competitive conditions are clearer and more robust. Highlighting these improvements would strengthen the manuscript.

-- Fig. S9 does not use the terms "competitive" and "non-competitive," which may confuse readers. Maintaining consistent terminology throughout the manuscript and supplementary materials would help avoid misunderstandings.

-- The purpose of the extensive references cited in Figure 5 is unclear. Adding clarification would enhance comprehension.

Declarations

Potential competing interests: No potential competing interests to declare.