Responses to Peer Reviewers' Comments

Reviewer D:

This research is very preliminary, the method is not a new development or have high novelty, even though this is what the author want to show and approved ; research that the in silico method can improve the qPCR validation system to be more effective.

but in fact, almost all researchers when doing qPCR optimization, do the same thing.

My personal suggestion for this research is will be more interesting if it focuses on why these genes were chosen in the beginning. The author developed the qPCR optimization method with in silico approach, its possible to be matrix for the new logarithm of broad in qPCR optimization application in the future.

> Answer: Target gene selection reasons have been added to the abstract (Line 11) and introduction section (Line 56-60).

This research needs to find novelty in method development if the emphasis of the research is on method development. However, if the aim of the author's research scope is to examine the expression of several genes in Rattus novergicus, then it will be much more meaningful, unfortunately it is not discussed further in the entire article.

> Answer: This article emphasizes the workflow of the primer design and optimization, accounting for the SYBR Green fluorophore optimum annealing temperature (revision has been made in the article title and lines 9th and 27th) to ensure the primer oligonucleotide molecular function and displaying the optimized and validated oligonucleotides for the selected target genes in *Rattus norvegicus*.

Reviewer E:

This article is good and comprehensive in designing ideal primers for use in qPCR and observed by electrophoresis.

The weakness of this article does not explain about:
1. no information about the target gene analyzed

Answer: Target gene selection reasons has been added to the abstract (Line 6) and introduction section (Line 56-60).

2. Some of the primers analyzed were not named in the journal

Answer: The unsuitable sets of primer sequences analyzed were considered to be not shown in the article. This consideration was taken as the enormous size of primer sets was found from published articles and generated via *in silico* design, and each primer set candidate was analyzed.

3. Can the design method be used for DNA because this article used cDNA as the template

Answer: This *in silico* primer design and analysis method could be used for quantification purposes, including DNA template. However, the clarity of method validation needs to be confirmed further. The primer design method for sequencing purposes would need specialized adjustment (Karst *et al*., 2021).

4. Why are the PCR products from electrophoresis almost the same size 100-150 bp?

Answer: Consideration was given to selecting qPCR amplicon lengths ranging from 100-150 bp for each target gene, with such shorter amplicon lengths exhibiting better amplification kinetics. Shorter amplicon length could ensure completed polymerization during the qPCR cycle, thus ensuring the relative fluorescence unit scanning and increasing the certainty of relative quantification of cDNA (Debode *et al*., 2017).

References:

Debode, F., Marien, A., Janssen, E., Bragard, C., Berben, G. 2017. The Influence of Amplicon Length on Real-Time PCR Results. *Biotechnol. Agrom. Soc. Environ.*, 21(1), pp. 3-11. doi: 10.25518/1780-4507.13461.

Karst, S. M., Ziels, R. M., Kirkegaard, R. H., Sorensen, E. A., McDonald, D., Zhu, Q., Knight, R., Albertsen, M. 2021. High-Accuracy Long-Read Amplicon Sequences using Unique Molecular Identifiers with Nanopore or PacBio Sequencing. *Nature Methods*, 18, pp. 165-169. doi: https://doi.org/10.1038/s41592-020-01041-y.