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TITLE:

Detection of Tilapia Lake Virus using conventional RT-PCR and SYBR Green RT-qPCR

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SUMMARY:

This protocol diagnoses Tilapia Lake Virus (TiLV) in tilapia tissues using RT-PCR methodologies. The entire method is described from tissue dissection to total RNA extraction, followed by cDNA synthesis and detection of TiLV by either conventional PCR or quantitative PCR using dsDNA binding a fluorescent binding dye.

ABSTRACT:

The aim of this method is to facilitate the fast, sensitive and specific detection of Tilapia Lake Virus (TiLV) in tilapia tissues. This protocol can be used as part of surveillance programs, biosecurity measures and in TiLV basic research laboratories. The gold standard of virus diagnostics typically involves virus isolation followed by complementary techniques such as reverse-transcription polymerase chain reaction (RT-PCR) for further verification. This can be cumbersome, time-consuming and typically requires tissue samples heavily infected with virus. The use of RT-quantitative (q)PCR in the detection of viruses is advantageous because of its quantitative nature, high sensitivity, specificity, scalability and its rapid time to result. Here, the entire method of PCR based approaches for TiLV detection is described, from tilapia organ sectioning, total ribonucleic acid (RNA) extraction using a guanidium thiocyanate–phenol–chloroform solution, RNA quantification, followed by a two-step PCR protocol entailing, complementary deoxyribonucleic acid (cDNA) synthesis and detection of TiLV by either conventional PCR or quantitative identification *via* qPCR using SYBR green I dye. Conventional

PCR requires post-PCR steps and will simply inform about the presence of the virus. The latter approach will allow for absolute quantification of TiLV down to as little as 2 copies and thus is exceptionally useful for TiLV diagnosis in sub-clinical cases. A detailed description of the two PCR approaches, representative results from two laboratories and a thorough discussion of the critical parameters of both have been included to ensure that researchers and diagnosticians find their most suitable and applicable method of TiLV detection.

INTRODUCTION:

The global capita fish supply reached a new record of 20 kg in 2014 and this was due to vigorous growth in aquaculture. Aquaculture remains one of the fastest growing animal food producing sectors worldwide and is the only animal food producing sector that is growing faster than the human population¹. Tilapine cichlids comprise the second most important fresh water fish farmed worldwide with a total global production of 6.4 million tons (MT) and an estimated value of \$9.8 billion US dollars in 2015². The top ten producers of tilapia are China (1.78 MT), Indonesia (1.12 MT) and Egypt (0.88 MT), followed by Bangladesh, Vietnam, the Philippines, Brazil, Thailand, Colombia and Uganda². It is expected that global tilapia production will be around 7.3 MT by 2030³. Tilapia have become such an important global food source not only because they are an inexpensive source of protein⁴ but also because they are easy to breed in capacity under a wide range of water and climate conditions^{5,6}. Just a few decades ago, it was believed that there were few commercially significant diseases threatening tilapia farming but this is no longer true. An emerging viral disease called tilapia lake virus disease (TiLVD) is the first ever critical disease epidemic found in tilapia and the entire industry is at risk. This disease has serious socio-economic consequences and is a direct threat on food security for millions of people in Africa⁷, Asia and South America. At the start of 2018, the World Organization for Animal Health (OIE) reported that the etiological agent of this disease, TiLV, had been officially detected on three continents covering eight countries⁸ and since this pathogen information card was updated there have been more reports of TiLV in Tanzania, Uganda⁹, Indonesia¹⁰, Taiwan¹¹ and Peru¹². TiLV is a novel single-stranded RNA virus described to be an orthomyxo-like virus because it contains a variety of characteristics reminiscent of other orthomyxoviruses such as Influenza or Infectious salmon anaemia virus (ISAV)¹³. It was first identified in the aftermath of massive losses of wild and farmed tilapia in the Lake of Galilee, Israel¹⁴. Thereafter, similar disease outbreaks referred to as summer mortalities and the one month mortality syndrome associated with TiLV infection were reported in Nile tilapia (*Oreochromis niloticus*) in Egypt¹⁵ and Nile and red hybrid tilapia (*Oreochromis* spp.) in Thailand¹⁶, respectively. Detection of aquatic animal viruses is historically carried out by growth and isolation of viruses in cell culture. Various cell lines have been tested for the propagation and isolation of TiLV including, E-11 cells derived from snakehead fish (*Ophiocephalus striatus*)^{17,18}, OmB and TmB originating from *Oreochromis mossambicus*¹⁸, and OnIB and OnIL originating from Nile tilapia (*O. niloticus*)¹⁹. While virus culture has the advantage that it provides material for further experiments, it has the disadvantage that it requires at least 4-7 days to observe the formation of cytopathic effects (CPE) and crucially, different piscine viruses, that are more fit to replicate may be propagated and produce similar CPE.

In the last few decades, there has been a move away from traditional, often time-consuming diagnostic methods such as cell culture, serology and antigen detection and replacement with faster and more sensitive nucleic acid based detection tests^{20,21}. This is evident by the fact that many qPCR assays have been developed as important diagnostic methods for a multitude of viral diseases in aquatic animals, such as for ISAV^{22,23}, viral haemorrhagic septicaemia virus (VHSV)^{24,25}, betanodavirus^{26,27}, salmonid alphavirus²⁸, fish iridovirus²⁹, Anguillid herpesvirus 1 (AngHV1)³⁰, and Lymphocystis disease virus (LCDV)³¹. Robust methods for diagnosis and pathogen surveillance are urgently required to reduce the spread of TiLV. Such methods should allow early detection of infection before clinical signs develop and detection of low virus loads. To date, different PCR protocols including RT-PCR^{14,32}, nested RT-PCR¹⁸, semi-nested RT-PCR³³, and RT-qPCR^{32,34} have been developed for the detection of TiLV in fish tissues. A comparison of RT-qPCR and virus isolation in susceptible cell lines for TiLV detection revealed that RT-qPCR was 1,000 times more sensitive than the virus isolation³². Although each published PCR protocol has reported different sensitivities for the detection of TiLV, most assays are highly sensitive with the detection limits of viral copies at 7.5 copies³³, 7 copies¹⁸ or 2 copies³² per reaction.

The aim of this methods article is to explain, in detail, how to perform TiLV detection assays, starting with tilapia tissue collection, to total RNA extraction, cDNA synthesis and then TiLV specific PCR based assays. Specifically, comprehensive protocols of both conventional RT-PCR and also SYBR green-based RT-qPCR have been described to appeal to a wide range of scientists aiming to detect TiLV. The former is less sensitive but is typically a cheaper detection option. The latter requires more elaborate infrastructure such as a quantitative PCR machine and more expensive reagents, but it has the advantages of being quantitative, fast and highly sensitive, meaning that it can be used for the detection of TiLV in sub-clinically infected fish. The RT-PCR and RT-qPCR protocols were performed in two different laboratories with distinct geographical isolates of TiLV and the included results highlight the sensitivity and reproducibility of the assays described here.

PROTOCOL:

The animal use protocol for this study was approved by the Kasetsart University Animal Ethics Committee under permit number ACKU 59-VET-016.

Note: Please refer to the **Table of Materials** for extended information concerning the reagents and equipment suggested for this protocol.

1. Tissue Sample Collection

1.1. Euthanize the fish using an overdose of clove oil (the volume depending on the size of fish and concentration of products, usually more than 3 mL/L). Immerse one-fourth of the forceps and mayo scissors into 95% (v/v) ethanol followed by burning the equipment using an alcohol burner to sterilize the equipment.

Note: Tricaine methanesulfonate (MS-222) can be used instead of clove oil.

1.2. Find the liver and cut off a small piece (approximately 20 mg) or collect 200 μ L of mucus using cover glass or surgical blade to remove mucus from anterior to posterior of the tilapia fish and place the samples into a 1.5 mL microcentrifuge tube.

1.3. Process samples immediately, store in a RNA stabilizing solution or move them to -80 °C until further use.

Note: The biggest task in working with RNA is preparing intact RNA molecules and keeping them undamaged throughout any subsequent handlings. The RNA backbone is innately more sensitive to damage than DNA. Extraction and isolation of total RNA from tissue cells necessitates careful laboratory technique; take all provisions to prevent RNase contamination by wearing gloves, using RNase free water, reagents, equipment, plastic ware, glassware, working space and by using filter tips for pipetting.

2. Guanidium Thiocyanate–Phenol–Chloroform Extraction of RNA

2.1. Add 1 mL of monophasic solution containing phenol and guanidine isothiocyanate into a tube containing tissue sample from Section 1.

CAUTION: This solution is very toxic and must be handled with care in a laminar flow hood with protective equipment and by wearing the proper protective eyewear, clothing and safety gloves.

2.2. Grind the tissue sample using a tissue pestle homogenizer until homogenous.

Note: Samples can also be homogenized using a power homogenizer combined with ceramic beads. Ensure that the tissue sample is completely homogenized before proceeding to the next step or stop the protocol here and store the samples at -80 °C until further use.

2.3. Add 200 μ L of chloroform for phase separation.

CAUTION: Chloroform is a potential narcotic and is extremely hazardous. It should be handled with care in a laminar flow hood with protective equipment as well as by wearing the proper protective eyewear, clothing and safety gloves. As a less toxic alternative, 1-Bromo-3-chloropropane can also be used.

Note: Scale the volumes up or down where appropriate. For example, if only 500 μ L of monophasic solution containing phenol and guanidine isothiocyanate was used, then only add 100 μ L of chloroform at this step.

2.3.1. Mix samples by hand for 15 s.

2.3.2. Incubate samples for 3 min at room temperature (RT).

2.4. Centrifuge for 15 min at 12,000 × g and 4 °C.

Note: There should be a clear separation into a lower organic phase, a white interphase and an upper aqueous phase containing RNA. This top phase is normally colorless but depending on the type and amount of homogenized tissue, it can have a light pink appearance.

2.5. Transfer the upper aqueous layer (approximately 500 µL) to a fresh microcentrifuge tube without disturbing the interphase.

Note: do not try to transfer the entire aqueous phase, leave a small amount to prevent any potential contamination of the RNA containing aqueous phase with the organic or interphase.

2.6. Add 1 volume of 100% isopropanol to precipitate the RNA.

2.6.1. Optionally, if very small amounts of tissue were used, then add 1 µL (5-10 µg) of RNase-free glycogen to each sample to promote efficient RNA precipitation. This will aid the identification of the RNA pellet in step 2.8.

Note: The glycogen acts as a carrier of the RNA and will prevent small amounts of RNA from sticking to the side of the tube.

2.6.2. Mix tubes well by inversion several times.

2.6.3. Store the samples for 2 h to overnight at -20 °C.

2.7. Centrifuge samples for 15 min at 12,000 × g and 4 °C.

2.8. Discard the supernatant, being careful not to dislodge the RNA pellet at the bottom of microcentrifuge tube.

2.9. Add 1 mL of 75% ethanol (v/v) and mix RNA samples by inverting the tube several times.

2.10. Centrifuge for 15 min at 10,000 × g and 4 °C.

Note: The protocol can be stopped here and the samples comprising the RNA pellet in 75% ethanol can be stored at -20 °C until further use.

2.11. Discard the supernatant, being watchful not to dislodge the RNA pellet at the bottom of microcentrifuge tube.

2.11.1. Optionally, repeat steps 2.9 – 2.11 using 70% ethanol (v/v). Thoroughly washing the RNA pellet will minimize any salt or contaminant carry-over that may interfere with sensitive downstream applications.

2.12. Draw out the remaining ethanol using a pipette and then air-dry the RNA pellet at room temperature for no longer than 5 to 10 min.

Note: over-dried pellets will be difficult to resuspend.

2.13. Add 30-60 μL of RNase-free water, pre-warmed to 55-60 $^{\circ}\text{C}$ to solubilize the RNA pellet.

2.14. Place the RNA on ice for immediate use or store at - 80 $^{\circ}\text{C}$ for later use.

3. Quantify RNA Concentration using a Micro-Volume Spectrophotometer

3.1. Switch the settings of the spectrophotometer to RNA.

3.2. Use 1-2 μL of RNase-free water as a blank.

3.3. Use 1-2 μL of each RNA sample to assess the RNA quantity.

3.4. Record the readings at 230 nm, 260 nm and 280 nm for each sample.

3.5. Dilute the RNA to 200 $\mu\text{g}/\text{mL}$ using RNase-free water.

4. Synthesis of complementary DNA (cDNA) using total RNA

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 MgCl_2 , 0.5 mM dNTPs, and 100 U of reverse transcriptase enzyme mix comprised of the reverse transcriptase, RNase inhibitor and mix of random primers and oligo (dT).

Note: The controls are a minus-reverse transcriptase sample (-RT) wherein the RT enzyme is replaced with nuclease-free water and a no template control (NTC) wherein nuclease-free water is added to the master-mix instead of RNA template.

4.2. Add 1 μg of the adjusted RNA (200 ng/ μL) of total RNA from protocol 2.

4.3. Incubate the samples in a thermocycler at 65 $^{\circ}\text{C}$ for 5 min followed by 42 $^{\circ}\text{C}$ for 60 min and 85 $^{\circ}\text{C}$ for 5 min.

4.4. Dilute the synthesized cDNA to a desired concentration by adding an appropriate volume of nuclease-free water and place the cDNA on ice for immediate use or store it at -20 $^{\circ}\text{C}$ for later use.

5. TiLV Conventional PCR

5.1. Use the cDNA, samples and controls, generated in protocol section 4 as templates for a PCR reaction using any of the established primer pairs detailed in **Table 1**, along with a DNA polymerase of choice.

Note: An additional no-template control (NTC) should be included here by substituting cDNA for nuclease-free water in the PCR reaction. A positive control, if available, should also be included comprising previously verified TiLV positive samples or the appropriate TiLV cDNA fragment cloned in to a plasmid.

5.2. Prepare a PCR master-mix according to the guidelines of the DNA polymerase system in use and the number of samples and controls to be tested. This mix should include the forward primer, reverse primer, dNTPs, MgCl₂ and the selected DNA polymerase, along with its buffer.

5.3. According to the guidelines of the selected DNA polymerase, combine the designated volume of master-mix with suggested amount of cDNA samples and control samples.

Note: Preparing a 0.5x reaction excess is often beneficial since some of the master-mix is lost during pipetting.

5.4. Perform PCR cycling conditions according to the guidelines of the utilized DNA polymerase system and using an appropriate annealing temperature for the primers in use (**Table 1**). Usually, such a program will involve an initial denaturation at 95 °C for 2-5 min, followed by 30-40 cycles of denaturation at 95 °C for 30 s, annealing at the recommended temperature for 30 s and elongation at 72 °C for 30 s, followed by a final elongation at 72 °C for 5-10 min.

5.5. Load 5-15 µL of each PCR reaction and an appropriate DNA ladder in to wells of a 1-2% agarose gel, depending on the expected PCR product size. Separate the amplified DNA by gel electrophoresis and stain the gel by ethidium bromide (EthBr) to facilitate visualisation of DNA bands of the expected size (**Table 1**) in a gel documentation machine using UV light.

CAUTION: EtBr is toxic; it should be handled with care by wearing the proper protective clothing and safety gloves.

[Enter Table 1 here]

6. TiLV Quantitative Polymerase Chain Reaction (qPCR)

6.1. Using a plasmid containing the appropriate TiLV genomic segment 3 cDNA as a standard, such as pTiLV³², prepare a duplicated or triplicated 10-fold serial dilution series.

6.2. Prepare a qPCR master-mix for all samples, standards and controls, taking into account that the reactions should be performed in duplicate or triplicate utilizing 0.4 µL of nuclease-free

water, 0.3 μ L of forward primer, 0.3 μ L of reverse primer and 5 μ L of 2x SYBR Green DNA polymerase master-mix per reaction.

6.2.1. Use the primers at a concentration of 10 μ M, and the primer information and the standard pTiLV as follows:

Forward primer: TiLV-112F (5'-CTGAGCTAAAGAGGCAATATGGATT-3')

Reverse primer: TiLV-112R (5'-CGTGCGTACTCGTTCAGTATAAGTTCT-3')

Standard pTiLV: 10 pg/ μ L

Note: If the total number of samples and controls is 10 and will be performed in triplicates, this equates to a qPCR master-mix comprising 12 μ L nuclease-free water, 9 μ L forward primer, 9 μ L reverse primer and 150 μ L of SYBR Green DNA polymerase master-mix. Commercially purchased 2x universal SYBR Green DNA polymerase master-mixes contain all the necessary components for the qPCR reaction, namely, the SYBR Green I dye, hot-start Taq DNA polymerase, dNTPs, MgCl₂ and passive reference dyes. Protect the SYBR Green master mix from light.

6.3. Dispense 6 μ L of the qPCR master-mix into qPCR strip tubes or a 96 well plate compatible with the qPCR machine in use.

6.4. Add 4 μ L of cDNA template, controls or serially diluted TiLV standards into the tubes or wells of the 96 well plate.

6.5. Close the qPCR tubes or seal the 96 well plate with a compatible plate cover for the qPCR machine in use

6.5.1. Gently spin down the qPCR tubes or 96 well plate using centrifuge to collect all the liquid in the bottom of the vessels.

6.6. Place the tubes or plate into the real-time thermal cycler.

6.6.1. Program the qPCR thermocycler to perform an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s for primer annealing and elongation, ending with a melting curve step of 65 °C to 95 °C with an increment of 0.5 °C/ 5 s.

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.

6.6.3. Open the lid of the RT-qPCR machine and place the qPCR strip into the assigned wells, then close the lid.

6.7. Perform the RT-qPCR assay with the selected conditions. The machine will start to run after the lid has reached the desired temperature. Collect the fluorescence of each sample after each extension step to monitor the progress of the reaction.

Note: The qPCR machine and related software will automatically calculate all parameters of the assay and display the amplification curves in real-time, while the standard curve and melting curve will be generated at the end of qPCR cycle.

6.8. Perform data analysis and acquisition by first ensuring that the melting curves for each sample and standard have one uniform peak at the expected temperature for the amplicon.

6.8.1. Evaluate the amplification curves of the samples and standards and set the threshold in a region where the amplification rate of the cDNAs is the same in all samples. This is normally performed automatically by the software but should be carefully checked.

6.8.2. Calculate the number of TiLV copies using the standard curve.

REPRESENTATIVE RESULTS

Following the protocol described in section 1, severely moribund red hybrid tilapia that had been reared for 45 d and displaying clinical signs of TiLV infection (**Figure 1A**) were euthanized by bathing in a high concentration of clove oil, which acts as an anesthetic. Reported clinical symptoms are variable but the common symptoms appear to be lethargy, skin erosion and discoloration, exophthalmia, detached scales, open wounds/Lesion and abnormal behaviour^{15,16,33,35,36}, some of these can be clearly seen in **Figure 1A**. The abdominal wall was removed to collect internal organs such as the liver, spleen or head kidney (**Figure 1B**). Mucus samples were also collected at this stage by gently scraping the skin from the anterior to posterior of the fish using a cover glass or surgical blade³⁷.

[Enter Figure 1 here]

Thereafter, the protocol detailed in section 2 for Guanidium Thiocyanate–Phenol–Chloroform extraction of total RNA was followed and RNA quantification as outlined in section 3 was performed to assess sample purity by calculation of the purity ratios and examination of spectral profiles (**Figure 2**). **Figure 2A** shows a representative result from a successful total RNA extraction procedure, while **Figure 2B** represents a poor RNA preparation. Nucleic acids have absorbance maxima at 260 nm while proteins have theirs at 280 nm. The ratio of the measurements at 260 nm and 280 nm indicate the purity of each sample and ratios of 1.9 to 2.1 indicate pure RNA as is the case for the sample in **Figure 2A**. Lower A₂₆₀/A₂₈₀ ratios observed in **Figure 2B** indicate possible protein or phenol contamination leftover from the RNA extraction procedure. Absorbance at 230 nm can be the result of sample contamination and the A₂₆₀/A₂₃₀ ratio is also calculated for this reason. This ratio should be in the range of 2.0–2.2 for pure RNA preparations as illustrated by a value of 2.03 for the sample in **Figure 2A**, while **Figure 2B** has a low A₂₆₀/A₂₃₀ ratio of 1.07 and the spectral profile shows a shift in the trough at 230 nm towards 240 nm which is indicative of residual guanidine or phenol in the sample. For the sample shown in **Figure 2B**, re-precipitating the RNA to remove the contamination may improve the purity of the sample.

[Enter Figure 2 here]

To detect TiLV by RT-PCR, pure samples such as the one represented in **Figure 2A** were reverse transcribed (protocol 4) into cDNA and used as a template for the PCR assay detailed in section 5 and representative results are shown in **Figure 3A**. Primers shown in bold in **Table 1** were used to amplify a 491 bp fragment of TiLV genomic segment 3¹⁴. The PCR products were separated by gel electrophoresis and stained with EtBr for visualization. **Figure 3A** shows the results of a two-step RT-PCR using 4 cDNA samples (S1-S4), derived from the liver of diseased tilapia isolated in Thailand, and in each sample, a clean single band of approximately 500 bp can be observed and thus, samples 1-4 are TiLV positive. The same PCR product was obtained from the positive control sample, comprising cDNA of TiLV segment 3 cloned into a plasmid³² while the no template control (NTC) did not yield PCR products. The assay in **Figure 3B** was performed using the same primers as in **Figure 3A** but in a different laboratory, using a one-step RT-PCR approach and with 5 RNA samples derived from the head kidney tissues of tilapia originating in Egyptian aquaculture¹⁵. It was determined using this detection assay that samples 1, 3 and 5 are TiLV positive while samples 2 and 4 are TiLV negative since no PCR product was found at the correct size. The negative controls, including two minus reverse transcriptase controls and two NTCs did not generate any PCR products. A one-step RT-PCR assay was also performed targeting tilapia ActinB gene. The amplicon size of 217 bp was generated in every sample (S1-S5) as expected³⁸. This assay served as a control for the integrity of the RNA samples as well as allowing for a semi-quantitative examination of the TiLV positive samples. Given that the generated Tilapia ActB product is relatively equal, then differences in the amount of TiLV specific PCR product generated can be interpreted as a true reflection of the amount of TiLV in a given tissue sample.

[Enter Figure 3 here]

Unlike the endpoint PCRs represented in **Figure 3**, qPCR assays which are explained in protocol 6, measure the amount of PCR product after each PCR cycle. The amplification of target DNA is detected using fluorescent molecules that interact with DNA generated from each round of reaction. Here, SYBR Green I dye was utilized, which intercalates with double-stranded DNA. The fluorescent signal is followed during the reaction and its intensity relates to the amount of product formed³⁹⁻⁴³. TiLV qPCR assays were carried out as described in protocol 6 in different labs using different SYBR Green reagents, qPCR machines and samples from different countries. The resulting amplification curves are shown in **Figure 4A** and **4B**. It can be observed that for each assay, the course of the experiment has four phases: the linear ground phase, early exponential phase, late exponential phase and the plateau phase. The linear ground phase occurs during the early cycles where DNA duplication cannot yet be identified due to DNA quantities producing insufficient signal/background ratio. Baseline fluorescence is calculated during this phase. Thereafter, target DNA starts to double in concentration with each cycle inducing the signal to become detectable above background and increase exponentially. The amplification efficiency (E) of a well-optimized qPCR assay is very high (near 100%) in the beginning of the reaction and remains stable during this early exponential phase of the amplification and it is at this point that quantification is performed, when reaction efficiency is

still steady. In later cycles the signal starts to plateau, and the intensity of the fluorescence is no longer correlated to the starting template copy number because the reaction components are exhausted⁴⁴. Saturation may also occur due to competition from re-annealing reactions, the changing concentration ratios of the components, or the amount of enzyme units to DNA substrate molecules. Possibly, such parameters account for the differences between the amplification curves for the assays shown in **Figure 4A** and **4B**. The included controls did not generate these characteristic amplification curves.

[Enter Figure 4 here]

At the end of the qPCR thermocycling on the different machines in each laboratory, the data was acquired and analyzed. **Figure 5A** and **5B** show representative melting curves from the assays performed in each laboratory. Each qPCR machine was programmed to perform a melting curve analysis at the end. This was achieved by incrementally increasing the temperature and monitoring the fluorescence as a function of the temperature. When the temperature is high enough to denature dsDNA, a large drop in fluorescence is recorded because the fluorophore molecule is released. The software of each qPCR instrument calculated the annealing temperature (T_m) from the melting curve data by plotting the negative first derivative vs temperature (**Figure 5**). It can be seen that in **Figure 5A** and **5B** that the products formed in the different sample sets have uniform melting transition at the expected temperature of approximately 80 °C for the assay. No other peaks at lower temperatures were observed. Due to their small size, the T_m of primer-dimers is typically lower than that of the target DNA sequence. Therefore, this difference between the T_m 's makes it easy to identify potential primer-dimers or other non-specific amplification products. The controls did not generate melt curves like TiLV positive samples and standards and can be seen as an almost horizontal line at the bottom of the charts in **Figure 5A** and **5B**.

[Enter Figure 5 here]

Most qPCR machines come with a software facilitating further evaluation the qPCR run and will quantify the samples by generating a standard curve by automatically plotting the cycle threshold (C_t) against the logarithm of the pTiLV standards' template copy number as shown in **Figure 6A** and **6B** for the two independent laboratories. Briefly, the C_t is the unit used for evaluating qPCR results. The C_t value denotes the number of cycles required to reach a set threshold fluorescence signal level. The greater the amount of starting template, the fewer cycles it takes to attain a detectable fluorescence level. Indeed, samples with a high load of TiLV will have lower C_t values than samples with a low load TiLV such as in fish with sub-clinical infection. To determine C_t values, background fluorescence levels are first deducted from raw data. Next, the software associated with the qPCR device will automatically select a fluorescence threshold by searching the data curves for each sample and incorporating a C_t representing where the sample crossed the threshold. This is done separately for each assay and each threshold should be carefully evaluated, ensuring that the threshold has been set in the logarithmic part of the amplification curves and at a place where all curves are parallel. Thus, the specific C_t acquired is a relative value and it is relative to the starting template copy

number⁴⁵, but it is also specific for the qPCR machine and reagents used, the efficiency of the PCR amplification and the sensitivity of detection. These parameters contribute to the differences observed using the same assay in **Figure 6**.

From the standard curves in **Figure 6**, regression analyses, including calculation of standard curve slopes (m) and intercepts, amplification efficiencies ($100 \times (10^{1/m} - 1)$)⁴⁶ and linearity of the reaction were performed. Standard curve analyses were also used to confirm sensitivity (limit of detection), repeatability and reproducibility of the assay. Theoretically, the amount of DNA is doubled with every PCR cycle, meaning that the efficiency (E) is equal to 100%. However, in practice such an ideal efficiency is seldom reached due to sub-optimal PCR conditions such as, DNA polymerase inhibition, contaminants, too much cDNA and pipetting errors⁴⁷. Typically, amplification E range from 90-110% for good assays, in **Figure 6A** an efficiency of 94.5% was calculated using 8 serially diluted pTiLV samples, while, the assay efficiency in the assay shown in **Figure 6B** using 7 serially diluted pTiLV samples was 101.2%. An efficiency of over 100% is usually due to the presence of PCR inhibitors in the assay. Linear regression analysis of the standard plot also allows for the calculation of the number of TiLV copies in each sample^{41,42,45}, as can be observed for the three TiLV samples shown in red in **Figure 6B** which is in line with the results for samples S1, S3 and S5 shown in **Figure 3B**.

[Enter Figure 6 here]

FIGURE AND TABLE LEGENDS:

Table 1. Published primer pairs for the amplification of TiLV cDNA using endpoint PCR. The primer set shown in bold were used to generate the representative results shown in **Figure 3A** and **3B**.

Figure 1. Tilapia dissection and sample collection. **A.** TiLV-infected red hybrid tilapia with skin lesions, redness around the mouth and operculum, skin erosion and corneal opacity. **B.** Sectioned red hybrid tilapia to allow for tissue collection from the liver (at the point of blue arrow), spleen or head kidney organs. The red-hybrid tilapia weighed 10 g, measured 5.5 cm in length and had been reared for 45 d.

Figure 2. Spectrophotometric quantification of total RNA extracted from diseased tilapia tissues. **A.** purity ratios and spectral profiles from a successful RNA preparation. **B.** As A, except representative of a poor RNA extraction procedure.

Figure 3. TiLV RT-PCR. **A.** cDNA samples produced from liver tissues of diseased tilapia, collected from Thailand were screen for TiLV infection using specific primers for segment 3 (shown in bold in **Table 1**) of TiLV using a 2-step RT-PCR assay. M = marker shown in base-pairs; S1-4 = samples 1-4; C1 = positive control using pTiLV as a PCR template; and C2 = no template control (NTC). **B.** One-step RT-PCR using the same primers as in A and samples from head kidney tissues of diseased tilapia collected from Egypt¹⁵. M = marker shown in base-pairs; S1 – S5 = samples 1-5. Controls C1-2 are minus reverse transcriptase controls and C3-4 are NTCs.

Bottom panel is a one-step RT-PCR using primers directed against tilapia ActinB³⁸ (see text for details) producing a PCR product of 217 base-pairs.

Figure 4. Amplification plots to show the accumulation of product over the duration of the real-time PCR assay. **A.** Amplification curves of TiLV-positive samples derived from Thailand, NTCs, and positive plasmid control using a SYBR-Green I 2-step qPCR assay. The chart was generated by plotting relative fluorescence (RFU) vs. cycle number. **B.** Amplification curves of TiLV positive samples derived from Egypt, as in Figure 3B and a NTC. The amplification curve is the fluorescence of the reporter signal normalized to the fluorescence of the passive ROX dye included in the assay (Rn) versus cycle number.

Figure 5. Melt curve analysis to ensure assay specificity and different PCR products can be differentiated by their melting features. **A.** Melt curve analysis of TiLV-positive samples originating from Thailand, negative control, and positive plasmid control. **B.** Melt curve analysis of TiLV positive samples derived from Egypt, pTiLV standards and a NTC. The charts in A and B both show the change in fluorescence divided by the change in temperature plotted against temperature to produce a clear picture of the melting dynamics.

Figure 6. RT-qPCR standard curves. Real-time PCR of 10-fold serial dilutions of pTiLV, the standard used in both laboratories. **A.** 8 serially diluted pTiLV samples were tested, all of known concentration and correlated to the number of TiLV copies/ reaction. The standard curve was generated by plotting log copy number vs. cycle threshold (C_t). The slope = -3.462, R² = 0.9992 and the efficiency is 94.47%. **B.** As in A, except 7 serially diluted pTiLV samples (green) were tested and the chart displays the threshold cycle on the y-axis and the copy number of TiLV (Quantity) on the x-axis. The y-intercept = 32.327, slope = -3.292, R² = 0.98 and the efficiency is 101.2%. For both standards curves in A and B, the slope, y-intercept and correlation coefficient values (R²) are utilized to understand the performance of the assay. Importantly, R² value should be close to 1 since it is a measure of the linearity of the standard curve. The slope is used to measure PCR efficiency wherein 100% efficiency corresponds to a slope of -3.32, see main text for the equation and further details. A good qPCR reaction generally has an efficiency between 90-110% correlating to a slope of between -3.58 and -3.10. The standard curve is used for absolute quantification of unknown TiLV positive samples and determines the exact number of TiLV copies / reaction, as is the case for the three TiLV positive samples colored red in B.

DISCUSSION:

TiLV was first reported in 2014 in Israel¹⁴ and since then it has been identified in multiple countries including Egypt, Colombia, India, Malaysia, Uganda, Tanzania and Thailand^{15,16,18,35,48}. Global awareness, particularly, in tilapia producing countries has placed more attention on the virus and various restrictions and control measures from government authorities have been implemented attempting to prevent the spread of TiLV. Here, a detailed protocol for TiLV detection in tilapia tissue, covering sample collection, RNA isolation, cDNA synthesis, PCR and qPCR assays has been explained. There are various aspects to these methods that warrant specific discussion. TiLV has been identified in fish spanning a variety sizes^{9,12,14,15,49} and species of tilapia so far, including farmed hybrid tilapia (*O. niloticus* x *O. aureus*)^{11,14}, Nile tilapia (*O.*

niloticus)^{9,10,14-16,33,35,36,49,50} and red tilapia (*Oreochromis sp.*)^{16,33,48,51}, as well as in wild Nile tilapia^{9,12}, black tilapia⁵¹, *T. zilli*^{14,15}, *S. galilaeus*, *O. aureus* and *T. simonis intermedia*¹⁴ and very recently TiLV was identified in wild carp (*Barbonymus schwanefeldii*)⁵². Tissue samples from internal organs (gill, spleen, liver, heart, head kidney) or mucus³⁷ can be collected from healthy as well as moribund tilapia regardless of age, size or species and processed for RNA isolation. The total RNA extraction protocol outlined here uses a monophasic solution of phenol and guanidinium thiocyanate, which is a chaotropic denaturing agent. The tissues are directly homogenized in this solution followed by the addition of chloroform and centrifugation to achieve phase separation wherein a clear RNA containing upper aqueous phase, an interphase and a lower organic phase is generated. The RNA is isolated from the aqueous phase by isopropanol precipitation, followed by washing of the recovered RNA to get rid of contaminants. Isolation of RNA by this methodology was pioneered by Piotr Chomczynski and Nicoletta Sacchi and was referred to as guanidinium thiocyanate-phenol-chloroform extraction^{53,54}. This type of reagent used for RNA extraction can be commercially purchased or made in the laboratory (see the **Table of Materials** for further information). This protocol takes slightly longer than column-based methods such as the silica-based purification, but in general, it is more cost effective and yields more RNA.

In this protocol, quantifying RNA using A260 values was outlined whereby spectrophotometry values can indicate RNA quality ($A_{260}/A_{280} = 1.9 - 2.1$). While this method will give a good indication of sample purity, it cannot absolutely inform about the quality of the extracted RNA. To properly determine whether the RNA is intact or partially degraded, samples can be separated by agarose gel electrophoresis wherein smearing of the EtBr stained 18S and 28S rRNA bands indicate RNA degradation. Further verification of RNA quality may include using a lab-on-a-chip instrument. Furthermore, it is also important to digest the purified RNA with DNase I to remove contaminating host genomic DNA, which depending on the downstream applications may lead to false results. If host gDNA is still contaminating the RNA sample to a large extent, an additional DNase I treatment can also be performed at the end of the RNA extraction procedure (see **Table of Materials**).

Complementary DNA synthesis can greatly affect the overall qPCR results and is an aspect of the method that can introduce variation. The cDNA protocol advocated here comprises of a commercially bought master-mix containing all components required for the reverse transcription reaction and is very fast and simple without the usual multi-step, pipetting and multi-temperature protocol. This is advantageous because it minimizes handling and promotes uniformity across all samples. The protocol uses both oligo(dT) and random primers making it applicable to different RNA templates and generating representative cDNA copies of sequences from the entire length of the RNAs in a population (viral and tilapia host mRNA) and in theory, every desired RNA species can then be measured by conventional PCR or qPCR from such a sample. This versatility is the main advantage of a 2-step RT-PCR approach; it provides a long-term pool that can be used for many different experiments. In the results, a one-step RT-PCR approach has been represented wherein sequence specific primers (**Table 1**) were used and the RT and PCR were performed in one tube (see material list). In general, sequence specific primers allow for a higher RT efficiency of the specific target RNA than using random priming,

but the specific target RNA is the only one that can be quantified in such a cDNA sample which may be the only aim of certain laboratories.

While conventional RT-PCR appears to be commonly used so far in the TiLV diagnosis^{9,13-18,33,35,48,55}. RT-qPCR has been shown to be a more powerful tool for the detection and quantification of small amounts of TiLV in fish tissues or mucus^{32,37}. In general, qPCR is widely used in clinical virology diagnostic labs due to its high sensitivity, specificity, good reproducibility, wide dynamic range and speed²¹. While qPCR may be initially more expensive to implement than conventional RT-PCR, it does offer many important advantages over conventional PCR; it has a faster turn-around time from sample to results and it does not require any post-PCR steps. This latter point means that there is minimal risk for laboratory contamination and it can be more easily adapted to high-throughput situations such as in the event of outbreaks. Furthermore, it is inherently more sensitive than conventional RT-PCR, which is of vital importance to detect low viral loads in sub-clinical infections²¹. This would require a nested PCR approach requiring reverse transcription, two further PCR reactions and then analysis by agarose gel electrophoresis. These many steps take a lot of time and increase the chances of errors or contamination. Nonetheless, due to its high sensitivity, RT-qPCR demands meticulous experimental design and a thorough understanding of quantification techniques for generating precise results^{56,57}.

The DNA binding fluorophore, SYBR Green I has been demonstrated in this protocol. It is a dsDNA unspecific DNA binding dye and thus the specificity of the assay lies entirely in the set of primers, which may generate false positives⁵⁸. Therefore, while the dsDNA melting curve analysis performed at the end of each PCR is an especially important part of the PCR reaction because it confirms that only one PCR amplicon of the ecorrect T_m is produced (this should also be achieved by gel electrophoresis when new assays are being implemented). The T_m of a DNA fragment is dependent on a variety of features such as its length, GC composition, sequence, strand complementarity, concentration as well as on buffer components and PCR enhancers. The melting curve analyses in the representative results shown from two laboratories did not reveal the presence of primer-dimers or other unwanted PCR products but if this is observed with other samples and/or experimental set-ups, then the assay should be re-optimized. More advanced qPCR technologies do not require such a melting curve step and indeed, since this methods paper was written, a TaqMan based TiLV RT-qPCR has been developed utilizing two primers and a probe making it highly TiLV specific³⁴.

Undoubtedly, the primers designed for RT-qPCR assays are fundamental to the success of assay and the primers here were designed based upon the publicly available TiLV genomic data at the time³². However, RNA viruses are well-known to exhibit high mutation rates and possible strains will escape the current diagnostic tests, as was observed for ISAV⁵⁹. It will always be difficult for such virus types to generate a universal pan-TiLV RT-qPCR assay and such assays will only be continually improved if more TiLV genomic data from far-reaching locations and time periods become available.

Finally, it is essential to run duplicate or if possible, triplicate reactions in both intra and inter qPCR assays. If the C_t values are very high, then the use of replicates is especially important to ascertain that the PCR reaction is reliable and reproducible. In general, if data from replicate reactions varies more than 0.5 cycles, the reactions should be repeated and if the C_t values consistently vary > 0.5 cycles in replicates, the assay should be re-optimized. The use of an integrated qPCR pipetting robot helps immensely with this issue, but it is a luxury tool. As with any experiment, the inclusion adequate and appropriate controls are of utmost importance to the development of robust molecular assays, especially in diagnostic laboratories where such assays have to be accredited. Controls should include positive (positive TiLV sample, TiLV plasmid standard) and negative controls (NTC and –RT) samples as well as the detection of endogenous tilapia housekeeping genes. Such controls cannot be underestimated and should be included in each assay to properly understand the quality of each step of the assay and to properly interpret the results.

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DISCLOSURES:

The authors have nothing to disclose.

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832

Figure 1

A



B

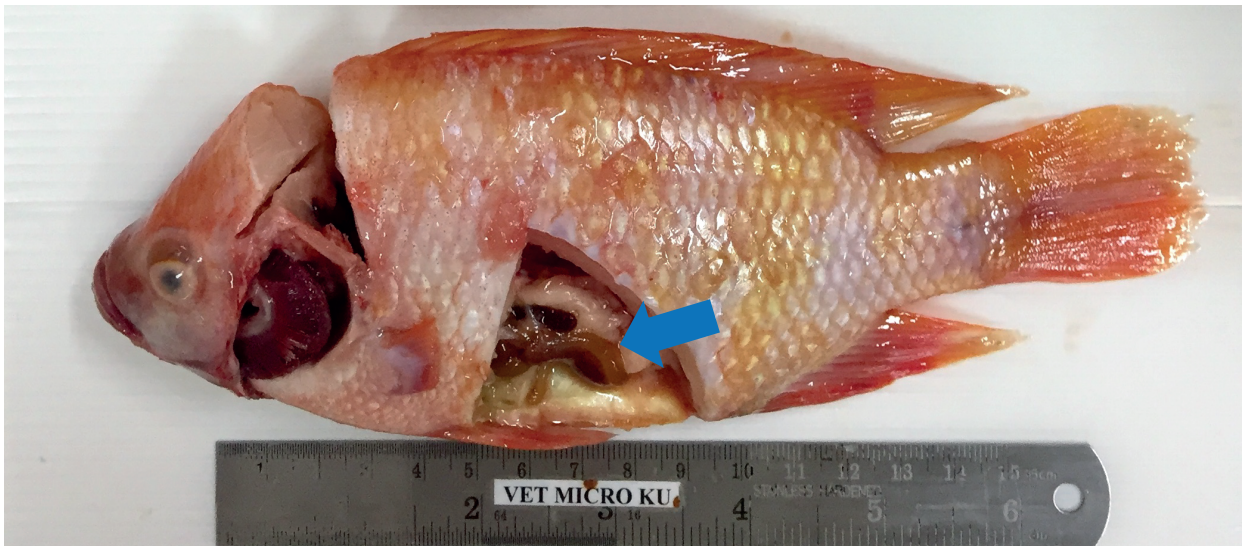
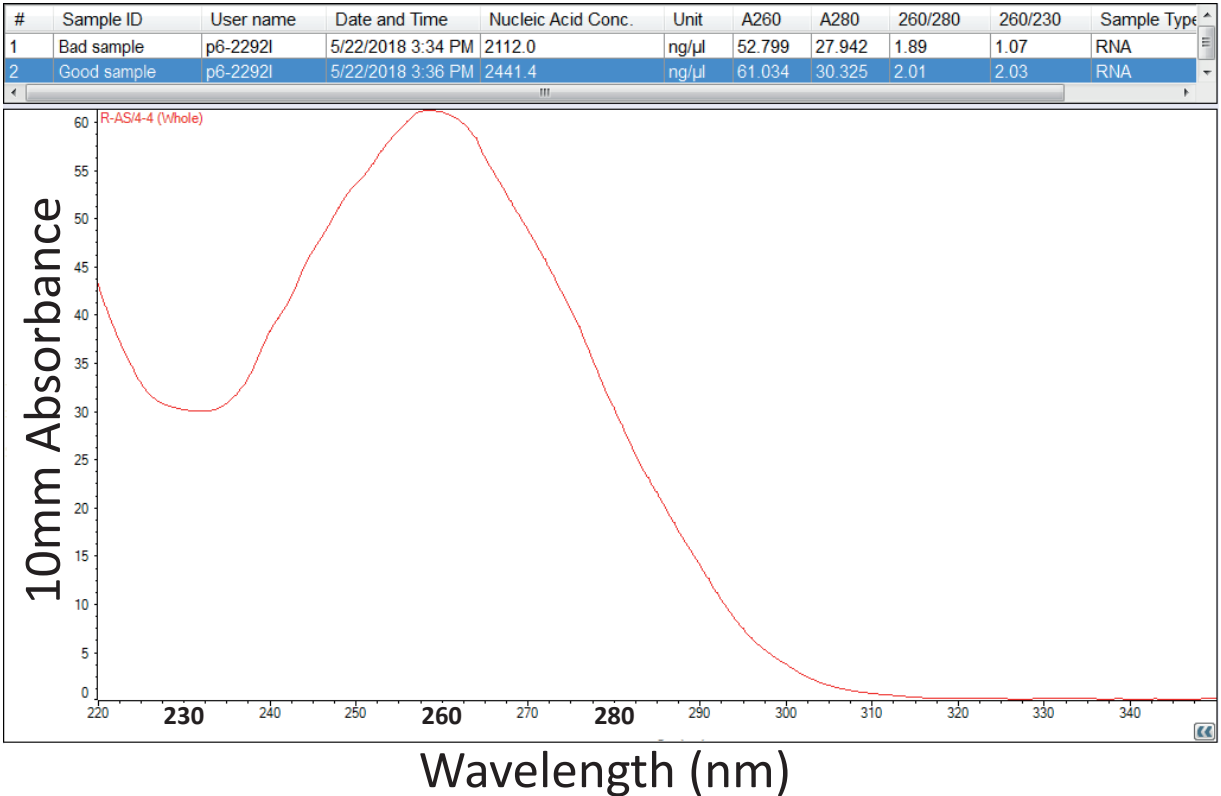


Figure 2

A



B

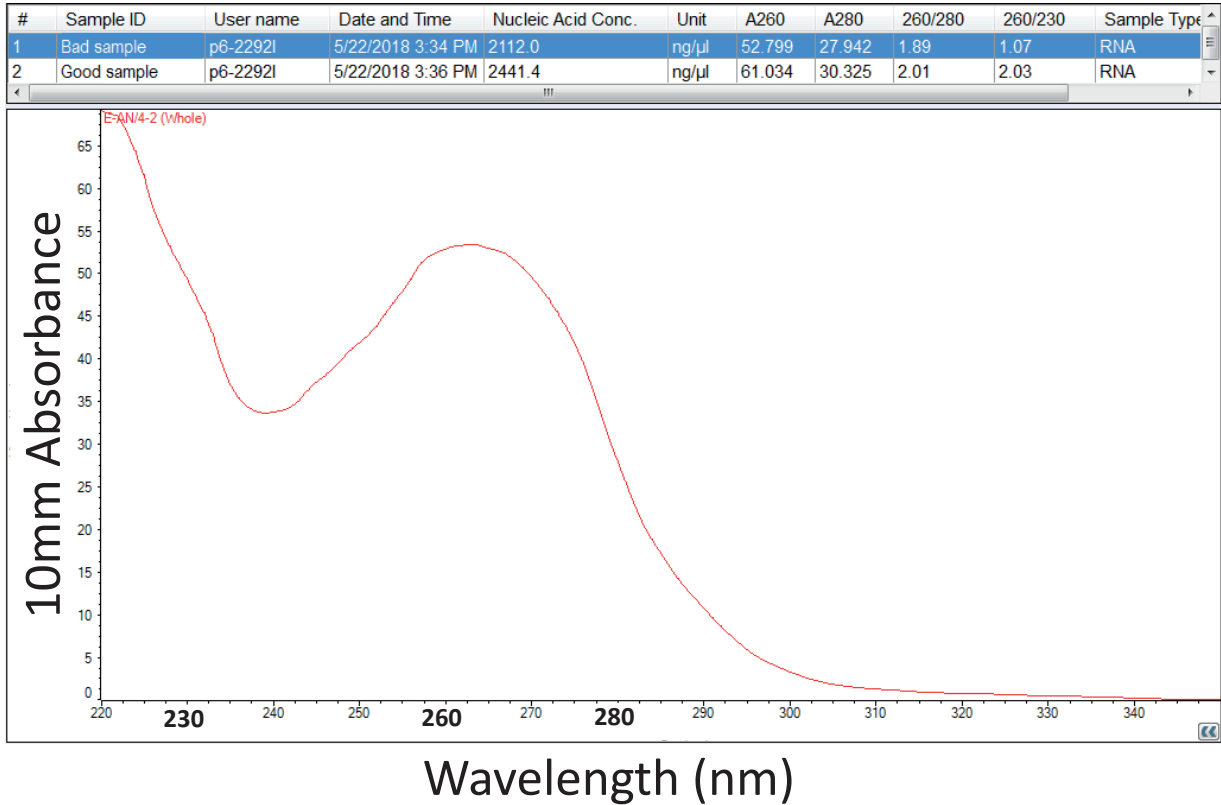


Figure 3

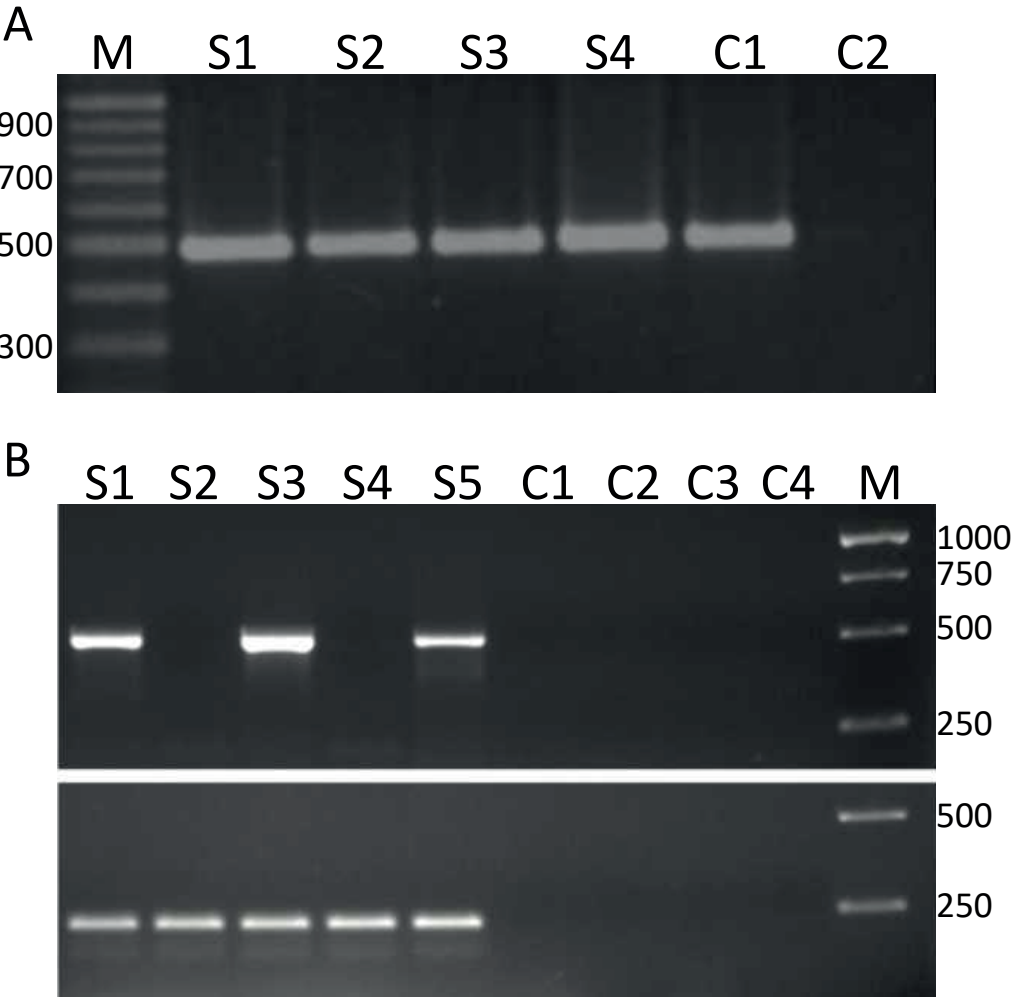
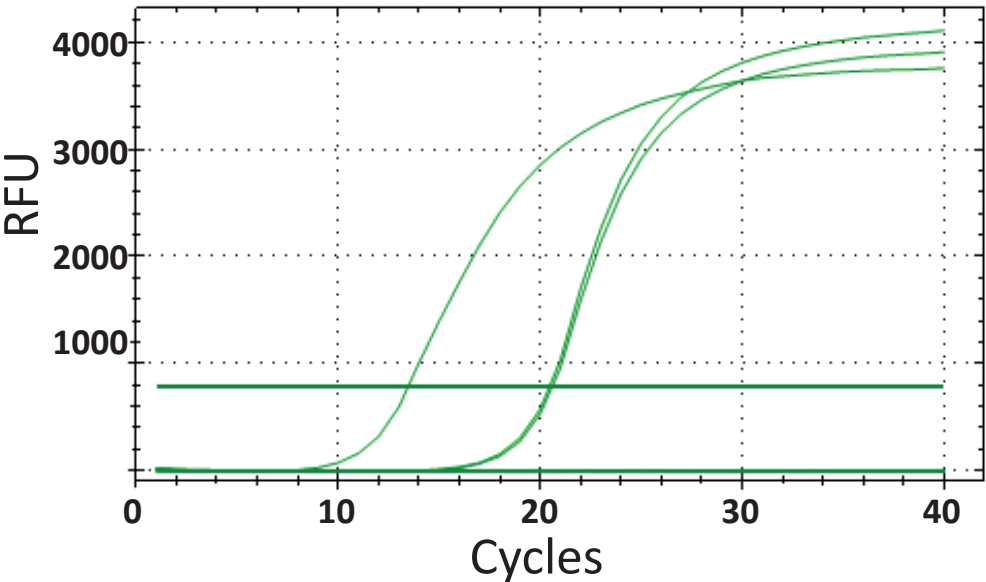


Figure 4

A



B

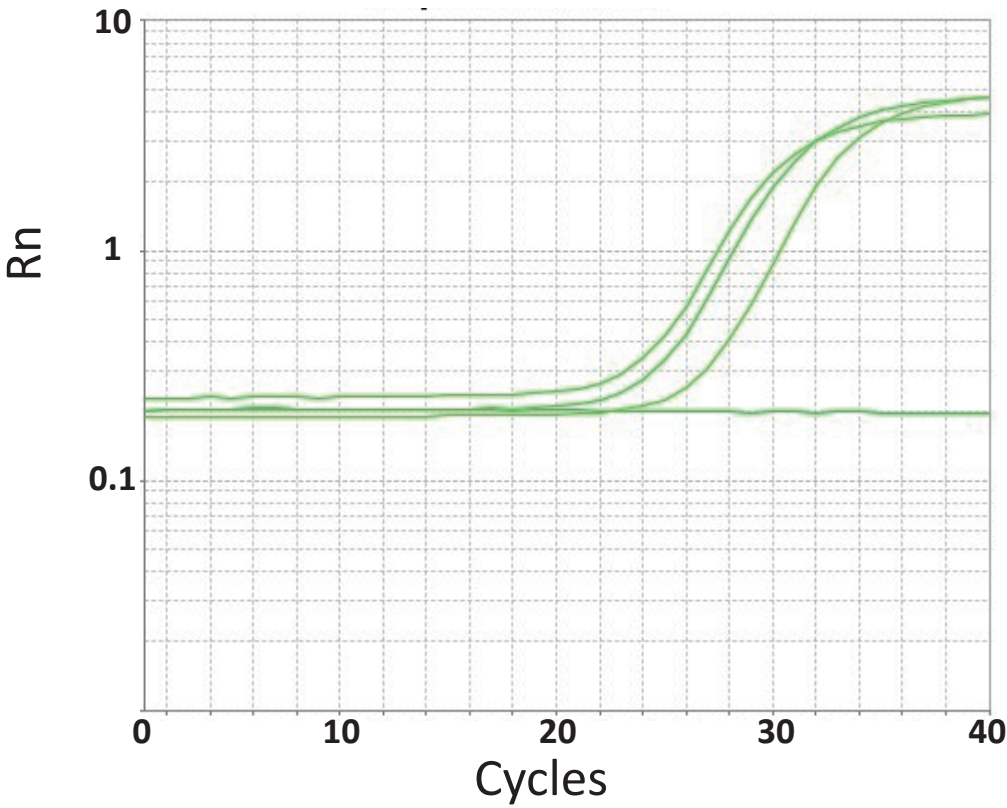
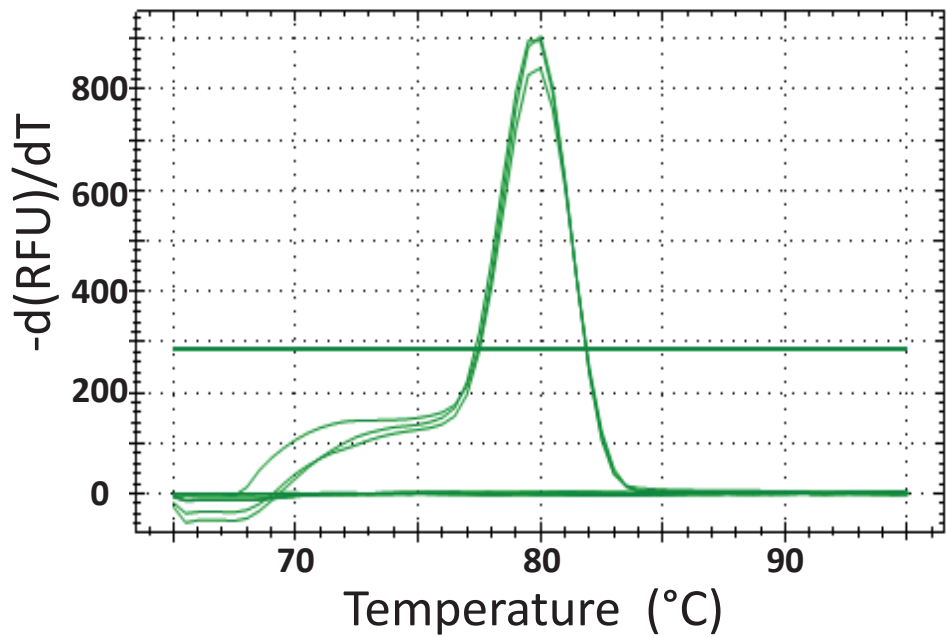


Figure 5

A



B

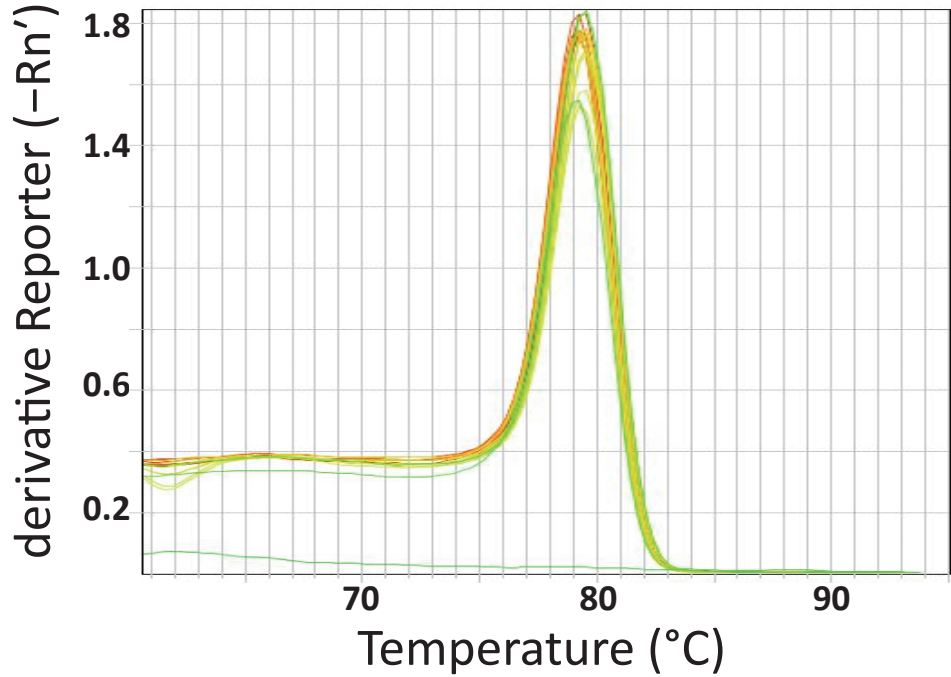
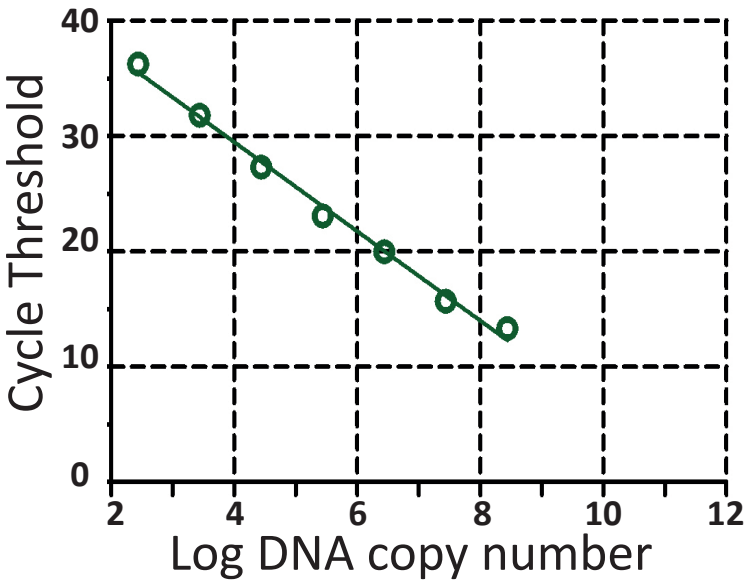
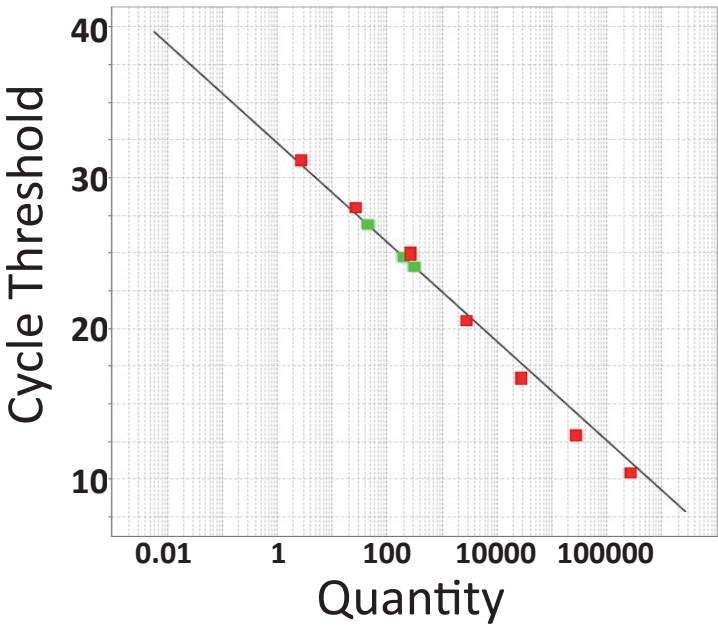


Figure 6

A



B



Target TiLV genome segment	Forward primer 5' - 3'	Reverse primer 5' - 3'	PCR product size (bp)	T _m °C	Original reference
1	CCAAACGTTATCTCTTAATTACGCAC	GCAAATATTTCTCTCATTCGCCT	1641	50	Surachetpong et al., 2017
1	CCTCATTCCTCGTTGTGTAAGT	AGGAGTTGCTGTTGGGTATAG	1000	62	Mugimba et al., 2018
2	ACTCTCTATTACCAAATACATTACT	TTACCATATATATAGTGAAGGC	1445	45	Surachetpong et al., 2017
2	GTCCAGGGCGGTATGTATTG	CTTACGGCTGACAAGTCTCTAAG	834	62	Mugimba et al., 2018
3	GTTGGGCACAAGGCATCCTA	TATCACGTGCGTACTCGTTCAGT	250	56	Eyngor et al., 2014
3	TATGCAGTACTTTCCCTGCC	TTGCTCTGAGCAAGAGTACC	491	57	Eyngor et al., 2014
3	ACCCCTTAATCCTTAATAGACCGTTA	CCCATAATCCTCTATTAGAACGTCGT	1352	50	Surachetpong et al., 2017
3	GTCGAGGCATTCCAGAAGTAAG	GAGCTAAGGGAACGGCTATTG	834	62	Mugimba et al., 2018
4	AGCAGCAGCAGGAGAAAGAG	ACCGTCCTGTTTCTGAATGG	358	60	Nicholson et al., 2017
4	CCAAAGTTTACTCCTATTACCCAGA	GCAAATCTTCTCCAATTACCGTCT	1250	50	Surachetpong et al., 2017
4	GCCCAATGGTCCCATATCT	GCCCAATGGTCCCATATCT	524	62	Mugimba et al., 2018
5	CCAAATGTTTCTTATCTCAGACTC	CTTTTCTCAGTTACCACTTTATG	1087	57	Surachetpong et al., 2017
5	CAACTCTTAGCCTCCGAATAC	CGTTCTGCACTGGGTTACA	696	62	Mugimba et al., 2018
6	CCAAATTTTACCTCTCGCAT	TCAAGCACTTAAACTGTACC	1027	45	Surachetpong et al., 2017
6	CCCACACGACAGGACATATAG	GAGTTGGCTTAGGGTGATAAGA	948	62	Mugimba et al., 2018
7	CTCTCTTGCATTGCATACCGT	GACCAATTATCCCTGCTTTCA	704	57	Surachetpong et al., 2017
7	TCCTTTAGGGATTGGCACTAAC	TTCCATCGACTGCTCCTAGA	486	62	Mugimba et al., 2018
8	ACCTCATCTACCTAACATTTCCA	TCATCATTACACAAATGGAGTAGCT	637	50	Surachetpong et al., 2017
8	CTTAAGGGCCATCCTGTCATC	TGGCTCAAATCCCAACTAA	476	62	Mugimba et al., 2018
9	TTGGTGATGTCACGATGGATA	AGTTCTATCGCCAGCCATGT	351	60	Nicholson et al., 2017
9	ACAAGTCCGATTACTTTTTCCGC	TCTTCTCACGTCCTTAAAGTCA	530	50	Surachetpong et al., 2017
9	GATATCCTCCACATGACCCTTC	GTACGTCACTTTGTGCCATTAC	261	62	Mugimba et al., 2018
10	AACCCTACTAACACCAAATATAGCT	CTTCCCTCTGACACCCTGT	450	50	Surachetpong et al., 2017
10	TCCTCTCTGTCCCTTCTGTT	CAGGATGAGTGTGGCAGATTAT	276	62	Mugimba et al., 2018

Reagents	Company	Catalog Number	Comments/Description	Step
Tissue collection				1
Tricaine methanesulfonate	Sigma-Aldrich	E10521	An alternative to clove oil.	1.1
RNAlater stabilization solution	Thermo Fisher Scientific	AM7020	For storing tissues if they cannot be processed immediately	1.3
RNA extraction				2
TRIreagent	Sigma-Aldrich			2.1
TRIzol	Thermo Fisher Scientific (Invitrogen)	15596026		
GENEzol	Geneaid	GZR100		
Trisure	Bioline	BIO-38032		
Homemade solution	-	-	94.53 g/L (800 mM) guanidine thiocyanate 30.45 g/L (400 mM) ammonium thiocyanate 8.20 g/L (100 mM) sodium acetate 380 mL/L (38 % v/v) phenol 50 mL/L (5 % v/v) glycerol 1.0 g/L (0.1 % w/v) 8-quinolinol, pH 5.0 Store up to 2 years at 4°C	
MagNA Lyser Green Beads	Roche	3358941001	An alternative tissue homogenization method used in conjunction with tissue lysing machines detailed below	2,2
Lysing Matrix D, 2 mL Tube	MP BIOMEDICALS	116913050		
Chloroform	Sigma-Aldrich	C2432		2.3
Chloroform	RCI Labscan	AR1027E-G2.5L		
1-Bromo-3-chloropropane	Sigma-Aldrich	B9673	A less toxic alternative to chloroform	
Isopropanol (GC) ≥ 99.8 %	Sigma-Aldrich	59300		2.6
Isopropanol (ACS, ISO Reag. Ph Eur)	Merck KGaA (EMSURE)	1.09634.2500		
Glycogen, molecular biology grade (e.g., Sigma, cat# 10510)	Thermo Fisher Scientific (Thermo Scientific)	R0551	Useful step if tissue starting material is small to maximise RNA precipitation	<i>optional</i>
Ethanol (purity (GC) ≥ 99.9 %)	Sigma-Aldrich (EMD Millipore)	1.00983		2.9
Ehtanol (ACS, ISO Reag. Ph Eur)	Merck (EMSURE)	1.00983.2500		
Nuclease-free water	Promega	P1193		2.13
Nuclease-free water	Multicell	809-115-CL		
Ambion TURBO DNA- <i>free</i> kit	Thermo Fisher Scientific (Invitrogen)	AM1907	Can be performed at the end of the RNA extraction protocol	<i>optional</i>
cDNA synthesis				4
ReverTra Ace qPCR RT MasterMix with gDNA remover	Toyobo	A1172K		4.1
ReverTra Ace qPCR RT Kit	Toyobo	FSQ-101		
AffinityScript Multiple Temperature Reverse Trans	Agilent Technologies	600107	An alternative option if the above kits are not possible	
PCR				5
DNA polymerase systems :				5.2
- Platinum II Hot-Start Green PCR Master Mix (2X)	Thermo Fisher Scientific (Invitrogen)	14001012a		
- GoTaq Mastermix	Promega	M7122		
Separate PCR mixture components:				
10mM dNTP Mix	Vivantis	NP2409		
25mM MgCl ₂	Thermo Fisher Scientific	R0971		
10X Taq Buffer with KCl	Thermo Fisher Scientific	00348114		

Taq DNA polymerase	Vivantis	PL1202		
- Verso 1-step RT-PCR ReddyMix with ThermoPrime Taq	Thermo Fisher Scientific	AB1454	One step RT-PCR exemplified in Figure 3B	
Gel electrophoresis:			For visulation of PCR products from steps 5.1-5.4	
Ethidium Bromide solution (10 mg/mL)	Thermo Fisher Scientific	17898		5.5
Tris/Acetic/EDTA (TAE) buffer:				
- Tris	Vivantis	PR0612-1KG		
- Acetic acid (glacial) (ACS, ISO, Reag. Ph Eur)	Merck KGaA (EMSURE)	1.00063.2500		
- Ethylenediaminetetraacetic acid (EDTA)	BIO-RAD	161-0729		
Agarose	Vivantis	PC0701-100G		
DNA ladders and markers	Vivantis	NL1405		
DNA gel loading dye (6X)	Thermo Fisher Scientific	R0611		
qPCR				6
PowerUP SYBR Green Master Mix	Thermo Fisher Scientific (Applied Biosystems)	A25779	Exemplified in Figures4-6B	6.2
iTaq Universal SYBR Green Supermix	BIO-RAD	1725120	Exemplified in the video and in Figures 4-6A	
Equipment				
Dounce tissue grinder pestle	Sigma-Aldrich	P1110	Protocol 2	2.2
MagNA Lyser Instrument	Roche	3358976001	An alternative tissue homogenizing option for protocol 2 which are used in conjunction with the lysing beads detailed above	
FastPrep-24 5G Homogenizer	MP BIOMEDICALS	116005500		
Refrigerated microcentrifuge	Eppendorf	Eppendorf 5427R	Protocol 2	2.4, 2.7 & 2.10
Refrigerated microcentrifuge	Eppendorf	Eppendorf 5418R		
Heat box	Labnet	AccuBlock Digital Dry Bath	Protocol 2	2.13
Microvolume spectrophotometer	Thermo Fisher Scientific (Applied Biosystems)	Nanodrop 2000	Protocol 3	3.1-3.4
PCR machine	BIO-RAD	T100 Thermal Cycler	Protocol 5	5.4
Power supply	BIO-RAD	PowerPac HC	Protocol 5	5.5
Horizontal gel electrophoresis	BIO-RAD	Mini ReadySub-Cell GT Cell	Protocol 5	
Mini microcentrifuge	Corning	#1704487edu	Useful to quickly spin down PCR reaction tubes in protocols 5 & 6	6.5.1
Microcentrifuge	LioFuge	LSE 6766 LM-60		
qPCR machine and software	Thermo Fisher Scientific	7500 Fast Real-Time PCR System with 7500 Software v2.0	Protocol 6	6.6-6,8

qPCR machine and software	BIO-RAD	CFX96 Touch Real-Time PCR Detection System with CFX Manager software
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General Materials

1.2

Mayo scissors		
Forceps		
Pipette	Rainin	Pipette-Lite XLS
Aerosol-barrier pipette tips	Sigma-Aldrich	Z333328, Z333336, Z333344

Nuclease-free 1.5-ml microcentrifuge tubes	Eppendorf
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Author(s):	Pamela Nicholson, Pattarasuda Rawiwan, Win Surachetpong

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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 ($\times g$) could not be selected. However, RCF, which stands for relative

centrifugal force, is measured in multiples of the standard acceleration on gravity at the Earth's surface and thus equates to $\times g$.

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 $\times g$ 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 $\mu\text{g}/\text{mL}$ 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 MgCl_2 , 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 °C 3 min
- 40 cycles of
 - 95 °C 10 s
 - 60 °C 30 s
- Increase the temperature from:
 - 65 °C to 95 °C with an increment of 0.5 °C / 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 from 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.*