Response to Editorial, video production and peer reviewer comments (JoVE58596)

Title: "Detection of Tilapia Lake Virus using conventional RT-PCR and SYBR Green RT-qPCR,"

*Dear Dr. Alisha DSouza,*

*We are grateful to the editorial and production team, as well as to the reviewers for their time and comments. The manuscript and video have been altered according to the collected comments and suggestions. The modifications have been incorporated into the manuscript via the Microsoft Word tracked change mode. Our specific answer to each comment is shown below.*

**Editorial and production comments:**

**Changes to be made by the Author(s) regarding the written manuscript:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

***Answer:*** *The manuscript in its entirety as well as its associated figures and tables have been proofread and any observed spelling or grammar mistakes have been corrected. The manuscript has also been updated given the 4 month period of time between submission and revision. This only involved very minor revisions and the addition of a new reference. A few sentences have been added to increase clarity or information to the protocol.*

2. Please revise lines 171-173, 178-179, 305-307, 336-338, 415-417, 428-430, 435-437, 453-455, 544-552, 590-595, and 601-603 to avoid previously published text.

***Answer:*** *We thank the editors for this comment and also apologise for this issue; it was not done intentionally and we have of course completely revised all of the lines stipulated above.*

*The revised lines are specified below:*

171-173 –see lines 187-189

178-179 –see lines 197-198

305-307 –see lines 342-343

336-338 –see lines 383-385

415-417 –see lines 468-470

428-430 –see lines 481-484

435-437 –see lines 489-492

453-455 –see lines 508-514

544-552 –see lines 610-623

590-595 –see lines 662-677

601-603 –see lines 682-686

*- 3. ?*

4. Figure 1B: It is unclear where the arrow is. Please use a different color for better contrast.

***Answer:*** *The arrow appears to be missing from Figure 1B. The arrow is in the revised high quality version of Figure 1 and is very clearly represented.*

5. Figures: Please line up the panels better. Some panels are off-set in Figure. Please ensure that the panels are of the same dimensions if possible.

***Answer:*** *The authors thank the editorial staff for this comment. We have improved the uniformity and quality of our figures by re-creating them in Adobe illustrator and then conversion to pdf.. Figures 4-6 show representative qPCR data originating from different qPCR instrument associated software and thus it was not possible to make them look completely uniform in style. The authors wanted to keep the authenticity of the data output styles to show the different types of qPCR machines, software and so on that can be used, in this way, we felt it was more “representative”.*

6. Please define all abbreviations before use.

***Answer:*** *This important point was also addressed in comment 1 when comprehensive proofreading of our article was undertaken. We have corrected all abbreviations that were made without any prior statement of definition starting from the long abstract..*

7. 1.1: Please specify the age and type of fish. Please do not highlight any steps describing euthanasia.

***Answer:*** *The authors have removed the highlighted text from protocol part 1.1 and 1.2 since it was not shown in the final cut of the video. Just recently it was shown that TiLV can infect carp too and since any fish displaying TiLV symptoms can be the starting material, the word fish remains in section 1.1. Importantly, a statement concerning the age and exact type of fish exemplified in the protocol has been included in the figure legend of Figure 1 and in the main text at L393-394. A discussion of the exact species and age of the fish typically used in such protocols has been discussed at L603-609.*

8. 6.8: Please write the text in the imperative tense.

***Answer:*** *We have altered the text in protocol section 6.8 and it is now written in the imperative tense.*

9. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

***Answer:*** *The authors specifically downloaded and used the JoVE style output provided on the JoVE website but had not updated their endnote library journal term lists. The full journal names are now included in the JoVE output style and thus shown in our references too. The references that are lacking volume or issues number are because the given reference does not have such a number.*

10. Table of Equipment and Materials: Please change “ml” to “mL”. Please include a space between all numbers and their corresponding units.

***Answer:*** *The authors have re-read all of the information in this table and have made the requested correction.*

**Changes to be made by the Author(s) regarding the video:**

1. Please increase the homogeneity between the written protocol and the narration in the video.

***Answer:*** *We have corrected the video to ensure that there is homogeneity between the protocol and narration.*

2. Titles of the manuscript and the video do not match.

***Answer:*** *Due to the limitation of the length of video (10 minutes), we had to leave out the conventional PCR part of the methodology paper and the parts that are shown in the video are highlighted in yellow in the main text. We felt it was important to keep both types of PCR approaches in the written manuscript since conventional PCR is still so widely used and it makes the manuscript as a whole applicable to more scientist. The fact that we decided to include the more sensitive and advanced part of the method in the video is reflected in the video title taking the name of a sub-section of our whole methodology to make it clear what will be featured in the video.*

3. 02:55, 04:37, etc.: Please use centrifugal force (x g) instead of rcf for centrifuge speed.

***Answer:*** *In this case, the centrifuge (model Eppendorf 5418R) only has RPM and RCF options. Therefore, centrifugal force () could not be selected. However, RCF, which stands for relative centrifugal force, is measured in multiples of the standards acceleration on gravity at the Earth’s surface and thus equates to.*

4. The details in the video are not the same as the details in the written manuscript. For example:

03:44-03:51: The video says “keep at -20 °C for 2 hours to precipitate the RNA” while the written manuscript states that overnight. The written manuscript states that “mix tubes well by inversion several times” while the video/voice do not show that.

***Answer:*** *We have corrected the narration to match what is written in the manuscript and vice versa, including the abovementioned examples.*

04:37: The video says 10,000 while the written manuscript state 12,000.

***Answer:*** *We have changed the centrifugal speed in the manuscript from 12,000 to 10,000 × g at step 2.10 and now the video and written protocol are in agreement.*

05:15: The video says “draw out the left over ethanol using the pipet” while the written manuscript states “Air-dry the RNA pellet at room temperature for no longer than 5 to 10 min”.

***Answer:*** *Both video and manuscript are now corrected as follows: “draw out the left over ethanol using the pipet then air-dry the RNA pellet at room temperature for no longer than 5 to 10 min”. This has been integrated at step 2.12.*

01:43-01:50, 06:20-06:26, 06:33, 08:40-09:20: Such details in the video are not mentioned in the written manuscript.

***Answer:*** *01:43-01:50 – We have added the following details to the manuscript at lines 157-158 & 172-173: “….. should be performed in a laminar flow hood with protective equipment”.*

*06:20-06:26 – We have added the following details to the manuscript at step 3.5, line 253: “dilute the RNA to 200 µg/mL using RNase-free water”.*

*06:33 – We have added the details of the chemical list and conditions for cDNA synthesis as follows:  
 Lines 257: 4.1) Prepare a RT-master-mix according to the number of samples and controls to be tested. This mix includes nuclease-free water, 1X reverse transcriptase buffer containing MgCl2, 0.5 mM dNTPs, 100U reverse transcriptase enzyme mix comprising of reverse transcriptase and RNase inhibitor and mix of random primers of oligo (dT).*

*Line 272: 4.3) Incubate the samples in a thermocycler at 65 °C for 5 min followed by 42 °C for 60 min and 85 °C for 5 min.*

*08:40-09:10 – We have added the details of methods mentioned in the video.*

*Line 365: 6.6.2) Select SYBR as a fluorophore dye, then select unknown as a sample type and insert a name into a sample name box.*

*Line 368: 6.6.3) Open the lid of the RT-qPCR machine and place the qPCR strip into the assigned wells, then close the lid.*

*Line 371: 6.7) Perform the RT-qPCR assay with the selected condition. The machine will start to run after the lid has reached the desired temperature. The fluorescence of the samples is collected after each extension step to monitor the progress of the reaction.*

5. 07:12: The video shows “95 °C 10 min” and “0.5 °C/s”, while the written manuscript says 40 cycles of 95 °C for 10 s and 0.5 °C/ 5 s, respectively. Please also change sec to s and include a space between all numbers and their corresponding units.

***Answer:*** *The authors apologise for these discrepancies and we have corrected the conditions shown in the video to match those written in the manuscript as follow:*

*Condition  
 - 1 cycle of:  
 - 95 ºC 3 min  
 - 40 cycles of  
 - 95 ºC 10 s  
 - 60 ºC 30 s  
 - Increase the temperature from:  
 - 65 ºC to 95 ºC with an increment of 0.5 ºC / 5 s*

6. The video must have a representative results section following the protocol. This section must have voice-over describing the results being shown.

***Answer:*** *We have added a voice-over describing the results of how the sample is interpreted as being positive or negative as well as a brief explanation of the melting curve.*

7. Please upload a revised high-resolution video here: http://www.jove.com/files\_upload.php?src=17871828

***Answer:*** *We have uploaded the revised video according to your guidelines.*

8. 1:11, 1:14, 1:17, 1:28, 1:33, 2:09, 3:20, 5:14, 8:00, - The edits here are jump cuts, which tend to have a jarring effect on the viewer. They should be smoothed out with crossfades instead.

***Answer:*** *The smooth out effect has been used instead of jumping cut.*

9. 9:20 - Before the results section, a numbered chapter title card that reads "Results" or "Representative Results" should be added.

***Answer:*** *A slide with the words “Representative Results” has been added as per the above suggestion.*

10. 10:10 - A numbered chapter title card reading, "Conclusion" should be added here.

***Answer:*** *A slide with the word “Conclusion” has been included to increase clarity in the video.*

11. 10:26 - The "www.JOVE.com" should be removed from this card, since it wasn't produced by JoVE. The rest of the credits can and should stay.

***Answer:*** *www.JOVE.com was removed.*

Video file issues

12. For future submissions, the video file name should include the article ID number (58596).

***Answer:*** *The video file name now includes the article ID number 58596 as instructed.*

**Reviewers' comments:**  
  
**Reviewer #1:**Manuscript Summary:  
The protocol, including the video, will be of interest to a wide range of scientists, mainly in tilapia-producing countries. It will be of particular value for inexperienced scientists or where setting up new diagnostic facilities as is happening in several countries/regions following the increased awareness of TiLV.  
  
Major Concerns:  
Validation of TiLV diagnostic tests are currently of major interest to bodies such as the OIE. This publication describes the use of the same protocol on different samples of different geographical origin in two different laboratories which is somewhat of relevance to test validation. It would, however have been very useful if the protocol could have been validated using the same samples in the two different laboratories and published with this protocol. Please include unless there are important reasons why this can't be done.

***Answer:*** *We are grateful to the reviewer for this comment and agree with the reviewer that the validation of TiLV diagnostics is desperately required but we believe that such validation assays are beyond the scope of this manuscript and accompanying video. The aim of this manuscript is purely methodology driven and we have offered a protocol starting with tilapia fish dissection to TiLV identification using all conventional RT-PCR primers as well as primers for SYBR Green RT-qPCR (all TiLV RT-PCR primers published according to the date of submission). The point of showing the representative results using the same primers but different samples and in different labs was to show that the methodology is reproducible. It is not the place or point of this manuscript to publish validation data concerning any previously published TiLV detection methods. The validation of TiLV diagnostic tools should involve inter and intra laboratory experiments using many TiLV isolates of diverse origin, including those coming from sub-clinical infections and a variety of different sample types (different tissues, mucous, water, and so on). Only this type of approach would give meaning to a validation study and it will most likely require a concerted international collaborative effort. The experiments involved in the validation of TiLV diagnostic assay, may use the methods described here but such a study will be results orientated. The authors believe that the fundamental methods described in this paper will still be useful for any future validated TiLV RT-PCR primers. On a more practical point, the TiLV isolates used here to generate representative results in the two laboratories do not exist in both labs and thus, comparing all samples in each lab using the protocol described here is not currently physically possible at the given time.*

Minor Concerns:  
Reagents: It's not necessarily obvious to the inexperienced reader/user which reagents you are referring to at all times in the protocol vs the Table of materials lists. Please clarify. E.g. Could you number the reagents in the material list and refer to those numbers? That would also at the same time clarify which reagents in the list are potential substitutes for one another.

***Answer:*** *The authors thank the reviewer for their suggestion and agree that this was not entirely clear. The JoVE regulations are that no specific brands, kits, reagents etc. should be stated in the manuscript and examples of what can be used should only be shown in the materials table. The numbering scheme offered by the reviewer was implemented to offset any disparities between the written protocol and those stated in the materials table but we felt that the numbering and constant referral to the materials table ultimately made the protocol longer and less clear. Therefore, a sentence has been added to the main text at L127 introducing the table of materials and the table has been expounded to make it very clear as to which reagents are suggested for which protocol part and sub-step. This was achieved by making ample use of the “comments/description” column and by introducing a column on the right-hand side headed “step” so that the reader knows what suggested reagent or equipment is intended for a particular step. The table also follows the order of the protocol.*  
  
"Optional"-comments: It may not be obvious to the reader when/why one would choose to perform **one of the optional steps. Please clarify.**

***Answer:*** *All optional steps have been reviewed and in each case, we have elaborated as to when and why one might want to perform each offered optional step. Specifically, alterations have been made at lines 204 and 230 to address this comment.*  
  
L99: Incomplete meaning of the sentence: "……. reducing the risk of TiLV." - are you referring to TiLV infection, TiLV spread, TiLV introduction etc? Please clarify.

***Answer:*** *We have altered the sentence at L100 of the revised manuscript to make it clear that we mean reducing the spread of TiLV*.

L383: Please clarify for the reader that these are samples from different countries and different tissues, not just samples analysed at different laboratories. (Not everybody will bother looking up the reference.) The figure legend describes this well but it should also be reflected in the text itself.

***Answer:*** *We thank the reviewer for this point and have added a sentence at L436 of the revised manuscript to make it clear that the samples shown in the representative results are from different countries and tissues types as well as being analysed in the different labs for the purpose of demonstrating the methodology.*L403: "TiLV qPCR assays were carried out as described in protocol 6 in different labs using different samples, SYBR Green reagents and qPCR machines". I'm assuming that these are the results form the same samples that were shown earlier or are these different samples? Please clarify as it is, again, of interest, with regards to validation.

***Answer:*** *Indeed, the same samples have been used in the assays used to generate the representative results in Figures 3A-6A and Figures 3B-6B and we have added a sentence at L455-456 of the revised manuscript to make it clear that the samples are from different countries.*  
  
Video:  
Generally the video is clear and informative. The video already uses a mixture of powerpoint slides and video images. Maybe a powerpoint slide clarifying which reagents could be added to make it clearer for the inexperienced viewers. Also it would make it more useful as a general teaching aid, particularly in countries where TiLV diagnostics are becoming an issue.

***Answer:*** *We thank the reviewer for their suggestion. The video was made according to the JoVE guidelines. Although the amount of reagents and details are important to clarify to the viewers, we can only include key information e.g. name of the reagents in the video due to the time limitation (10 minutes) of the video. The viewers can find detailed information of the protocol and reagents in the main text of manuscript and its accompanying figures and tables.*

For teaching purposes in particular it would be useful if the video explicitly could mention the inclusion of positive and negative controls.

***Answer:*** *There is a graph showing positive and negative controls in the video. Specifically, the positive sample has been compared to negative control sample in the same graph. To take on board the comment of the reviewer, we have added a voice-over to explicitly describe the results concerning what a positive control, negative control, and positive sample look like and where the cut-off point is.*  
  
The non-lethal sampling option is also mentioned which will be new to many viewers. Could this sample option also be shown in the video?

***Answer:*** *The non-lethal sampling procedure produces the same result and thus it is not necessary to show this result in the video, especially due to the limitation on the length of video. Furthermore, the non-lethal sampling method was previously published: “Non-lethal sampling for Tilapia Lake Virus detection by RT-qPCR and cell culture” by Liamnimitr et al. (2018) in which the only difference to the method described here is that the mucus is scraped the mucus and this is then used as a sample for RNA isolation. This has been referenced.*

**Reviewer #2:**   
Manuscript Summary:  
This article/video reports on "Detection of Tilapia Lake Virus using conventional RT-PCR and SYBR Green RTqPCR". This is very interesting information to all scientists and authorities who are working with this emerging diseases around the world.  
  
Major Concerns:  
No.  
  
Minor Concerns:  
In conclusion part of the video, perhaps the authors can elaborate more about the important of the techniques before concluded the whole video/study.

***Answer:*** *At the end of the video, we have added a slide to elaborate the importance of this technique for people who are interested in an alternative and fast-running method.*