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OPEN PEER REVIEW

In Vitro Chondrogenic Differentiation of Human Adipose-Derived Stem Cells by Diacerein

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About this report

This open peer review (OPR) report shows the public comments made on this article during peer review, by the reviewers, the associate editor and the editor-in-chief, for every revision round, together with the authors' replies. Comments appear only with the consent of the people who wrote them. Reviewers who chose to sign their report are named; the others are shown as "Referee". Comments are edited for language only, and confidential remarks to the editors are not included.

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1 Original submission

1 comment · 21 Jun 2023

Referee 1 Reviewer

MAJOR REVISION 21 Jun 2023

Items that needed to be corrected and clarified are marked in the text of the article.

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Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor and Reviewers,

Thank you very much for your letter dated 21th June 2023 regarding our manuscript entitled "Chondrogenic Differentiation by Diacerein in Mesenchymal Stem Cells" (Manuscript code: "137803"). We have modified the manuscript according to the reviewers' comments. The modification/addition in the manuscript text is highlighted in yellow. The point-wise answers to the reviewers' queries are provided.

We hope that the reviewers will find our responses to their comments satisfactory, and we are willing to finish the revised version of the manuscript including any further suggestion that the reviewers may have. We want to extend our appreciation for taking the time and effort necessary to provide such insightful guidance.

Yours Sincerely,

Morteza Daliri Joupari,

Corresponding author

Reviewers' Comments:

Reviewer 1:
Reviewer Comments on Manuscript (ID No. 137803): Chondrogenic Differentiation by Diacerein in Mesenchymal Stem Cells
The above submitted manuscript presents and discusses the effect of diacerein on chondrogenic differentiation of human MSCs by evaluation the expression levels of genes related to chondrogenesis and also by immunocytochemical analysis.
Some major information is needed for further evaluation of the result, as below:

1) The NCBI accession No. (NCBI Reference Sequence) of the mRNAs of mentioned genes

Answer: We have provided the NCBI Accession Number of mRNAs, as requested by the reviewer.

SOX9 Accession: NM_001348025.3 GI: 1890341613
COL2A1 Accession: NM_001844.5 GI: 1519243785
ACAN Accession: NM_001369268.1 GI: 1634229886
RUNX2 Accession: NM_001369405.1 GI: 1609559034
TGFB1 Accession: NM_000660.7 GI: 1653962350

2) The size (bp) of expected amplicon for each gene in real time PCR

Answer: We have provided the size of amplicon for each gene to address the editors' comments:

Gene	Amplicon Size (bp)
SOX9	176
COL2A1	138
ACAN	182
RUNX2	149
TGFB1	121

There are also some minor remarks, as below:
1) The suggested title: in vitro Chondrogenic Differentiation of Human Adipose-Derived Stem Cells by Diacerein
Answer: We greatly appreciate the Reviewer's suggestion. We have changed the title of the manuscript based on your suggestion.

2) It is recommended that the authors explain about the other genes (COL2A1, ACAN, RUNX2, and TGFB1) as they have explained about SOX9 in "Introduction" section.
Answer: We added some sentences in the Introduction section (Page 4, line 80-87) about other genes involved in chondrogenesis to address the reviewer's concern.

''This process is associated with the expression of specific cartilage genes, which consist of genes for cartilage extracellular matrix ingredients, particularly collagen type II 1 (Col2a1), aggrecan (ACAN), and cartilage matrix protein, in different induction kinetics. Mediators of chondrogenic differentiation and cartilage development include transforming growth factor beta (TGFβ), sex-determining region Y-box 9 (SOX9), and runt-related transcription factor 2 (RUNX2). The regulation of the expression of these factors and genes is done at the level of transcription, spatially and temporally, so that during chondrogenesis, they have distinct and dynamic expression patterns.''

3) The source of Fat should be mentioned in line 89.
Answer: Thanks for your kind reminders. We have revised the sentence as follows: (Page 5, line 106)

''Human fat was harvested from the donor abdomen in six distinct areas, typically through a 2 mm-diameter cannula, into a 10 cc syringe.''

4) Recheck all of the numbers that must be superscript.
Answer: We apologize for this issue. We have corrected superscript numbers throughout the manuscript.

5) The number of the procedure in line 156 should be inserted.
Answer: Thank you very much for pointing this out. We have added the number of the procedure (Page 8, line 173).

6) It is highly recommended that all procedures in "Methods" section be cited to proper references.
Answer: We thank you for this useful suggestion. We have added references to Method procedures to address the reviewer's comment.

Reviewer 2:
Items that needed to be corrected and clarified are marked in the text of the article.

1. The title of the article needs to be clarified according to its content
Answer: We have changed the title of the manuscript to "In vitro Chondrogenic Differentiation of Human Adipose-Derived Stem Cells by Diacerein" based on Reviewer 1 suggestion.

2. Please add some information about different types of stem cells and the advantage of adipocyte cells and why did you choose this type of stem cell?

Answer: Thank you very much for pointing this out. We have added some information as follows: (Page 2, 3, line 43-51)

''The most commonly identified sources for MSC isolation consist of adipose tissue, bone marrow, peripheral blood, umbilical cord blood, Wharton's jelly, and fetal tissues. The remarkable expansion capacity of these cells in vitro allows them to quickly reach the expected number for in vivo therapy. Due to characteristics such as greater proliferative potential, ease of access and harvest, and less invasiveness, the isolation of MSCs from adipose tissue has received more attention than bone marrow and other tissues. In addition, fibrosis reduction, vessels increase, and collagen structure modification in transplanted tissues are created as a result of the transplantation of adipose tissue to different organs. For this reason, they were extensively studied for defective pancreatic islet cells, tendons, and damaged articular cartilage.''

3. What do you mean by 10-4 M?
Answer: It means the concentration of diacerein in the culture media. It was 10^{-4}.

4. In your study the differentiation of the cell with diacerein is three weeks, why did you choose 48hours for cell cytotoxicity assay exposure?
Answer: It's a typo, it should be 72 h. The meaning of writing 48 h is that 48 hours after refreshing the culture medium. We apologize for the confusion. This problem has been corrected in the text of the manuscript (Page 7, line 153).

5. HOW much MTT did you add?
Answer: 5 mg in 1 ml DMSO. We have mentioned this in the manuscript (Page 7, line 154).

''…, the media was replaced with DMEM containing 10% FBS and 10% of MTT (5 mg/ml) in all the wells.''

6. For what? Rewrite your legend
Answer: We have revised Table 1 legend as requested by reviewer (Page 9, line 185).

Revised legend: ''Primer sets for quantitative RT-PCR, related to gene expression analysis.''

7. $P<0.05$
Answer: Thank you. We have corrected it (Page 10, line 202).

8. Add a little bit more data about staining
Answer: Thank you for your nice comment. As suggested by the reviewer, we have added more information about staining (Page 10, 11, line 213-217).

''Figure 3 shows differentiated isolated stem cells to adipose and osteocyte cells. Differentiation into osteocytes was confirmed by extracellular matrix mineralization of differentiated cells in the presence of Alizarin Red (Figure 3B). In addition, the accumulation of lipid droplets in cells indicated the adipogenic differentiation of stem cells by Oil Red O staining (Figure 3C). There was no difference in the staining of control cultures in normal growth media (Figure 3A).''

9. Change the Y-axis to percentage of viable cells compare to control group
Answer: We appreciate for this comment. Following your suggestion, the Y-axis in Figure 4 has been revised to cell viability percentage.

10. What do you mean?
Answer: We are sorry for this confusion. We revised the sentences in 4.2. Section to make them clearer (Page 11, line 225-227).

''Three different concentrations were chosen as a trial test; 10^{-4} M, 10^{-5} M and 10^{-6} M. Highest nontoxic concentration of DMSO as the solver was 1%.''

Highest nontoxic concentration of DMSO as the solver was 1% according to our pilot study and previously research.

11. Clarify this phrase
Answer: We used three different concentrations as a trial test; 10^{-4} M, 10^{-5} M and 10^{-6} M. As revealed in this study, the 10^{-4} M concentration was toxic for cells compare to control with a highly significant difference ($p<0.001$). Diacerein with 10^{-5} M concentration was the highest concentration that was not toxic for cells with no significant difference to control ($p = 0.865$) and also diacerein with 10^{-6} M ($p = 0.679$) had no cytotoxicity effect on cells. For this reason, 10^{-5} M concentration was chosen as the highest nontoxic concentration for the chondrogenesis study. On the other hand, highest nontoxic concentration of DMSO as the solver was 1% according to our pilot study.

12. In the method section, the exposure time was 48 hours. Please correct the exposure time
Answer: We deeply apology for this typo error and corrected it. It should be 72 h. Thank you.

13. Improve your discussion by comparing your result with other related studies
Answer: We have followed the reviewer's comments and discussed these aspects as much as possible.

''It also has been suggested that an increase in the tissue inhibitor of metalloproteinase-1 (TIMP-1) and a decrease in the generation of pro-matrix metalloproteinases (MMPs), which play a role in cartilage destruction, are caused by diacerein at the level of gene expression (40, 41). (Page 14, line 303-306).
Studies have shown that diacerein's efficacy is associated with the reduction of IL-1β-induced inflammatory pathways which are mainly involved in cartilage degradation (34, 43). (Page 15, line 309-311).
Altogether, our findings are in agreement with previous reports demonstrating the efficiency of diacerein to promote chondrogenic differentiation by up-regulating the expression regulator proteins during chondrogenesis (COL2, SOX9, and ACAN) (48, 49). (Page 15, line 320-323). ''

14. How does your scientific finding in this article change the position of diacerein? Its local use, in the stages of cell therapy...?
Answer: Side effects of oral usage are the main problem for diacerein but due to its molecular pathway we decided to check that we could use it as a local drug to have drugs effect while we remove the side effects. We found that diacerein has a chondrogenic effect on stem cells so it can be used as combining with other techniques such as hydrogels to treat osteoarthritis.

15. Which percentage of DMSO did you use?
Answer: 1 percentage of DMSO. According to the previously research[1] and our pilot study we used 1% of DMSO as a highest nontoxic concentration.

[1] Pal, R., Mamidi, M.K., Das, A.K. et al. Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells. Arch Toxicol 86, 651–661 (2012). https://doi.org/10.1007/s00204-011-0782-2

16. In the specific range that you studied

Answer: Thank you for pointing this out. We added ‘’in the specific range’’ in the desired sentence in Conclusion section (Page 15, line 328).

‘’Evidences in this paper proved that specific concentration of diacerein has potentiality to initiate chondrogenesis without using any other factors or special media and also has no toxic effect on cells in the specific range.’’

2 Revision 1

0 comments

No public comments in this round.

3

Revision 2

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor and Reviewer,

Thank you for giving us the opportunity to submit a revised draft of our manuscript titled “Chondrogenic Differentiation by Diacerein in Mesenchymal Stem Cells”. We appreciate the time and effort that the editor and the reviewer have dedicated to providing valuable feedback on our manuscript. We are grateful to the reviewer for their insightful comments on and valuable improvements to our paper. We have been able to incorporate changes to reflect most of the suggestions provided by the reviewer. We have highlighted the changes within the manuscript.

Here is a point-by-point response to the reviewer’s comments and concerns. We hope that the reviewer will find our responses to their comments satisfactory, and we are willing to finish the revised version of the manuscript including any further suggestions that the reviewer may have.

Yours Sincerely,

Corresponding author

Reviewers' Comments:

Reviewer 2:
Reviewer Comments on Manuscript (ID No. 137803): In vitro Chondrogenic Differentiation of Human Adipose-Derived Stem Cells by Diacerein [Revision 1]
The above submitted manuscript presents and discusses the effect of diacerein on chondrogenic differentiation of human MSCs by evaluation the expression levels of genes related to chondrogenesis and also by immunocytochemical analysis.

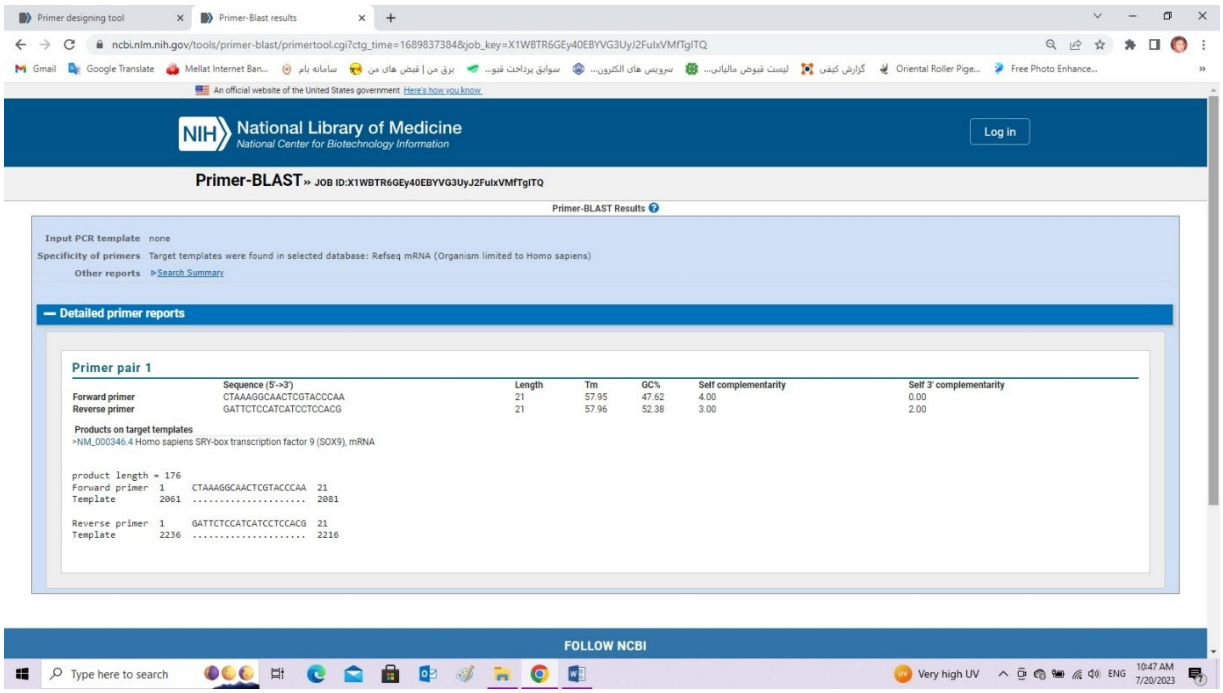
There are some remarks, as below.

Major remarks:
• Based on given primers for RUNX2 (NM_001369405.1) in Table 1, there is no potential amplicon with the size of 149bp in the sequence of RUNX2 mRNA. It is highly recommended that the manuscript be revised and all the sections and phrases related to RUNX2 must be omitted.
Answer: Our sincerest apologies for the problem you faced. Following your request, we noticed some errors in our data information- we apologize for not noticing them earlier. We left the primer design and manufacturing to a company. Unfortunately, the company had provided us with incomplete and wrong information. Finally, we received the correct and complete information. We hope the manuscript has been improved accordingly. We are sincerely sorry for the mistake and for any trouble it may have caused. We greatly appreciate your understanding and patience in this matter.

We have added the corrected information for RUNX2 as follows:
Primers: Forward: 5'-TAGGCGCATTTCAGGTGCTT-3',
Reverse: 5'-TGCATTCGTGGGTTGGAGAA-3'
NCBI Accession Number: NM_001015051.4
Amplicon size: 105 bp

• The designed primers for SOX9 are not specific to RNA and there is potential amplicon on genomic DNA with the same size (176bp), at the same Tm (59°C). If there is any genomic DNA contamination during RNA extraction, this potential amplicon affects the result of real time PCR. It is highly recommended that the manuscript be revised and all the sections and phrases related to SOX9 should be omitted.

Answer: While we appreciate the reviewer's feedback, we respectfully disagree. We think designed primers for SOX9 are specific. We also have done the extraction steps carefully and we have tried our best to not have any DNA contamination. We are sure about this matter.



Minor remarks:

• zinc finger protein 606, not SRY-box transcription factor 9 (SOX9) is the Official Full Name of the given accession number (NM_001348025.3) and the given primers in table 1 is not in the sequence of NM_001348025.3. please recheck NCBI accession No. (NCBI Reference Sequence) of the mRNA of SOX9 (However, in the previous remark, it is recommended that the sections related to SOX9 be omitted from manuscript).

Answer: We greatly appreciate the Reviewer's comment and we deeply apology for this error. The correct NCBI Accession Number is NM_000346.4 for Homo sapiens SRY-box transcription factor 9 (SOX9).

• The names of the primers of ACAN should be corrected. The sequence of forward primer is 5'TGCGGGTCAACAGTGCCTATC3' and the sequence of reverse primer is 5'CACGATGCCTTTCACCACGAC3'.

Answer: Thank you very much for the reminder. We have made revisions accordingly in Table 1.

• It is recommended that the NCBI accession numbers and the amplicon sizes of all genes be added to Table 1.

Answer: We appreciate the Reviewer's suggestion. We have added the suggested content to the manuscript (Table 1).

3 Revision 2

0 comments

No public comments in this round.

4

Revision 3

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor and Reviewer,

Thank you for giving us the opportunity to submit a revised draft of our manuscript titled "Chondrogenic Differentiation by Diacerein in Mesenchymal Stem Cells". We appreciate the time and effort that the editor and the reviewer have dedicated to providing valuable feedback on our manuscript. Here is a point-by-point response to the reviewer's comment and concern. We hope that the reviewer will find our response to its comment satisfactory, and we are willing to finish the revised version of the manuscript including any further suggestions that the reviewer may have.

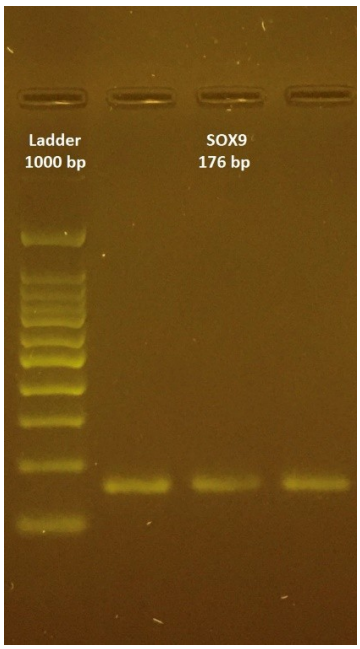
Yours Sincerely,

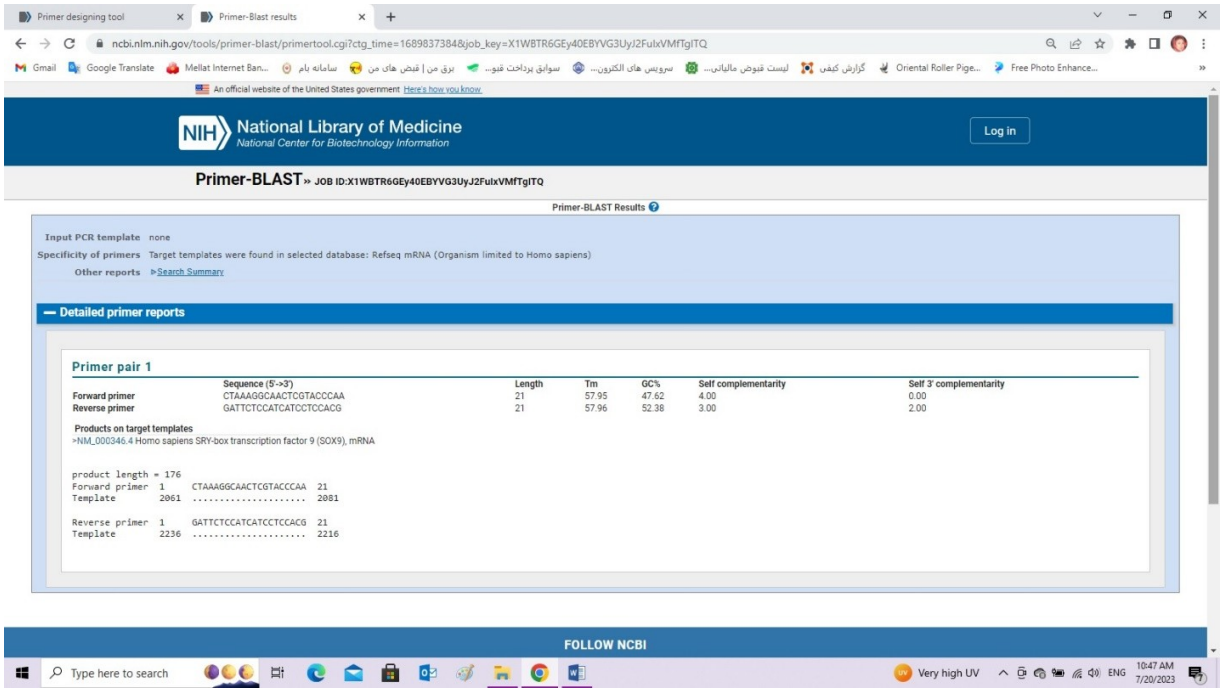
Corresponding author

Reviewers' Comment:

Reviewer 2:
As it was mentioned in previous peer reviewing of this manuscript, the designed primers for SOX9 are not specific to RNA and there is potential amplicon on genomic DNA with the same size (176bp), at the same Tm (59°C) (based on MFE primer 3.1 result). If there is any genomic DNA contamination during RNA extraction, this potential amplicon affects the result of real time PCR. It is highly recommended that the manuscript be revised and all the sections and phrases related to SOX9 be omitted. Otherwise, it is recommended to design new specific primers and carry out the test again.

Answer: While we appreciate the reviewer's feedback, we respectfully disagree. We used DNase treatment. Also, we performed the extraction steps carefully and the NanoDrop and gel electrophoresis results showed that there is no DNA contamination. The image of the gel will be sent to you below.




Primer designing tool
Primer-Blast results
ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time=1689837384&job_key=X1WBTR6GEy40EBYVG3UyJ2FulxVMfTgITQ
An official website of the United States government Here's how you know
National Library of Medicine
National Center for Biotechnology Information
Log in
Primer-BLAST » JOB ID:X1WBTR6GEy40EBYVG3UyJ2FulxVMfTgITQ
Primer-BLAST Results
Input PCR template none
Specificity of primers Target templates were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)
Other reports Search Summary
Detailed primer reports
Primer pair 1
Sequence (5'->3') Length Tm GC% Self complementarity Self 3' complementarity
Forward primer CTAAAGGCAACTCGTACCCAA 21 57.95 47.62 4.00 0.00
Reverse primer GATTCTCCATCATCCTCCACG 21 57.96 52.38 3.00 2.00
Products on target templates
>NM_000346.4 Homo sapiens SRY-box transcription factor 9 (SOX9), mRNA
product length = 176
Forward primer 1 CTAAAGGCAACTCGTACCCAA 21
Template 2061 2081
Reverse primer 1 GATTCTCCATCATCCTCCACG 21
Template 2236 2216
FOLLOW NCBI
Type here to search
Very high UV
ENG
10:47 AM
7/20/2023

4 Revision 3

0 comments

No public comments in this round.

5

Revision 4

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

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Dear Editor and Reviewer,

Thank you for giving us the opportunity to submit a revised draft of our manuscript titled “Chondrogenic Differentiation by Diacerein in Mesenchymal Stem Cells”. We appreciate the time and effort that the editor and the reviewer have dedicated to providing valuable feedback on our manuscript. Here is a point-by-point response to the reviewer’s comment and concern. We hope that the reviewer will find our response to its comment satisfactory, and we are willing to finish the revised version of the manuscript including any further suggestions that the reviewer may have.

Yours Sincerely,

Corresponding author

Reviewers' Comment:

Reviewer 2:
It was expected that the authors mentioned the DNase treatment of extracted RNAs in previous revision, not in Revision 3 of manuscript. Without DNase treatment, the result of this real time PCR is not reliable (and performing the extraction steps carefully and observing single bond of expected DNA amplicon on gel do not confirm the purity of extracted RNAs).
Generally, in order to design primers for Real time PCR, the primer pair should be separated by at least one intron on the corresponding genomic DNA. otherwise, using DNase treatment has been recommended.
As the authors mentioned they have carried out DNase treatment, it is highly recommended that DNase treatment of extracted RNA be added to method section of this manuscript.

Answer: We appreciate the Reviewer’s helpful comment, and revised the manuscript accordingly. We added this sentence to the RT-PCR section: “The RNA samples were treated with DNase to eliminate possible residues of genomic DNA”.

5 Revision 4

0 comments

No public comments in this round.