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REPORT OF THE VIRTUAL MEETING OF THE OIE *AD HOC* GROUP ON INFECTION WITH TILAPIA LAKE VIRUS[1]

September 2019–September 2021

The *ad hoc* Group on Infection with tilapia lake virus (the *ad hoc* Group) continued its work electronically between September 2019 and September 2021. This work was delayed due to SARS-COV 2 restrictions. The list of participants is attached as Annex 1.

The terms of reference for this *ad hoc* Group were to assess TiLV diagnostics and validation. Since it was convened in November 2017, it has been working to:

- evaluate published and unpublished methods for detection of TiLV;
- describe the level of validation of each method and determine additional validation requirements;
- recommend any additional assays that may need to be developed; and
- facilitate the sourcing and distribution of well-characterised positive control material for method evaluation, implementation and two inter-laboratory comparability studies.

This report details the *ad hoc* Group test validation work, its conclusions and recommendations based on two rounds of inter-laboratory comparability testing. Estimates for accuracy and precision obtained during these rounds are limited and further validation using field samples is required to obtain more robust estimates for Diagnostic Sensitivity (DSe) and Diagnostic Specificity (DSp).

A first round of the OIE inter-laboratory comparability panel for tilapia lake virus PCR was carried out in 2019 and the results are presented in Annex 2. The second round was carried out in 2021 and the results are presented in Annex 3. Testing in both rounds was performed anonymously using blinded samples.

Results and recommendations

The TiLV inter-laboratory panel testing was undertaken in two stages. Round 1 involved two laboratories and four molecular assays and Round 2 involved seven laboratories and four molecular assays. The recommendations of the *ad hoc* Group are based on results of testing for both Rounds.

During two inter-laboratory comparaibility rounds, three real-time PCR assays and one conventional nested PCR were evaluated for their ability to reliably detect TiLV in an inter-laboratory comparison using a panel of 30 samples. All assays perfomed as expected and could detect TiLV with various levels of accuracy. The overall good precision of results indicates a good robustness and ruggedness of the assays when evaluated in a total of seven laboratories in North America, South America, Africa, Europe, and Australia. Specificity of all assays was close to 100% in all laboratories.

The two real-time probe-based PCR assays being the Cefas RT-qPCR and Hong RT-qPCR assays had the highest sensitivity, repeatability and robustness of all assays evaluated and are the recommended assays for detecting TiLV.

[1] Note: This report should be read in conjunction with the September 2021 report of the OIE Aquatic Animal Health Standards Commission because this report provides its considerations and comments. It is available at https://www.oie.int/en/what-we-do/standards/standards-setting-process/aquatic-animals-commission

The Sybr-based PCR assay (Tattiyapong RT-qPCR) produced acceptable results but would require optimisation of reaction components and conditions and definition of assay interpretation criteria, prior to implementation for routine use. However, this assay still exhibited acceptable sensitivity, repeatability and robustness and would be an approriate assay for use in laboratories that do not have access to real-time probe-based assays.

The conventional nested PCR assay (Dong RT-nPCR) was the least sensitive assay, which is not unusual when conventional and real-time assays are compared. Noting the reduced senstivity, this assay would be acceptable for use in laboratories that do not have real-time test capability.The Dong RT-nPCR assay would also be approriate for confirmatory testing of samples screened positive by real-time PCR for the purpose of confirmation of TiLV by sequence analysis, when investigating clinical disease or as follow-up testing of samples screened positive by real-time PCR from surveillance testing of apparently healthy animals. The comparative sensitivity should be determined as samples screening positive by real-time PCR may generate C_T values above the limits of detection of the RT-nPCR.

During initial assessment of published information and evaluation of real-time assays at CSIRO Australian Centre for Disease Preparedness Fish Disease Laboratory (AFDL), the Waiyamitra RT-qPCR demonstrated reduced sensitivity based on limit of detection studies so this assay was excluded from the suite of assays used during inter-laboratory comparability panel testing. This assay was included in the evaluation by one laboratory during panel testing for Round 1 and one laboratory during panel testing for Round 2. Results obtained using the Waiyamitra assay were mostly comparable to those obtained by these laboratories using the Cefas and Hong RT-qPCR assays, so the Waiyamitra assay may also be an additional real-time RT-qPCR that could be used for screening for the presence of TiLV.

Based on results provided in this report it is the opinion of the *ad hoc* Group that all four tests would allow criterion 3 of Chapter 1.2, Criteria for listing aquatic animal diseases, of the *Aquatic Code* to be fulfilled.

Acknowledgements

The *ad hoc* Group wished to thank all participants for their expertise to review published and un-published methods, sharing TiLV isolates to produce panel samples and willingness to collaborate in both inter-laboratory comparison rounds, which provided the results for the assessment in this report.

…/Annexes

Annex 1

VIRTUAL MEETING OF THE OIE *AD HOC* GROUP ON INFECTION WITH TILAPIA LAKE VIRUS

September 2019–September 2021

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Annex 2

OIE INTER-LABORATORY COMPARABILITY PANEL FOR TILAPIA LAKE VIRUS PCR (ROUND 1) FEBRUARY TO SEPTEMBER 2019

Summary

The ACDP Fish Disease Laboratory (AFDL) received TiLV samples from Peru and Thailand but only specimens from Thailand were viable and were grown successfully for propagation of TiLV and production of positive PT samples. AFDL performed testing for adventitious agents, assessed stability and homogeneity – and performed preliminary validation experiments to assess analytical sensitivity (ASe), analytical specificity (ASp) and repeatability. Due to unforeseeable circumstances only 2 laboratories, AFDL and the Centre for Environment and Fisheries and Aquaculture Science (CEFAS) were able to perform molecular tests on the inter-laboratory comparability panel, which consisted of 30 gamma-irradiated samples. Detailed testing and results reporting instructions were provided along with the inter-laboratory comparability panel. Results allowed preliminary evaluation and comparison of different priority assays for fitness for purpose and are presented in detail in this report. Based on preliminary results, the real-time qPCR (RT-qPCR) from Hong *et al.* was the most sensitive and reliable test.

Recommendations

Although OIE headquarters was highly supportive to contact and re-contact *ad hoc* group members to remind them about their obligation and relevance of testing the panel of samples using different molecular tests, only 2 laboratories were able to carry out the inter-laboratory comparison experiment. As a result, the information generated can be considered as preliminary. *Ad-hoc* group members need to be contacted (preferably by OIE) to evaluate the rate of participation to determine whether another inter-laboratory comparability round will be performed. As a consequence, there will be additional costs and time but in return a project extension will allow to corroborate preliminary findings and turn estimates for accuracy and precision more robust. It may be necessary to contact alternative collaborating laboratories and it may be possible to obtain further TiLV isolates at AFDL, which would be a significant step to further assess DSe.

Amplification of TiLV and generation of positive control material received from ad hoc group members

The Thai isolate (designated 18-03492) was confirmed as TiLV after testing with the TiLV RT-nPCR assay described by Dong *et al.* (2017), with modifications to be consistent with AFDL test protocols, and sequence analysis of the 415bp gel purified RT-PCR amplicon. By BLAST search through the NCBI database, the 375 bp primer-trimmed amplicon shared the highest nucleotide identity of 94.7% with two isolates from Israel; Tilapia lake virus isolate Til-4-2011 segment 3 (KU751816.1) and Tilapia lake virus clone 7450 (KJ605629.1). The isolate tested negative after adventitious agent testing for Koi Herpes Virus (KHV), Nervous necrosis virus (NNV) and Infectious spleen and kidney necrosis virus/Red seabream iridovirus (ISKNV/RSIV). TiLV was amplified in E-11 cell cultures and the end-point dilution determined using 10-fold dilutions with each of the TiLV molecular tests to ensure the material was suitable for preparation of the IL. Comparison of the ASe, ASp and repeatability was assessed for molecular assays. Clarified cell culture supernatant was then gamma-irradiated at 50kGy and tested to determine the degree of degradation of the TiLV RNA by the gamma-irradiation.

Inter-laboratory comparability panel

The inter-laboratory comparability panel consisted of 20 positive and 10 negative samples that included:

- 10-fold dilution series (6 samples) to enable estimates of efficiency of real-time molecular assays;
- Strong positive (at least 2 samples);
- Medium positive (at least 2 samples);
- Weak positive (at least 2 samples);
- 10-fold dilution of medium and weak positive;
- Positive samples with various viral concentrations to make up the 20 positive samples;
- Negative samples consisting of supernatant of uninfected cell culture (10 samples).

Samples were provided as a gamma-irradiated cell culture supernatant with a volume of 50µL to be extracted and tested. Multiple aliquots of each of the different samples were stored at -80°C. Participating laboratories received the blinded samples as numbered tubes with each panel ideally tested twice. Not explicitly stating the planned composition of the panel was not a test of the capability of the *ad hoc* Group member's laboratories, it is simply that it is good laboratory practice to provided "blinded" samples to participants undertaking this kind of test evaluation. Results were reported back to the Chair of the *ad ho*c Group for collation and reporting back as uncoded results to the participating laboratories for discussion. Use of duplicate samples and 10-fold dilutions of samples will enable statistical analysis to determine repeatability and reproducibility, if enough laboratories participate.

Homogeneity testing was undertaken using 10 aliquots of each different concentration, with a coefficient of variation of <5% indicating satisfactory homogeneity of samples when tested with the TiLV Hong RT-qPCR (Appendix 1). Stability testing, using three aliquots of each sample, was undertaken with aliquots tested after holding for 1 week, 2 weeks and 4 weeks at temperatures of -20°C, 4°C, 25°C and 37°C. This was done to make sure there were no stability issues with transport delays of the inter-laboratory comparability panel to *ad hoc* Group member's laboratories. There was no significant effect on stability of samples held at 20°C, 4°C and 25°C for 4 weeks (Appendix 2-7). Material held at 37°C was stable for 1 week but C_T values had increased when tested after 2 weeks and 4 weeks. Stability testing was also planned to be undertaken when all laboratories had reported results to check the stability of stored aliquots at AFDL.

Initially, the inter-laboratory comparability panel used different dilutions of a single TiLV isolate. If additional TiLV isolates were obtained from different geographical locations, additional testing to determine ASp and ASe would be required. A second round of inter-laboratory comparability panel testing was planned.

According to the results from the literature review and discussions with AHG members, the molecular assays to be evaluated in Round 1 were:

1. Unpublished RT-qPCR test from Dr David Stone (CEFAS)
2. Real-time probe-based assay (RT-qPCR) which is unpublished and has been provided to the *ad hoc* Group by Dr Hong Liu
3. Real-time Sybr assay (RT-qPCR) described by Tattiyapong *et al.*, 2017.
4. Conventional semi-nested assay (RT-nPCR) described by Dong *et al.*, 2017. This test uses primers designed by Eyngor *et al.*, 2014 with modifications described by Tsofack *et al.*, 2017.

Results

TiLV inter-laboratory comparability panel testing results – Tilapia lake virus real-time RT-qPCR assays

Testing of IL was undertaken by AFDL and CEFAS. Laboratory 2 reported results for tests conducted on different days (Test 1 and Test 2). Results were reported as a quantitative C_T value and a qualitative interpretation (positive or negative). The following tables include the compiled test results provided by AFDL and CEFAS for the TiLV Hong RT-qPCR (Table 1), TiLV CEFAS RT-qPCR (Table 2) and TiLV Tattiyapong SYBR Green RT-qPCR (Table 3). AFDL also tested the panel with the TiLV Waiyamitra RT-qPCR (Table 4).

TiLV inter-laboratory comparability panel testing results – Tilapia lake virus conventional nested PCR

Testing of the TiLV inter-laboratory comparability panel was undertaken by AFDL and CEFAS. Results were reported as a qualitative interpretation (positive or negative). Laboratory 2 reported results for tests conducted on different days (Test 1 and Test 2). Compiled test results provided by AFDL and CEFAS for the TiLV Dong RT-nPCR are included in Table 5.

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Table 1. *Laboratory qualitative and quantitative results for TiLV Hong RT-qPCR*

Samples		Laboratory 1 Reported Results		Laboratory 2 Reported Results			
				Test 1		Test 2	
		Mean C_T	Interpretation	Mean C_T	Interpretation	Mean C_T	Interpretation
1	Negative	Und	NEG	Und	NEG	Und	NEG
2	Strong positive 2	23.9	POS	29.4	POS	29.1	POS
3	Strong positive 2	23.9	POS	30.0	POS	29.1	POS
4	Weak positive 2	33.9	POS	40.6	IDT	39.1	POS
5	Weak positive 2	34.0	POS	39.2	POS	39.3	POS
6	Negative	Und	NEG	Und	NEG	Und	NEG
7	Weak positive 2	33.9	POS	39.1	POS	38.5	POS
8	Weak positive 2	33.8	POS	38.8	POS	39.1	POS
9	Moderate positive 1	27.3	POS	32.6	POS	32.5	POS
10	Weak positive 1	32.5	POS	38.0	POS	38.3	POS
11	Strong positive 1	20.5	POS	25.9	POS	25.7	POS
12	Negative	Und	NEG	Und	NEG	Und	NEG
13	Negative	Und	NEG	Und	NEG	Und	NEG
14	Negative	Und	NEG	Und	NEG	Und	NEG
15	Negative	Und	NEG	Und	NEG	Und	NEG
16	Moderate positive 1	27.1	POS	33.2	POS	36.7	POS
17	Moderate positive 1	27.2	POS	26.0	POS	34.3	POS
18	Negative	Und	NEG	Und	NEG	Und	NEG
19	Weak positive 1	32.6	POS	38.8	POS	38.4	POS
20	Weak positive 1	32.7	POS	38.0	POS	38.0	POS
21	Strong positive 1	20.8	POS	25.8	POS	25.5	POS
22	Strong positive 2	23.9	POS	33.9	POS	28.2	POS
23	Moderate positive 2	30.6	POS	36.5	POS	35.9	POS
24	Negative	Und	NEG	Und	NEG	Und	NEG
25	Strong positive 1	20.6	POS	26.0	POS	25.7	POS
26	Weak positive 1	32.6	POS	38.5	POS	37.3	POS
27	Moderate positive 2	30.6	POS	36.2	POS	36.2	POS
28	Moderate positive 2	30.6	POS	36.7	POS	38.2	POS
29	Negative	Und	NEG	Und	NEG	Und	NEG
30	Negative	Und	NEG	Und	NEG	Und	NEG

Table 2. *Laboratory qualitative and quantitative results for TiLV CEFAS RT-qPCR*

Samples		Laboratory 1 Reported Results		Laboratory 2 Reported Results			
				Test 1		Test 2	
		Mean C_T	Interpretation	Mean C_T	Interpretation	Mean C_T	Interpretation
1	Negative	Und	NEG	Und	NEG	Und	NEG
2	Strong positive 2	27.1	POS	29.7	POS	29.3	POS
3	Strong positive 2	27.2	POS	30.2	POS	29.4	POS
4	Weak positive 2	38.4	POS	Und	NEG	40.1	IDT
5	Weak positive 2	37.0	POS	Und/39.8	IDT	Und/41.2	IDT
6	Negative	Und	NEG	Und	NEG	Und	NEG
7	Weak positive 2	37.6	POS	Und	NEG	Und/39.7	IDT
8	Weak positive 2	37.4	POS	Und/39.7	IDT	39.6	POS
9	Moderate positive 1	30.5	POS	32.6	POS	32.8	POS
10	Weak positive 1	36.5	POS	37.7	POS	37.5	POS
11	Strong positive 1	23.9	POS	26.0	POS	25.9	POS
12	Negative	Und	NEG	Und	NEG	Und	NEG
13	Negative	Und	NEG	Und	NEG	Und	NEG
14	Negative	Und	NEG	Und	NEG	Und	NEG
15	Negative	Und	NEG	Und	NEG	Und	NEG
16	Moderate positive 1	30.3	POS	33.5	POS	34.9	POS
17	Moderate positive 1	30.4	POS	34.9	POS	34.8	POS
18	Negative	Und	NEG	Und	NEG	Und	NEG
19	Weak positive 1	36.0	POS	39.3	POS	Und	NEG
20	Weak positive 1	35.4	POS	38.2	POS	37.4	POS
21	Strong positive 1	24.2	POS	26.2	POS	25.6	POS
22	Strong positive 2	27.2	POS	29.4	POS	28.8	POS
23	Moderate positive 2	34.1	POS	36.9	POS	36.5	POS
24	Negative	Und	NEG	Und	NEG	Und	NEG
25	Strong positive 1	23.9	POS	27.1	POS	25.4	POS
26	Weak positive 1	36.7	POS	38.0	POS	37.0	POS
27	Moderate positive 2	33.7	POS	36.8	POS	36.0	POS
28	Moderate positive 2	33.7	POS	37.7	POS	36.4	POS
29	Negative	Und	NEG	Und	NEG	Und	NEG
30	Negative	Und	NEG	Und	NEG	Und	NEG

Table 3. *Laboratory qualitative and quantitative results for TiLV Tattiyapong SYBR Green RT-qPCR*

Samples		Laboratory 1 Reported Results		Laboratory 2 Reported Results			
				Test 1		Test 2	
		Mean C_T	Interpretation	Mean C_T	Interpretation	Mean C_T	Interpretation
1	Negative	38.9*	NEG	Und	NEG	Und	NEG
2	Strong positive 2	19.0	POS	26.5	POS	26.4	POS
3	Strong positive 2	19.1	POS	26.9	IDT	26.5	POS
4	Weak positive 2	29.1	POS	36.7	POS	34.6	IDT
5	Weak positive 2	29.2	POS	Und	NEG	Und/36.4	IDT
6	Negative	35.4*	NEG	Und	NEG	Und	NEG
7	Weak positive 2	29.3	POS	Und/35.2	IDT	Und/34.8	IDT
8	Weak positive 2	29.2	POS	Und	NEG	Und	NEG
9	Moderate positive 1	22.2	POS	30.5	POS	29.7	IDT
10	Weak positive 1	28.0	POS	34.1	IDT	34.5	IDT
11	Strong positive 1	15.9	POS	26.3	IDT	22.9	POS
12	Negative	Und	NEG	Und	NEG	Und	NEG
13	Negative	39.9*	NEG	Und	NEG	Und	NEG
14	Negative	35.9*	NEG	Und	NEG	Und	NEG
15	Negative	37.5*	NEG	37.3	IDT	Und	NEG
16	Moderate positive 1	22.1	POS	30.2	POS	32.5	IDT
17	Moderate positive 1	22.4	POS	33.0	POS	30.9	IDT
18	Negative	40.2*	NEG	Und	NEG	Und	NEG
19	Weak positive 1	28.1	POS	35.0	POS	36.1	POS
20	Weak positive 1	28.4	POS	34.2	IDT	34.6	IDT
21	Strong positive 1	16.0	POS	22.7	IDT	23.3	IDT
22	Strong positive 2	19.3	POS	25.9	IDT	26.2	IDT
23	Moderate positive 2	25.8	POS	33.5	IDT	33.1	IDT
24	Negative	Und	NEG	Und	NEG	Und	NEG
25	Strong positive 1	15.8	POS	24.1	POS	22.8	POS
26	Weak positive 1	28.1	POS	34.4	POS	33.8	IDT
27	Moderate positive 2	25.8	POS	32.9	IDT	33.2	IDT
28	Moderate positive 2	26.0	POS	33.7	IDT	34.4	IDT
29	Negative	35.6*	NEG	Und	NEG	Und	NEG
30	Negative	35.7*	NEG	Und	NEG	Und	NEG

*non-specific amplification

Table 4. *Laboratory qualitative and quantitative results for TiLV Waiyamitra RT-qPCR*

Samples		**AFDL Reported Results**	
		Mean C_T	**Interpretation**
1	Negative	Und	NEG
2	Strong positive 2	23.1	POS
3	Strong positive 2	23.2	POS
4	Weak positive 2	36.5	POS
5	Weak positive 2	36.9	POS
6	Negative	Und	NEG
7	Weak positive 2	39.4	POS
8	Weak positive 2	38.1	POS
9	Moderate positive 1	26.7	POS
10	Weak positive 1	33.4	POS
11	Strong positive 1	20.2	POS
12	Negative	Und	NEG
13	Negative	Und	NEG
14	Negative	Und	NEG
15	Negative	Und	NEG
16	Moderate positive 1	26.6	POS
17	Moderate positive 1	26.6	POS
18	Negative	Und	NEG
19	Weak positive 1	33.3	POS
20	Weak positive 1	33.4	POS
21	Strong positive 1	20.4	POS
22	Strong positive 2	23.4	POS
23	Moderate positive 2	30.5	POS
24	Negative	Und	NEG
25	Strong positive 1	20.3	POS
26	Weak positive 1	33.0	POS
27	Moderate positive 2	30.3	POS
28	Moderate positive 2	30.2	POS
29	Negative	Und	NEG
30	Negative	Und	NEG

Table 5. *Laboratory qualitative results for TiLV Dong RT-nPCR*

Samples		Laboratory 1 Reported Results	Laboratory 2 Reported Results	
			Test 1	Test 2
		Interpretation	Interpretation	Interpretation
1	Negative	NEG	NEG	NEG
2	Strong positive 2	POS	POS	POS
3	Strong positive 2	POS	POS	POS
4	Weak positive 2	POS	NEG	NEG
5	Weak positive 2	POS	IDT	POS
6	Negative	NEG	NEG	NEG
7	Weak positive 2	POS	POS	NEG
8	Weak positive 2	POS	POS	IDT
9	Moderate positive 1	POS	POS	POS
10	Weak positive 1	POS	IDT	POS
11	Strong positive 1	POS	POS	POS
12	Negative	NEG	NEG	IDT
13	Negative	NEG	NEG	NEG
14	Negative	NEG	NEG	NEG
15	Negative	NEG	NEG	NEG
16	Moderate positive 1	POS	POS	POS
17	Moderate positive 1	POS	POS	POS
18	Negative	NEG	NEG	NEG
19	Weak positive 1	POS	POS	POS
20	Weak positive 1	POS	POS	POS
21	Strong positive 1	POS	POS	POS
22	Strong positive 2	POS	POS	POS
23	Moderate positive 2	POS	POS	POS
24	Negative	NEG	NEG	NEG
25	Strong positive 1	POS	POS	POS
26	Weak positive 1	POS	POS	POS
27	Moderate positive 2	POS	POS	POS
28	Moderate positive 2	POS	POS	POS
29	Negative	NEG	NEG	NEG
30	Negative	NEG	NEG	NEG

Appendix 1. Homogeneity test results for dilutions used to prepare the panels at AFDL, tested using the TILV Hong RT-qPCR

Replicate	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6
1	20.79	24.16	27.47	30.77	32.91	34.14
2	20.84	24.20	27.31	30.69	32.82	34.21
3	20.72	24.10	27.42	30.76	32.82	34.27
4	20.81	24.12	27.31	30.96	32.74	34.15
5	20.74	24.16	27.44	30.76	33.04	34.27
6	20.71	24.09	27.44	30.84	33.06	34.12
7	20.58	24.00	27.42	30.67	33.10	34.52
8	20.60	24.12	27.39	30.75	32.91	34.48
9	20.69	24.13	27.41	30.84	32.86	34.20
10	20.65	24.06	27.37	30.74	32.95	34.34
Average	**20.714**	**24.114**	**27.398**	**30.778**	**32.921**	**34.244**
Coefficient of variation	**0.29%**	**0.00%**	**0.17%**	**0.24%**	**0.29%**	**0.00%**
PASS/FAIL	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**

Appendix 2. Stability test results for the Strong 1 panel aliquots, tested using the TILV Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Strong 1	20.71	-20°C	20.50	21.56	20.88
Strong 1		-20°C	20.49	21.51	20.91
Strong 1		-20°C	20.47	21.50	20.99
Strong 1		4°C	20.49	21.55	21.15
Strong 1		4°C	20.50	21.57	21.09
Strong 1		4°C	20.50	21.64	21.13
Strong 1		25°C	20.82	21.82	22.37
Strong 1		25°C	20.78	21.92	21.75
Strong 1		25°C	20.95	21.90	21.82
Strong 1		37°C	21.51	24.37	27.83
Strong 1		37°C	21.75	24.97	27.82
Strong 1		37°C	21.99	24.93	29.49

Appendix 3. Stability test results for the Strong 2 panel aliquots, tested using the TILV Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Strong 2	24.11	-20°C	23.86	24.95	24.51
Strong 2		-20°C	23.91	24.84	24.47
Strong 2		-20°C	23.97	24.89	24.47
Strong 2		4°C	23.91	24.90	24.50
Strong 2		4°C	23.79	24.99	24.34
Strong 2		4°C	23.95	24.98	24.47
Strong 2		25°C	24.11	25.30	25.95
Strong 2		25°C	24.30	25.33	25.50
Strong 2		25°C	24.19	25.44	25.51
Strong 2		37°C	25.41	27.78	30.87
Strong 2		37°C	25.04	29.04	32.41
Strong 2		37°C	25.32	27.69	31.18

Appendix 4. Stability test results for the Medium 1 panel aliquots, tested using the TILV Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Medium 1	27.4	-20°C	27.17	28.10	27.65
Medium 1		-20°C	27.24	28.19	27.76
Medium 1		-20°C	27.21	28.14	27.76
Medium 1		4°C	27.25	28.21	27.84
Medium 1		4°C	27.23	28.26	27.87
Medium 1		4°C	27.30	28.28	27.92
Medium 1		25°C	27.62	28.92	28.68
Medium 1		25°C	27.39	28.90	29.23
Medium 1		25°C	27.54	28.88	29.18
Medium 1			28.29	31.22	33.58
Medium 1		37°C	28.50	31.18	35.14
Medium 1		37°C	28.50	30.51	35.41

Appendix 5. Stability test results for the Medium 2 panel aliquots, tested using the TILV Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Medium 2	30.78	-20°C	30.58	31.62	31.18
Medium 2		-20°C	30.65	31.63	31.20
Medium 2		-20°C	30.58	31.55	31.09
Medium 2		4°C	30.74	31.67	31.16
Medium 2		4°C	30.62	31.55	31.11
Medium 2		4°C	30.58	31.55	31.17
Medium 2		25°C	30.99	31.87	32.51
Medium 2		25°C	31.02	32.31	32.04
Medium 2		25°C	31.37	31.68	32.24
Medium 2		37°C	31.92	34.41	36.25
Medium 2		37°C	32.40	34.27	38.14
Medium 2		37°C	32.43	34.26	39.03

Appendix 6. Stability test results for the Weak 1 panel aliquots, tested using the TILV Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Weak 1	32.92	-20°C	32.99	32.93	32.79
Weak 1		-20°C	33.13	33.08	33.05
Weak 1		-20°C	32.98	33.03	33.00
Weak 1		4°C	33.12	33.17	33.04
Weak 1		4°C	33.20	33.16	33.33
Weak 1		4°C	33.05	33.11	33.26
Weak 1		25°C	33.48	33.72	34.30
Weak 1		25°C	33.43	33.62	34.20
Weak 1		25°C	33.20	33.40	34.62
Weak 1		37°C	34.16	37.05	Neg
Weak 1		37°C	34.61	36.79	38.36
Weak 1		37°C	34.70	36.34	39.36

Appendix 7. Stability test results for the Weak 2 panel aliquots, tested using the TILV Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Weak 2	34.24	-20°C	34.33	34.48	34.23
Weak 2		-20°C	34.66	34.34	34.65
Weak 2		-20°C	34.49	34.50	34.22
Weak 2		4°C	34.31	34.33	34.50
Weak 2		4°C	34.42	34.53	34.79
Weak 2		4°C	34.49	34.40	34.50
Weak 2		25°C	35.09	35.12	35.94
Weak 2		25°C	34.92	35.05	35.75
Weak 2		25°C	35.11	34.89	Neg/40.05
Weak 2		37°C	35.77	37.22	36.02
Weak 2		37°C	35.89	36.84	Neg
Weak 2		37°C	35.74	37.52	Neg/40.0

Annex 3

OIE INTER-LABORATORY COMPARABILITY PANEL FOR TILAPIA LAKE VIRUS PCR (ROUND 2)

January to August 2021

Summary

The objective of **TiLV inter-laboratory comparability panel testing (Round 2)** was to assess the accuracy and precision of four molecular assays and to determine and compare the reliability to produce true positive and true negative results on a panel of 20 positive and 10 negative samples and remain unaffected by internal and external variables, e.g. different operators, laboratory environments in different regions and countries. The objective of the comparison was not to determine laboratory proficiency (PT) but rather assay reliability.

Overall quantitative and qualitative results of Round 2 showed a high degree of precision and accuracy for the Cefas and Hong assays. Based on results from a lower number of participants evaluating the Sybr and Dong assays, both assays showed a reduced reliability to detect weak or moderate positive samples in some laboratories. The overall good precision of results indicates a good robustness and ruggedness of the assays when evaluated in a total of seven laboratories in North America, South America, Africa, Europe, and Australia. Specificity of all assays was close to 100% in all laboratories. Almost all negative samples tested negative in all assays, which is also an indicator for efficient workflow and absence of cross-contamination. Only two negative samples tested false-positive in two laboratories in the Hong (Lab 2, Sample 26) and Sybr (Lab 6, Sample 14) assays. Results from this project with a limited number of samples (n=30) indicate that all tests can detect TiLV in positive samples and not detect TiLV in negative samples.

Due to SARS-CoV 2 restrictions Round 2 was delayed to 2021, the composition of laboratories had to change, and four new participants joined the group. Nevertheless, the overall results between the Round 1 and Round 2 are comparable indicating acceptable reliability of the assays.

Instructions for participants for the second Round were identical as for Round 1, which included a document with information about samples and testing, an Excel file for results entry and a previous email advising that this panel contained the ACDP worksheets for the assays. Results for quantitative assays were entered as C_T values in duplicate and mean values and interpreted as positive and negative. Conventional RT-nPCR were reported as positive or negative. Assay and extraction details were provided in an Excel spreadsheet together with results.

According to the results from literature review and inter-laboratory comparison from Round 1, molecular assays to be evaluated in this second round were:

1. Unpublished RT-qPCR test from Dr David Stone (CEFAS).
2. Real-time probe-based assay (RT-qPCR) which is unpublished and has been provided to the *ad hoc* Group by Dr Hong Liu.
3. Real-time Sybr assay (RT-qPCR) described by Tattiyapong *et al.*, 2017.
4. Conventional semi-nested assay (RT-nPCR) described by Dong *et al.*, 2017. This test uses primers designed by Eyngor *et al.*, 2014 with modifications described by Tsofack *et al.*, 2017.

Based on encouraging results for the TiLV inter-laboratory comparability panel testing for Round 1 undertaken in 2019 by AFDL and CEFAS, throughout 2020 ongoing communication occurred between participating laboratories to co-ordinate the best time for laboratories to be able to undertake testing. This co-ordination was required due to laboratory access restrictions due to the global SARS-CoV-2 pandemic. The pandemic also resulted in transport issues due to a reduction in airline flights which interfered with shipping of the panels.

Materials and Methods

Schedule

The Round 2 inter-laboratory comparability panel, consisting of 30 samples, was sent to 7 participants in April 2021 (Table 1). Detailed instructions to perform the assays and an Excel spreadsheet to enter results and technical information for reporting were provided. Results were requested to be received in June 2021 for analysis. The aim was to include results in the progress report for the TiLV OIE meeting in September 2021. Participants were requested to test a single extraction in duplicate and report individual results, mean C_T values (where appropriate) and result interpretation (positive, negative, indeterminate).

Table 1. *List of participating laboratories in Round 2*

Australia Peter Mohr ACDP Fish Diseases Laboratory CSIRO Australian Centre for Disease Preparedness Geelong, Victoria, Australia|	**Brazil** Marcelo Fernandes Camargos Laboratório Federal de Defesa Agropecuária Ministério da Agricultura, Pecuária e Abastecimento Pedro Leopoldo, Brazil
China (People's Rep. of) Hong Liu The National Key laboratory of Aquatic Animal Diseases, Animal and Plant Inspection and Quarantine Technical Centre, Shenzhen City, Guangdong province, China	**Denmark** Argelia Cuenca EURL for Fish and Crustacean Diseases Technical University of Denmark Kgs. Lyngby, Denmark
South Africa Marco Romito ARC-Onderstepoort Veterinary Institute Onderstepoort, South Africa	**United Kingdom** David Stone CEFAS Barrack Road, Weymouth, Dorset, UK
United States Janet Warg National Veterinary Services Laboratories Ames, Iowa, Unites States	

Samples

The Round 2 TiLV inter-laboratory comparison panel included 30 samples of different analyte concentration of 200 µL each (9 x negative, 9 x weak positive, 6 x moderate positive, 6 x strong positive, Table 2). Samples were shipped on dry ice, using a commercial international courier company with cold chain maintained throughout. A 1 mL aliquot of strong positive material was also included in case laboratories wanted to undertake any preliminary assay evaluation prior to testing the panels.

Homogeneity testing was determined using 10 aliquots of each different concentration, with a coefficient of variation of <5% indicating satisfactory results. To assess homogeneity, the samples were tested with the TiLV Hong RT-qPCR. All aliquoted samples for each dilution passed homogeneity testing (Appendix 2.1). Stability testing was planned to be undertaken when all laboratories had reported results to check the stability of stored aliquots at AFDL. Due to time constraints this was not possible. However, testing of samples (3 samples/dilution) prior to construction of Round 2 panels was conducted. All samples returned results that were equivalent with results obtained from homogeneity testing, therefore indicating acceptable stability (i.e. no sample degradation had occurred) during sample storage between Rounds 1 & 2.

Table 2. *Distribution of negative, weak, moderate, and strong positive samples and negative samples in the TiLV inter-laboratory comparison panel. During panel preparation, C_T values were used to arbitrarily classify samples as negative and weak (31-35), moderate (25-31) and strong (20-25) positives.*

1	Moderate positive 1	16	Weak positive 2
2	Weak positive 1	17	Negative
3	Negative	18	Strong positive 1
4	Negative	19	Weak positive 2
5	Negative	20	Moderate positive 2
6	Strong positive 2	21	Moderate positive 2
7	Strong positive 1	22	Weak positive 1
8	Weak positive 1	23	Strong positive 2
9	Negative	24	Moderate positive 2
10	Negative	25	Weak positive 2
11	Moderate positive 1	26	Negative
12	Weak positive 2	27	Weak positive 1
13	Moderate positive 1	28	Strong positive 1
14	Negative	29	Strong positive 2
15	Weak positive 2	30	Negative

Statistical analysis of results

Results were received and stored in Excel files. Statistical analysis was performed using comparison of multiple variable graphs and Youden analysis in MedCalc version 20.009 - © 2021 MedCalc Software Ltd, https://www.medcalc.org/.

Results

Six countries returned results for the CEFAS RT-qPCR and Hong RT-qPCR (hydrolysis probe-based real-time) assays. Five countries provided results for the Tattiyapong RT-qPCR (Sybr Green-based) assay and Dong RT-nPCR (conventional nested RT-PCR-based) assay. Laboratories have been coded (1, 2, 3, 4, 5, 6 & 7) with laboratory code provided when the coded report is emailed to participants to maintain confidentiality.

Summary results for the three real-time assays are provided in Figure 1, which shows the distribution of results (C_T values) for the 7 laboratories for the three real-time assays for the 30 samples in the panel. Coding is CEFAS RT-qPCR (Cefas) and Hong RT-qPCR (Hong) and Tattiyapong RT-qPCR (Sybr).

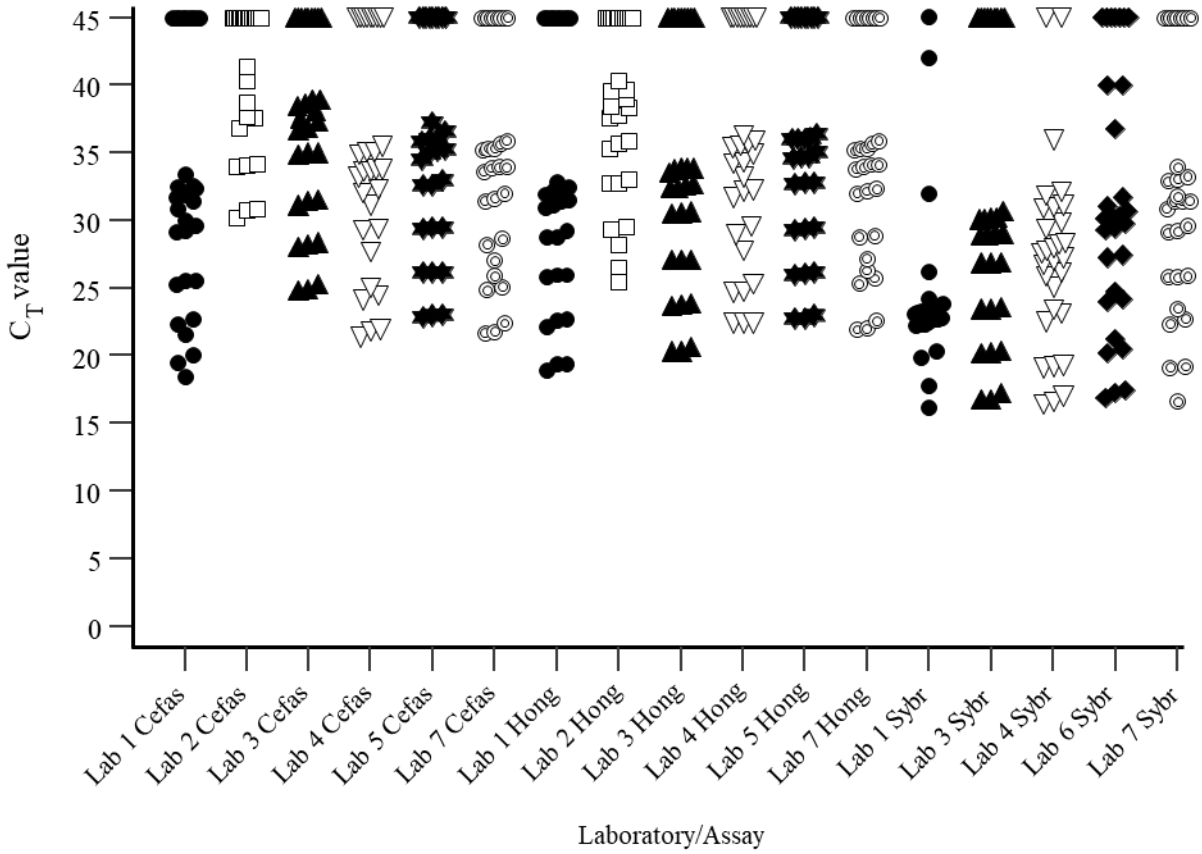

Figure 1. *Distribution of C_T values for each laboratory for the three TiLV real-time assays used in the evaluation.*

Specific comments on the performance of each of the assays are provided below.

CEFAS RT-qPCR assay

Six laboratories provided results for the CEFAS RT-qPCR assay (Laboratories 1, 2, 3, 4, 5, 7). Results are presented in Table 3.

5/6 participants had 100% agreement for accuracy and good overall precision (Laboratories 1, 3, 4, 5, 7). Laboratory 2 produced consistently higher C_T values, demonstrating a decrease in sensitivity and reduced agreement for this laboratory (11/30 false negative results and 2/30 indeterminate results). However, disagreement was only observed due to false-negative results on positive samples with no false-positive results on negative samples. Typically, most of the false-negative results occurred in weak or moderate positive samples. Under these circumstances the assay may miss a weak or moderate positive result. The reduced sensitivity of the performance of the CEFAS RT-qPCR assay in Laboratory 2 may be addressed by changes to the reagents used in Laboratory 2. The overall agreement of results provided by other laboratories (100% agreement between 5 laboratories) indicate acceptable reliability of the CEFAS RT-qPCR assay. With Coefficient of Variation (CV) values of less than 5% after analysis of C_T values for weak, moderate, and strong replicate samples the CEFAS assay showed satisfactory repeatability (Appendix 1.1.).

Hong RT-qPCR assay

Six laboratories provided results for the Hong RT-qPCR assay (Laboratories 1, 2, 3, 4, 5, 7). Results are presented in Table 4.

5/6 laboratories had 100% agreement for accuracy and good overall precision (Laboratories 1, 3, 4, 5, 7). Laboratory 2 produced consistently higher C_T values, demonstrating a decrease in sensitivity and reduced agreement for this laboratory (4/30 false negative results, 7/30 indeterminate results). Disagreement was mostly observed due to false-negative results on positive samples with **one false-positive result** for a negative sample. Typically, most of the false-negative results occurred in weak or moderate positive samples. Under these

circumstances the assay may miss a weak or moderate positive result. The reduced sensitivity of the performance of the Hong RT-qPCR assay in Laboratory 2 may be addressed by changes to the reagents used in Laboratory 2. The overall agreement of results provided by other laboratories (100% agreement between 5 laboratories) indicated the acceptable reliability of the Hong RT-qPCR assay. With CV values of less than 5% after analysis of C_T values for weak, moderate, and strong replicate samples the Hong assay showed satisfactory repeatability (Appendix 1.2.).

Tattiyapong RT-qPCR Sybr assay

Five laboratories provided results for the Tattiyapong RT-qPCR Sybr assay (Laboratories 1, 3, 4, 6, 7). Results are presented in Table 5.

Laboratories 3, 4 and 7 had 100% agreement for accuracy and good overall precision. Lab 1 had low sensitivity in this assay (e.g. 8/30 false negative results and 2/30 indeterminate results). Lab 6 had 2 false negative results, and **one false positive result**. Several laboratories commented that they do not use Sybr-based real-time assays as their preference is to use probe-based assays, due to the increased specificity afforded by the probe in these assays. In these laboratories, the Tattiyapong RT-qPCR Sybr assay was also implemented with little or no optimisation which would be required if the assay was going to be implemented for routine use. Comments were also received that the Tattiyapong RT-qPCR Sybr assay also required more rigorous analysis of results, to ensure the fluorescence was attributable to the pathogen and not due to non-specific amplification. The overall agreement of results provided by the laboratories (100% agreement between 3 laboratories) indicated the acceptable reliability of the Tattiyapong RT-qPCR Sybr assay, in terms of sensitivity. However, laboratories implementing this assay would need to undertake optimisation prior to implementing the assay for routine use and establish additional criteria for result acceptance (e.g. melt curve analysis). Although the CV was higher for the Sybr assay (compared to CEFAS and Hong assays) the CV values of less than 8% after analysis of C_T values for weak, moderate, and strong replicate samples the Sybr assay showed satisfactory repeatability (Appendix 1.3).

Dong RT-nPCR assay

Six laboratories tested the panel using the Dong RT-nPCR assay (Laboratories 1, 2, 3, 4, 5, 7). Results are presented in Table 6.

Laboratories 3, 4 and 5 had 100% agreement for accuracy and good overall precision. Laboratory 1 had 2 indeterminate results and 2/30 false-negative results on positive samples. Laboratory 2 had low sensitivity in the Dong RT-nPCR assay and produced 12/30 false-negative results on positive samples. Laboratory 7 had 3 indeterminate results and 4/30 false-negative results on positive samples. Based on the results provided the Dong RT-nPCR has a decreased sensitivity at detection of weak positive samples, compared to the real-time assays used in this inter-laboratory comparison.

Table 3. Laboratory quantitative and quantitative results for the CEFAS RT-qPCR assay (*mean C_T calculated from a single C_T value, highlighted results are incorrectly classified).

Samples		LAB 1		LAB 2		LAB 3		LAB 4		LAB 5		LAB 7	
		Mean C_T	INT	Mean C_T	INT	Mean C_T	INT	Mean C_T	INT	Mean C_T	INT	Mean C_T	INT
1	Moderate positive 1	25.22	POS	37.65	POS	31.14	POS	29.28	POS	29.44	POS	27.04	POS
2	Weak positive 1	29.97	POS	45	NEG	37.56	POS	33.62	POS	35.22	POS	33.61	POS
3	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
4	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
5	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
6	Strong positive 2	21.52	POS	33.96	POS	28.03	POS	24.99	POS	26.16	POS	25.92	POS
7	Strong positive 1	18.48	POS	30.76	POS	24.81	POS	21.81	POS	22.84	POS	21.77	POS
8	Weak positive 1	30.80	POS	45	NEG	36.86	POS	33.81	POS	34.48	POS	33.95	POS
9	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
10	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
11	Moderate positive 1	25.55	POS	36.85	POS	31.56	POS	27.65	POS	29.33	POS	28.22	POS
12	Weak positive 2	31.88	POS	41.40*	NEG	38.93	POS	33.65	POS	36.05	POS	35.65	POS
13	Moderate positive 1	25.60	POS	37.58	IDT	31.49	POS	29.32	POS	29.52	POS	28.61	POS
14	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
15	Weak positive 2	32.33	POS	45	NEG	38.49	POS	35.11	POS	36.59	POS	35.35	POS
16	Weak positive 2	32.55	POS	45	NEG	38.97	POS	35.50	POS	35.84	POS	35.33	POS
17	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
18	Strong positive 1	20.05	POS	30.18	POS	24.95	POS	21.97	POS	22.98	POS	21.67	POS
19	Weak positive 2	33.37	POS	45	NEG	38.61	POS	34.86	POS	35.98	POS	35.19	POS
20	Moderate positive 2	29.57	POS	38.74*	IDT	34.99	POS	32.34	POS	32.95	POS	31.98	POS
21	Moderate positive 2	29.08	POS	45	NEG	34.96	POS	31.13	POS	32.55	POS	31.42	POS
22	Weak positive 1	31.48	POS	45	NEG	37.33	POS	33.24	POS	35.19	POS	33.95	POS
23	Strong positive 2	22.72	POS	34.01	POS	28.23	POS	24.44	POS	26.09	POS	24.85	POS
24	Moderate positive 2	29.28	POS	40.36*	NEG	34.81	POS	32.01	POS	32.59	POS	31.60	POS
25	Weak positive 2	32.49	POS	45	NEG	38.16	POS	35.06	POS	37.24	POS	35.85	POS
26	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
27	Weak positive 1	31.66	POS	45	NEG	36.67	POS	33.83	POS	34.91	POS	33.84	POS
28	Strong positive 1	19.51	POS	30.81	POS	25.31	POS	21.38	POS	23.08	POS	22.40	POS
29	Strong positive 2	22.35	POS	34.21	POS	28.38	POS	24.08	POS	26.15	POS	25.09	POS
30	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG

Table 4. Laboratory quantitative and quantitative results for Hong RT-qPCR assay (highlighted results are incorrectly classified).

Samples		LAB 1		LAB 2		LAB 3		LAB 4		LAB 5		LAB 7	
		Mean C_T	INT	Mean C_T	INT	Mean C_T	INT	Mean C_T	INT	Mean C_T	INT	Mean C_T	INT
1	Moderate positive 1	25.80	POS	32.71	POS	27.07	POS	29.60	POS	29.32	POS	27.16	POS
2	Weak positive 1	31.57	POS	38.29	IDT	32.56	POS	34.21	POS	35.10	POS	33.82	POS
3	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
4	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
5	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
6	Strong positive 2	22.66	POS	29.53	POS	23.67	POS	25.30	POS	26.12	POS	26.26	POS
7	Strong positive 1	19.34	POS	28.24	POS	20.35	POS	22.44	POS	22.71	POS	21.92	POS
8	Weak positive 1	31.39	POS	40.34	NEG	32.44	POS	34.27	POS	34.70	POS	33.93	POS
9	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
10	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
11	Moderate positive 1	25.93	POS	32.76	POS	27.11	POS	27.78	POS	29.28	POS	28.81	POS
12	Weak positive 2	32.38	POS	39.56	IDT	33.83	POS	35.59	POS	35.97	POS	35.64	POS
13	Moderate positive 1	25.93	POS	32.96	POS	27.12	POS	28.96	POS	29.43	POS	28.84	POS
14	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
15	Weak positive 2	31.91	POS	38.95	IDT	33.89	POS	35.40	POS	36.35	POS	35.17	POS
16	Weak positive 2	32.30	POS	39.68	IDT	33.56	POS	36.32	POS	35.99	POS	35.35	POS
17	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG
18	Strong positive 1	18.86	POS	25.37	POS	20.36	POS	22.44	POS	22.96	POS	22.02	POS
19	Weak positive 2	32.86	POS	37.58	IDT	33.87	POS	35.69	POS	36.14	POS	35.35	POS
20	Moderate positive 2	29.20	POS	35.36	POS	30.57	POS	31.71	POS	32.70	POS	32.02	POS
21	Moderate positive 2	28.79	POS	35.63	POS	30.50	POS	32.22	POS	32.71	POS	32.15	POS
22	Weak positive 1	30.96	POS	35.85	POS	32.43	POS	33.24	POS	34.56	POS	34.12	POS
23	Strong positive 2	22.61	POS	38.36	IDT	23.89	POS	24.69	POS	25.97	POS	25.35	POS
24	Moderate positive 2	28.80	POS	45	NEG	30.64	POS	32.19	POS	32.78	POS	32.34	POS
25	Weak positive 2	32.21	POS	37.73	IDT	33.88	POS	35.88	POS	36.07	POS	35.92	POS
26	Negative	45	NEG	26.51	POS	45	NEG	45	NEG	45	NEG	45	NEG
27	Weak positive 1	31.14	POS	45	NEG	32.65	POS	34.	POS	34.69	POS	34.13	POS
28	Strong positive 1	19.35	POS	29.32	POS	20.68	POS	22.45	POS	22.76	POS	22.60	POS
29	Strong positive 2	22.10	POS	45	NEG	23.83	POS	24.72	POS	26.02	POS	25.71	POS
30	Negative	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG	45	NEG

Table 5. Laboratory quantitative and quantitative results for Tattiyapong Sybr RT-qPCR assay (*mean C_T calculated from a single C_T value, highlighted results are incorrectly classified).

Samples		LAB 1		LAB 3		LAB 4		LAB 6		LAB 7	
		Mean C_T	INT	Mean C_T	INT	Mean C_T	INT	Mean C_T	INT	Mean C_T	INT
1	Moderate positive 1	23.27	POS	23.50	POS	23.37	POS	24.21	POS	25.77	POS
2	Weak positive 1	23.55	NEG	28.86	POS	27.58	POS	30.16	POS	31.34	POS
3	Negative	23.48	NEG	45	NEG	35.96	NEG	45	NEG	45	NEG
4	Negative	23.20	NEG	45	NEG	45	NEG	45	NEG	45	NEG
5	Negative	23.16	NEG	45	NEG	30.99	NEG	40.00	NEG	45	NEG
6	Strong positive 2	32.04	POS	20.13	POS	19.12	POS	21.24	POS	23.45	POS
7	Strong positive 1	17.73	POS	16.80	POS	17.02	POS	17.43	POS	19.17	POS
8	Weak positive 1	23.66	NEG	29.02	POS	28.26	POS	29.26	POS	31.46	POS
9	Negative	23.43	NEG	45	NEG	29.82	NEG	40.00	NEG	45	NEG
10	Negative	45.11	NEG	36.89*	NEG	45	NEG	36.79	NEG	45	NEG
11	Moderate positive 1	22.29	POS	23.50	POS	22.50	POS	24.73	POS	25.88	POS
12	Weak positive 2	22.64	NEG	30.16	POS	26.66	POS	45	NEG	32.92	POS
13	Moderate positive 1	22.32	POS	23.62	POS	23.11	POS	23.95	POS	25.79	POS
14	Negative	22.81	NEG	45	NEG	32.08	NEG	30.29	POS	45	NEG
15	Weak positive 2	26.21	POS	30.22	POS	29.38	POS	30.22	POS	33.06	POS
16	Weak positive 2	23.10	NEG	30.11	POS	28.28	POS	30.63	POS	33.94	POS
17	Negative	23.40	NEG	45	NEG	30.89	NEG	45	NEG	45	NEG
18	Strong positive 1	16.12	POS	16.79	POS	16.62	POS	16.82	POS	19.11	POS
19	Weak positive 2	22.63	NEG	30.26	POS	27.75	POS	31.71	POS	31.80	POS
20	Moderate positive 2	23.61	POS	26.86	POS	26.16	POS	45	NEG	29.58	POS
21	Moderate positive 2	22.35	IDT	26.83	POS	25.87	POS	27.44	POS	29.11	POS
22	Weak positive 1	23.18	NEG	28.98	POS	26.99	POS	29.77	POS	30.91	POS
23	Strong positive 2	20.32	POS	20.23	POS	19.17	POS	20.52	POS	22.36	POS
24	Moderate positive 2	23.80	IDT	26.97	POS	25.06	POS	27.23	POS	29.17	POS
25	Weak positive 2	23.85	NEG	30.74	POS	27.22	POS	31.02	POS	33.26	POS
26	Negative	24.17	NEG	36.14*	NEG	31.86	NEG	45	NEG	45	NEG
27	Weak positive 1	22.46	NEG	29.01	POS	27.99	POS	29.46	POS	31.46	POS
28	Strong positive 1	23.27	POS	17.22	POS	16.41	POS	17.29	POS	16.61	POS
29	Strong positive 2	19.84	POS	20.42	POS	19.29	POS	20.19	POS	22.71	POS
30	Negative	42.00	NEG	45	NEG	31.16	NEG	24.21	NEG	45	NEG

Table 6. Laboratory qualitative results for Dong RT-nPCR assay (highlighted results are incorrectly classified).

Samples		LAB 1	LAB 2	LAB 3	LAB 4	LAB 5	LAB 7
		INT	INT	INT	INT	INT	INT
1	Moderate positive 1	POS	NEG	POS	POS	POS	POS
2	Weak positive 1	POS	NEG	POS	POS	POS	NEG
3	Negative	NEG	NEG	NEG	NEG	NEG	NEG
4	Negative	NEG	NEG	NEG	NEG	NEG	NEG
5	Negative	NEG	NEG	NEG	NEG	NEG	NEG
6	Strong positive 2	POS	POS	POS	POS	POS	POS
7	Strong positive 1	POS	POS	POS	POS	POS	POS
8	Weak positive 1	POS	NEG	POS	POS	POS	POS
9	Negative	NEG	NEG	NEG	NEG	NEG	NEG
10	Negative	NEG	NEG	NEG	NEG	NEG	NEG
11	Moderate positive 1	POS	POS	POS	POS	POS	POS
12	Weak positive 2	POS	NEG	POS	POS	POS	NEG
13	Moderate positive 1	POS	POS	POS	POS	POS	POS
14	Negative	NEG	NEG	NEG	NEG	NEG	NEG
15	Weak positive 2	POS	NEG	POS	POS	POS	NEG
16	Weak positive 2	POS	NEG	POS	POS	POS	NEG
17	Negative	NEG	NEG	NEG	NEG	NEG	NEG
18	Strong positive 1	POS	POS	POS	POS	POS	POS
19	Weak positive 2	NEG	NEG	POS	POS	POS	IDT
20	Moderate positive 2	POS	NEG	POS	POS	POS	IDT
21	Moderate positive 2	IDT	NEG	POS	POS	POS	POS
22	Weak positive 1	POS	NEG	POS	POS	POS	POS
23	Strong positive 2	POS	POS	POS	POS	POS	POS
24	Moderate positive 2	IDT	POS	POS	POS	POS	POS
25	Weak positive 2	POS	NEG	POS	POS	POS	IDT
26	Negative	NEG	NEG	NEG	NEG	NEG	NEG
27	Weak positive 1	NEG	NEG	POS	POS	POS	POS
28	Strong positive 1	POS	POS	POS	POS	POS	POS
29	Strong positive 2	POS	POS	POS	POS	POS	POS
30	Negative	NEG	NEG	NEG	NEG	NEG	NEG

Youden plots

The Youden plot is a graphical method to analyse inter-laboratory data, where all laboratories have analysed two samples. The plot visualises between-laboratory variability. For Round 2, sample 1 and sample 2 represent moderate positive and weak positive dilutions from a positive TiLV specimen. Each point in the plot corresponds to the results of one laboratory and is defined by a first response variable on the vertical axis (sample 1) and a second response variable 2 (sample 2) on the horizontal axis. A horizontal median line is drawn for sample 2 and a vertical median line is drawn for sample 1. The intersection of the two median lines is called the Manhattan median. A 45-degree reference line is drawn through the Manhattan median. Laboratories with results in the upper right or lower left quadrant tend towards systematic errors and laboratories in the upper left and lower right quadrant tend towards random errors.

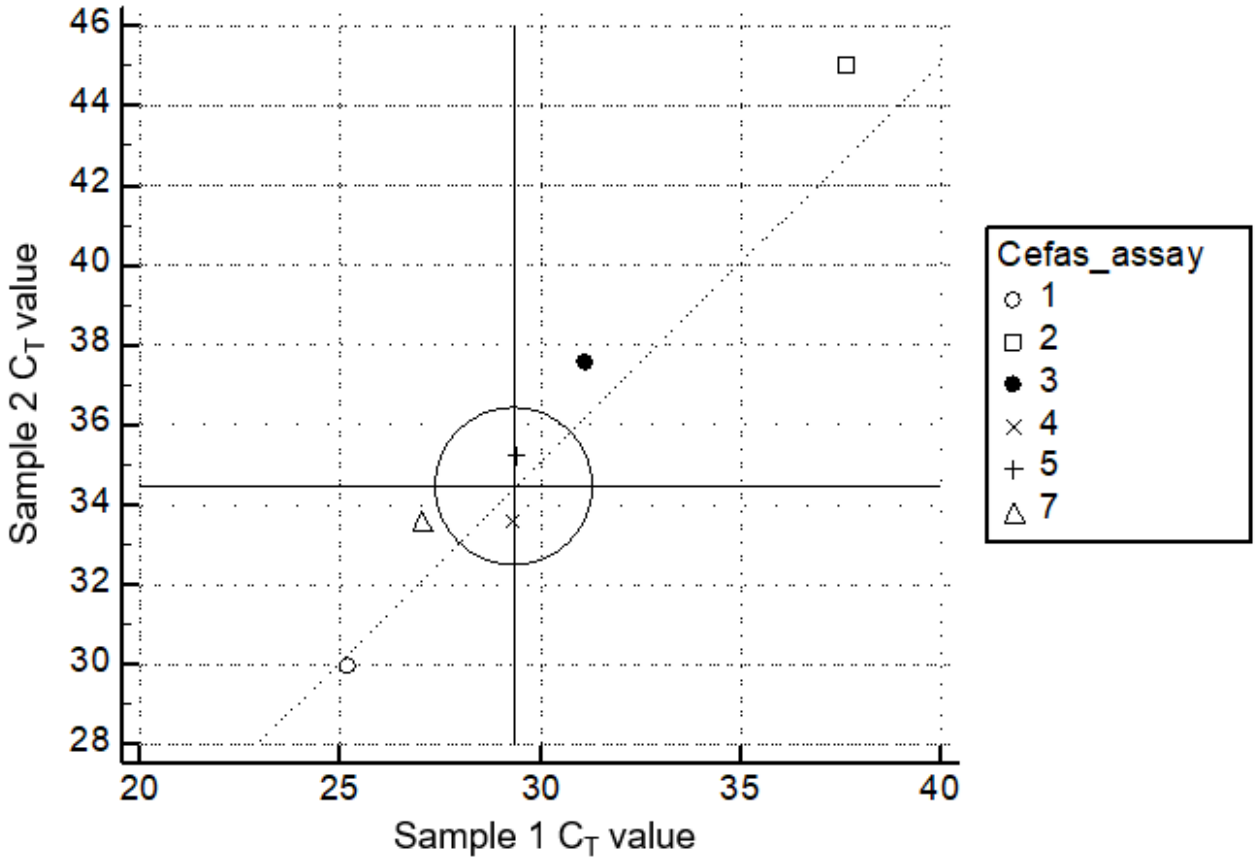

Figure 2. *Youden plot for CEFAS RT-qPCR assay using a weak positive (sample 1) and moderate positive (sample 2).*

Laboratory 2 had the highest C_T values (systematic) and laboratory 1 the lowest C_T values (highest Se). Lab 4 and 5 were within the 95% coverage probability circle and produced C_T values which were closest to the Manhattan median. Lab 7 produced a low value for sample 1 and a close-to-median value for sample 2 which could be an indication for a random error. Most results are scattered along the 45 reference line indicating a systematic relationship between results for sample 1 and 2.

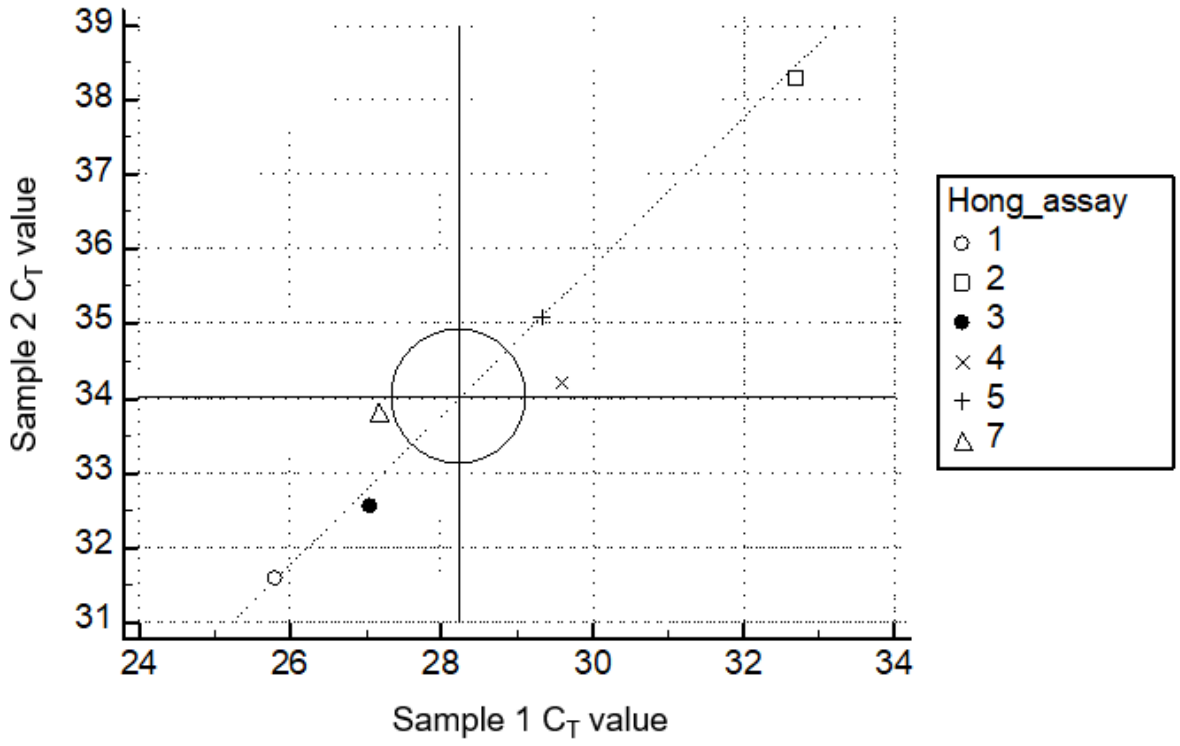

Figure 3. *Youden plot for the Hong RT-qPCR assay using a weak positive (sample 1) and moderate positive (sample 2).*

Laboratory 2 had the highest C_T values (systematic) and laboratory 1 the lowest C_T values (highest Se). The other labs are outside the 95% coverage probability area but close to the Manhattan mean. Most results are scattered along the 45 reference line indicating a systematic relationship between results for sample 1 and 2.

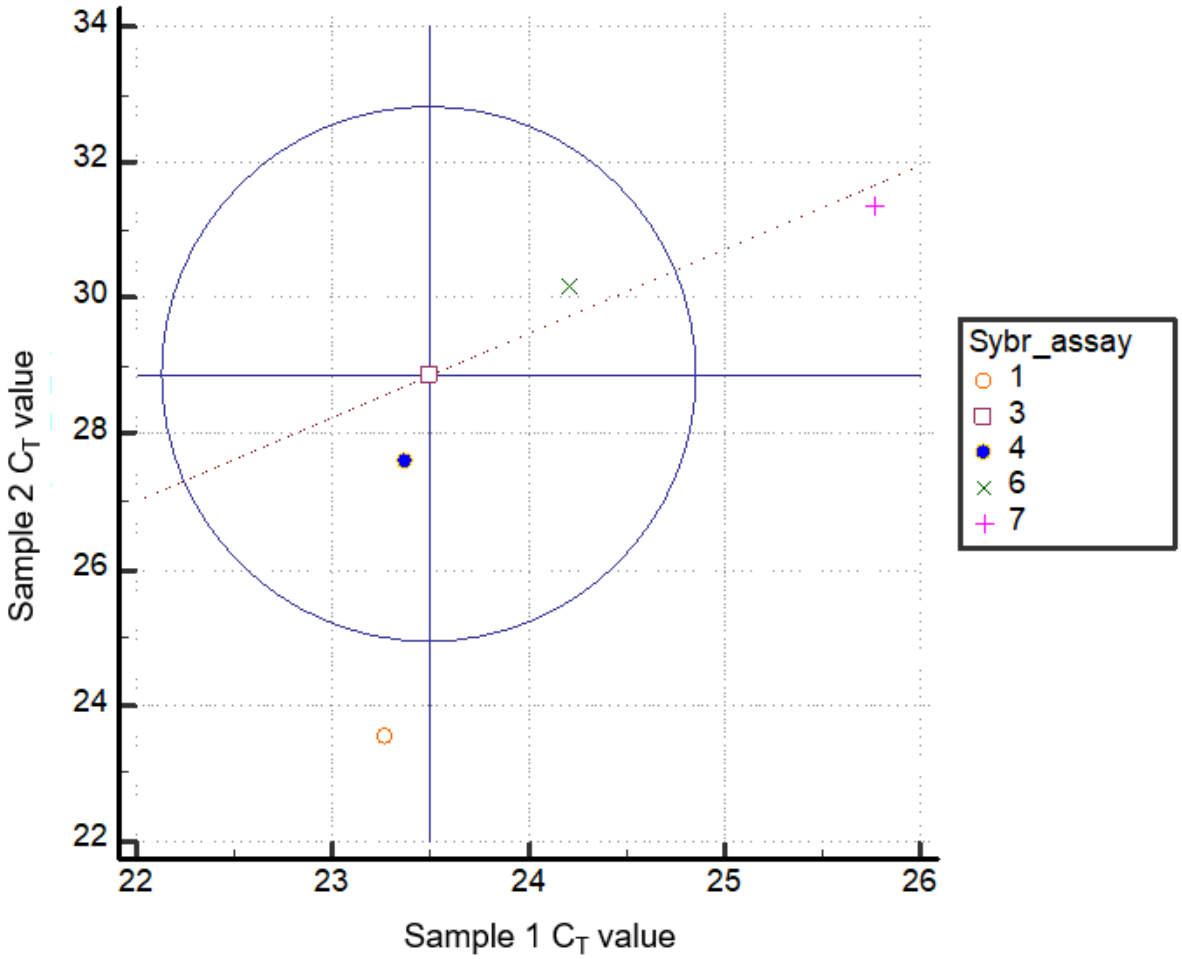

***Figure 4**. Youden plot for Tattiyapong RT-qPCR assay using a weak positive (sample 1) and moderate positive (sample 2).*

Laboratories 3, 4 and 6 are within the 95% coverage probability circle and laboratory 3 has produced median values of both samples. The circumference of the 95% circle is larger than for the Cefas and Hong assays because of the higher degree of variation of the Sybr assay. Lab 1 consistently produces low C_T values indicating a high Se. In contrast, laboratory 7 produced high C_T values for sample1 and close to median for sample 2 indicating random variation. The tilt of the diagonal indicates disproportional results for samples 1 and 2.

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Appendixes

Appendix 1.1. Repeatability of CEFAS TiLV RT-qPCR (results expressed in C_T values)

Sample	ID	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 7
7	Strong positive 1	18.48	30.76	24.81	21.81	22.84	21.77
28	Strong positive 1	19.51	30.81	25.31	21.38	23.08	22.4
18	Strong positive 1	20.05	30.18	24.95	21.97	22.98	21.67
6	Strong positive 2	21.52	33.96	28.03	24.99	26.16	25.92
23	Strong positive 2	22.72	34.01	28.23	24.44	26.09	24.85
29	Strong positive 2	22.35	34.21	28.38	24.08	26.15	25.09
1	Moderate positive 1	25.22	37.65	31.14	29.28	29.44	27.04
11	Moderate positive 1	25.55	36.85	31.56	27.65	29.33	28.22
13	Moderate positive 1	25.6	37.58	31.49	29.32	29.52	28.61
20	Moderate positive 2	29.57	38.74*	34.99	32.34	32.95	31.98
21	Moderate positive 2	29.08	45	34.96	31.13	32.55	31.42
24	Moderate positive 2	29.28	40.36*	34.81	32.01	32.59	31.6
2	Weak positive 1	29.97	45	37.56	33.62	35.22	33.61
8	Weak positive 1	30.8	45	36.86	33.81	34.48	33.95
22	Weak positive 1	31.48	45	37.33	33.24	35.19	33.95
27	Weak positive 1	31.66	45	36.67	33.83	34.91	33.84
12	Weak positive 2	31.88	41.40*	38.93	33.65	36.05	35.65
15	Weak positive 2	32.33	45	38.49	35.11	36.59	35.35
16	Weak positive 2	32.55	45	38.97	35.5	35.84	35.33
19	Weak positive 2	33.37	45	38.61	34.86	35.98	35.19
25	Weak positive 2	32.49	45	38.16	35.06	37.24	35.85
3	Negative	45	45	45	45	45	45
4	Negative	45	45	45	45	45	45
5	Negative	45	45	45	45	45	45
9	Negative	45	45	45	45	45	45
10	Negative	45	45	45	45	45	45
14	Negative	45	45	45	45	45	45
17	Negative	45	45	45	45	45	45
26	Negative	45	45	45	45	45	45
30	Negative	45	45	45	45	45	45

(*mean C_T calculated from a single C_T value)

Appendix 1.2. Repeatability of Hong TiLV RT-qPCR (results expressed in C_T values)

Sample	ID	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 7
7	Strong positive 1	19.34	28.24	20.35	22.44	22.71	21.92
28	Strong positive 1	19.35	29.32	20.68	22.45	22.76	22.6
18	Strong positive 1	18.86	25.37	20.36	22.44	22.96	22.02
6	Strong positive 2	22.66	29.53	23.67	25.3	26.12	26.26
23	Strong positive 2	22.61	38.36	23.89	24.69	25.97	25.35
29	Strong positive 2	22.1	45	23.83	24.72	26.02	25.71
1	Moderate positive 1	25.8	32.71	27.07	29.6	29.32	27.16
11	Moderate positive 1	25.93	32.76	27.11	27.78	29.28	28.81
13	Moderate positive 1	25.93	32.96	27.12	28.96	29.43	28.84
20	Moderate positive 2	29.2	35.36	30.57	31.71	32.7	32.02
21	Moderate positive 2	28.79	35.63	30.5	32.22	32.71	32.15
24	Moderate positive 2	28.8	45	30.64	32.19	32.78	32.34
2	Weak positive 1	31.57	38.29	32.56	34.21	35.1	33.82
8	Weak positive 1	31.39	40.34	32.44	34.27	34.7	33.93
22	Weak positive 1	30.96	35.85	32.43	33.24	34.56	34.12
27	Weak positive 1	31.14	45	32.65	34	34.69	34.13
12	Weak positive 2	32.38	39.56	33.83	35.59	35.97	35.64
15	Weak positive 2	31.91	38.95	33.89	35.4	36.35	35.17
16	Weak positive 2	32.3	39.68	33.56	36.32	35.99	35.35
19	Weak positive 2	32.86	37.58	33.87	35.69	36.14	35.35
25	Weak positive 2	32.21	37.73	33.88	35.88	36.07	35.92
3	Negative	45	45	45	45	45	45
4	Negative	45	45	45	45	45	45
5	Negative	45	45	45	45	45	45
9	Negative	45	45	45	45	45	45
10	Negative	45	45	45	45	45	45
14	Negative	45	45	45	45	45	45
17	Negative	45	45	45	45	45	45
26	Negative	45	26.51	45	45	45	45
30	Negative	45	45	45	45	45	45

Appendix 1.3. Repeatablity of Tattiyapong SYBR-Green RT-qPCR (results expressed in C_T values)

Sample	ID	Lab 1	Lab 3	Lab 4	Lab 6	Lab 7
7	Strong positive 1	17.73	16.8	17.02	17.43	19.17
28	Strong positive 1	23.27	17.22	16.41	17.29	16.61
18	Strong positive 1	16.12	16.79	16.62	16.82	19.11
6	Strong positive 2	32.04	20.13	19.12	21.24	23.45
23	Strong positive 2	20.32	20.23	19.17	20.52	22.36
29	Strong positive 2	19.84	20.42	19.29	20.19	22.71
1	Moderate positive 1	23.27	23.5	23.37	24.21	25.77
11	Moderate positive 1	22.29	23.5	22.5	24.73	25.88
13	Moderate positive 1	22.32	23.62	23.11	23.95	25.79
20	Moderate positive 2	23.61	26.86	26.16	45	29.58
21	Moderate positive 2	22.35	26.83	25.87	27.44	29.11
24	Moderate positive 2	23.8	26.97	25.06	27.23	29.17
2	Weak positive 1	23.55	28.86	27.58	30.16	31.34
8	Weak positive 1	23.66	29.02	28.26	29.26	31.46
22	Weak positive 1	23.18	28.98	26.99	29.77	30.91
27	Weak positive 1	22.46	29.01	27.99	29.46	31.46
12	Weak positive 2	22.64	30.16	26.66	45	32.92
15	Weak positive 2	26.21	30.22	29.38	30.22	33.06
16	Weak positive 2	23.1	30.11	28.28	30.63	33.94
19	Weak positive 2	22.63	30.26	27.75	31.71	31.8
25	Weak positive 2	23.85	30.74	27.22	31.02	33.26
3	Negative	23.48	45	35.96	45	45
4	Negative	23.2	45	45	45	45
5	Negative	23.16	45	30.99	40	45
9	Negative	23.43	45	29.82	40	45
10	Negative	45.11	36.89*	45	36.79	45
14	Negative	22.81	45	32.08	30.29	45
17	Negative	23.4	45	30.89	45	45
26	Negative	24.17	36.14*	31.86	45	45
30	Negative	42	45	31.16	24.21	45

(*mean C_T calculated from a single C_T value)

Appendix 2.1. Homogeneity test results for dilutions used to prepare the panels at ACDP, tested using the Hong RT-qPCR.

Replicate	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6
1	20.79	24.16	27.47	30.77	32.91	34.14
2	20.84	24.20	27.31	30.69	32.82	34.21
3	20.72	24.10	27.42	30.76	32.82	34.27
4	20.81	24.12	27.31	30.96	32.74	34.15
5	20.74	24.16	27.44	30.76	33.04	34.27
6	20.71	24.09	27.44	30.84	33.06	34.12
7	20.58	24.00	27.42	30.67	33.10	34.52
8	20.60	24.12	27.39	30.75	32.91	34.48
9	20.69	24.13	27.41	30.84	32.86	34.20
10	20.65	24.06	27.37	30.74	32.95	34.34
Average	**20.71**	**24.11**	**27.40**	**30.78**	**32.92**	**34.24**
Coefficient of variation	**0.29%**	**0.00%**	**0.17%**	**0.24%**	**0.29%**	**0.00%**
PASS/FAIL	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**

Appendix 2.2. Stability test results for the Strong 1 panel aliquots, tested using the Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey.

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Strong 1	20.71	-20°C	20.50	21.56	20.88
Strong 1		-20°C	20.49	21.51	20.91
Strong 1		-20°C	20.47	21.50	20.99
Strong 1		4°C	20.49	21.55	21.15
Strong 1		4°C	20.50	21.57	21.09
Strong 1		4°C	20.50	21.64	21.13
Strong 1		25°C	20.82	21.82	22.37
Strong 1		25°C	20.78	21.92	21.75
Strong 1		25°C	20.95	21.90	21.82
Strong 1		37°C	21.51	24.37	27.83
Strong 1		37°C	21.75	24.97	27.82
Strong 1		37°C	21.99	24.93	29.49

Appendix 2.3. Stability test results for the Strong 2 panel aliquots, tested using the Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey.

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Strong 2	24.11	-20°C	23.86	24.95	24.51
Strong 2		-20°C	23.91	24.84	24.47
Strong 2		-20°C	23.97	24.89	24.47
Strong 2		4°C	23.91	24.90	24.50
Strong 2		4°C	23.79	24.99	24.34
Strong 2		4°C	23.95	24.98	24.47
Strong 2		25°C	24.11	25.30	25.95
Strong 2		25°C	24.30	25.33	25.50
Strong 2		25°C	24.19	25.44	25.51
Strong 2		37°C	25.41	27.78	30.87
Strong 2		37°C	25.04	29.04	32.41
Strong 2		37°C	25.32	27.69	31.18

Appendix 2.4. Stability test results for the Medium 1 panel aliquots, tested using the Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey.

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Medium 1	27.40	-20°C	27.17	28.10	27.65
Medium 1		-20°C	27.24	28.19	27.76
Medium 1		-20°C	27.21	28.14	27.76
Medium 1		4°C	27.25	28.21	27.84
Medium 1		4°C	27.23	28.26	27.87
Medium 1		4°C	27.30	28.28	27.92
Medium 1		25°C	27.62	28.92	28.68
Medium 1		25°C	27.39	28.90	29.23
Medium 1		25°C	27.54	28.88	29.18
Medium 1		37°C	28.29	31.22	33.58
Medium 1		37°C	28.50	31.18	35.14
Medium 1		37°C	28.50	30.51	35.41

Appendix 2.5. Stability test results for the Medium 2 panel aliquots, tested using the Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey.

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Medium 2	30.78	-20°C	30.58	31.62	31.18
Medium 2		-20°C	30.65	31.63	31.20
Medium 2		-20°C	30.58	31.55	31.09
Medium 2		4°C	30.74	31.67	31.16
Medium 2		4°C	30.62	31.55	31.11
Medium 2		4°C	30.58	31.55	31.17
Medium 2		25°C	30.99	31.87	32.51
Medium 2		25°C	31.02	32.31	32.04
Medium 2		25°C	31.37	31.68	32.24
Medium 2		37°C	31.92	34.41	36.25
Medium 2		37°C	32.40	34.27	38.14
Medium 2		37°C	32.43	34.26	39.03

Appendix 2.6. Stability test results for the Weak 1 panel aliquots, tested using the Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey.

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Weak 1	32.92	-20°C	32.99	32.93	32.79
Weak 1		-20°C	33.13	33.08	33.05
Weak 1		-20°C	32.98	33.03	33.00
Weak 1		4°C	33.12	33.17	33.04
Weak 1		4°C	33.20	33.16	33.33
Weak 1		4°C	33.05	33.11	33.26
Weak 1		25°C	33.48	33.72	34.30
Weak 1		25°C	33.43	33.62	34.20
Weak 1		25°C	33.20	33.40	34.62
Weak 1		37°C	34.16	37.05	Neg
Weak 1		37°C	34.61	36.79	38.36
Weak 1		37°C	34.70	36.34	39.36

Appendix 2.7. Stability test results for the Weak 2 panel aliquots, tested using the Hong RT-qPCR. Results presented as the average with unacceptable results shaded in grey.

Sample	Homogeneity	Temperature	1 week	2 weeks	4 weeks
Weak 2	34.24	-20°C	34.33	34.48	34.23
Weak 2		-20°C	34.66	34.34	34.65
Weak 2		-20°C	34.49	34.50	34.22
Weak 2		4°C	34.31	34.33	34.50
Weak 2		4°C	34.42	34.53	34.79
Weak 2		4°C	34.49	34.40	34.50
Weak 2		25°C	35.09	35.12	35.94
Weak 2		25°C	34.92	35.05	35.75
Weak 2		25°C	35.11	34.89	Neg/40.05
Weak 2		37°C	35.77	37.22	36.02
Weak 2		37°C	35.89	36.84	Neg
Weak 2		37°C	35.74	37.52	Neg/40.0