IQ REAL™ TSV Quantitative System

Real-Time PCR System for

Taura Syndrome Virus (TSV)

in-vitro use only

No contagious material included

Instruction Manual

Cat. No. IQR-TSV, 200 assays

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Made in Taiwan

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I. Introduction

PCR technique has been commonly employed in the aquaculture industry for disease diagnosis and prevention. With the growing demand for a quantitative research tool and requirement for a high throughput screening system, real-time PCR is receiving more attention and becoming more important in the industry.

GeneReach has developed IQ REAL™ TSV Quantitative System, a real-time based diagnostic system. It not only meets the demands, but its advanced designs, listed below, also provides a more accurate, more sensitive, and more flexible real-time system to the shrimp farming industry.

◆ Advantages of IQ REAL™ TSV Quantitative System:

1. TaqMan® assay strategy: This system can tolerate higher concentration of sample load than SYBR Green. Consequently, it is able to detect the samples with very low viral/shrimp cell ratio.
2. Dual color design: This system can quantify both viral (FAM) and shrimp genomic nucleic acid (VIC) by a single reaction (Appendix A). This built-in internal control avoids the false negative results from failed extraction or inhibition.
3. Absolute and comparative quantitation: This system can quantify the viral amount by both absolute quantitative method (Q_{ab}, viral amount/ul) and comparative quantitative method (Q_{cp}, viral amount/10,000 shrimp cells). The Q_{cp} method can reflect the real infectious level and provide constant result for a sample within wide range of concentration. (Appendix B).
4. No standard required: There is no need to make a calibration curve for Q_{cp} method. Since a unique $\Delta\Delta C_t$ strategy is introduced to interpret the final result (Appendix B), there will be no waste for standards, as the reagents are only for the use of samples.

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II. System Components: **stored at -20℃**

Real-Time PreMix	4 vials, 1040 ul/vial, include reaction buffer, dNTPs, specific primers, and fluorescent probes
Dual P(+) Standard	1 vial, 100 ul/vial, 10^6 copies/ul for both TSV and internal control plasmid standard
Yeast tRNA	1 vial, 500 ul/vial, 40 ng/ul
IQzyme DNA Polymerase	1 vial, 400 ul/vial, 2 U/ul
RTzyme Mix	1 vial, 200 ul/vial

III. Extraction Reagents: storage condition as per label

Silica-Extraction Kit	1 kit, includes
Silica	8 ml, 1 g/ml
GT Buffer	280 ml
DEPC ddH_2O	200 ml

IV. Sample Preparation and Nucleic Acid Extraction

1. Sample preparation by Silica-Extraction Kit

   a. Add shrimp sample into a 1.5ml V shape tube with 900 ul GT Buffer. Grind well with a disposable grinder.

   b. Centrifuge at 12000g (12000 rpm, r = 5 ~ 7 cm) for 3 minutes.

   c. Add 40 ul of silica into a fresh 1.5ml V shape tube. Mix the silica well just before adding.

   d. After centrifuge, transfer 600 ul of the upper clear solution into the 1.5ml tube with silica which is prepared in step c. Then vortex to mix well.

   e. Centrifuge at 12000g for 15 seconds (no more than 20 seconds). Then decant the upper solution.

   f. Wash the silica pellet by 500 ul GT Buffer. Vortex until the silica pellet is thoroughly suspended. It is faster to break apart the pellet by pipette tip before vortex.

   g. Centrifuge at 12000g for 15 seconds (no more than 20 seconds). Then decant the upper solution.

   h. Add 1 ml of 70% ethanol to wash the silica pellet. Vortex until the silica pellet is thoroughly suspended. It is faster to break apart the pellet by pipetting prior to vortex.

   i. Centrifuge at 12000g for 15 seconds (no more than 20 seconds). Then, decant the ethanol. Pipette the remained 70% ethanol away.

   j. Add 1 ml of DEPC ddH_2O to re-suspend the silica pellet. Vortex until the silica pellet is thoroughly suspended. Incubate the tube at 55°C for 10 minutes. Vortex and then centrifuge at 12000g for 2 minutes.

   k. Transfer 500 ul of the upper solution to a fresh 1.5ml tube, and it is ready for further reaction.

2. Sample size

Due to the capacity limitation of the Silica and GT Buffer, the sample size from the different sample sources and different sizes of shrimp should be adjusted to fit the capacity and to avoid inhibition. The following sample sizes are recommended.

SIZE OF SHRIMP	SAMPLE SOURCE	SAMPLE SIZE
>30 g	gill	half piece
	pleopod	half leg
	hepatopancreas	~ 10 mm^3
Between 10 g to 30 g	gill	one piece
	pleopod	one leg
	hepatopancreas	~ 10 mm^3
Between 2 cm to 10 g	gill	few pieces
	pleopod	few legs
	hepatopancreas	~ 10 mm^3
Around 1 to 2 cm	head	~ 1/2 to 1 head
PL 1 to PL 12	whole animal	~ 10 to 20 pieces
< PL	whole animal	~ 50 ul

V. Amplification Reaction Protocol

The following amplification condition applies to 0.2ml thin-well tube or 96-well plate. Please refer to the instruction manual of the real-time machine for the choice of proper reaction tubes.

1. Reaction Condition:

   42°C, 30 minutes; then 93°C, 15 seconds; 60°C, 1 minute, repeat 40 cycles.

   (This universal program can be applied for the demand on multi-viral detection.)

2. Reagent preparation: Real-Time reaction reagent mixture: 23 ul/reaction

   Mix the following:

Real-Time PreMix	20 ul
RTzyme Mix	1 ul
IQzyme DNA polymerase 2 U/ul	2 ul

3. Reaction procedure:

   a. Choose quantitative assay system. Both absolute (Q_{ab}) and comparative Q_{cp}) quantitations (Appendix B) are available for IQ REAL™ Quantitative System. Calibration curve is essential for Q_{ab}, and at least 4 standards (10^5, 10^4, 10^3 and 10^2) are required. Oppositely, it is not necessary to make a calibration curve for Q_{cp} method. However, a 10^4 standard for Q_{cp} is recommended to monitor the performance of both viral and genomic amplification. Q_{cp} method is only for shrimp samples, it cannot be applied to the samples without shrimp nucleic acid, such as pond water, pond dirt, and other crustaceans. Q_{cp} data format still can be interpreted under Q_{ab} selection.

b. Standard preparation: **Yeast tRNA** (40 ng/ul) is recommended as the diluent for the **Dual P(+) Standard** dilution. The prepared standards should be stored at -20°C, and the shelf life is 1 week after dilution.

   i. Q_{ab}:

   (i) Add 2 ul **Dual P(+) Standard** (10^6 copies/ul) into 18 ul **Yeast tRNA** (40 ng/ul). Mix well to be 10^5 copies/ul standard.

   (ii) Use the same ratio to dilute the 10^5 copies/ul standard to 10^4, 10^3, and 10^2 copies/ul standard.

   (iii) At least 4 standards (10^5, 10^4, 10^3 and 10^2) are required for calibration curve.

   ii. Q_{cp}: Use the above procedure to prepare 10^4 copies/ul standard.

c. Prepare Real-Time reaction reagent mixture required according to the sample and standard number.

d. Pipette 23 ul of the Real-Time reaction reagent mixture into each reaction tube.

e. Add 2 ul of the sample or standard into each reaction tube.

f. Perform the Real-Time PCR reaction.

VI. Data Interpretation:

1. Absolute quantitation (Q_{ab}):

   a. Choose the correct wavelength of fluorophores (FAM for viral detection, VIC for internal control, TAMRA for quencher dye, see Appendix A) under the instruction of Real-Time machine manufacturers.

   b. Key in the quantities of standards, for both viral and internal control.

   c. Choose FAM to quantify the viral amount. Define the threshold. And then, determine the threshold cycle number (C_t) of samples and standards. (Appendix C)

   d. Make the calibration curve, log (quantity) vs. C_t, by at least 4 standards. Check the R square (R^2) of the calibration curve to evaluate the linear correlation. The qualified range of R square is between 0.95 to 1. If not, try to re-define the threshold.

   e. Interpolate the C_t of each sample into the calibration curve to get its log(quantity), and then, convert into its original quantity.

   f. Choose VIC to quantify the cell number (internal control). Follow step c to e.

2. Comparative quantitation (Q_{cp}):

a. Choose correct wavelength of fluorophores (FAM for viral detection, VIC for internal control, TAMRA for quencher dye, see Appendix A) under the instruction of Real-Time machine manufacturers.

b. Define the threshold of 10^4 standard for both FAM and VIC. And then, determine the threshold cycle number of FAM (C_{tF}) and VIC (C_{tV}).

c. Calculate the ΔC_{t0}: $\Delta C_{t0} = C_{tF} - C_{tV}$

d. If 10^4 standard did not add into the reaction, the ΔC_{t0} value can be determined from previous experiment. For the first time user, 10^4 standard is crucial.

e. Determine the C_{tF} and C_{tV} of each sample. The smaller the ΔC_{tSn} is, the higher concentration the S_n will be.

For sample 1: $\Delta C_{tS1} = C_{tFS1} - C_{tVS1}$

For sample 2: $\Delta C_{tS2} = C_{tFS2} - C_{tVS2}$

:

For sample n: $\Delta C_{tSn} = C_{tFSn} - C_{tVSn}$

The above calculations are only for the positive samples. For those samples which do not pass the VIC threshold after the whole amplification, the tests are failed because of failed extraction or inhibition. When VIC passes the threshold after reaction but FAM does not, it will be negative.

f. Calculate the $\Delta\Delta C_t$ of each sample.

$$\Delta\Delta C_{tSn} = \Delta C_{t0} - \Delta C_{tSn}$$

g. The quantity of sample n (Q_n) can be calculated as following:

$$Q_n = 10{,}000 \text{ X } 2^{\Delta\Delta CtSn} \text{ copies/10,000 shrimp cells}$$

VII. Appendix

1. Information of fluorophore: FAM, VIC, and TAMRA

Name	Excitation λ_{max} (nm)	Emission λ_{max} (nm)
FAM	495	520
VIC	520	546
TAMRA	544	576

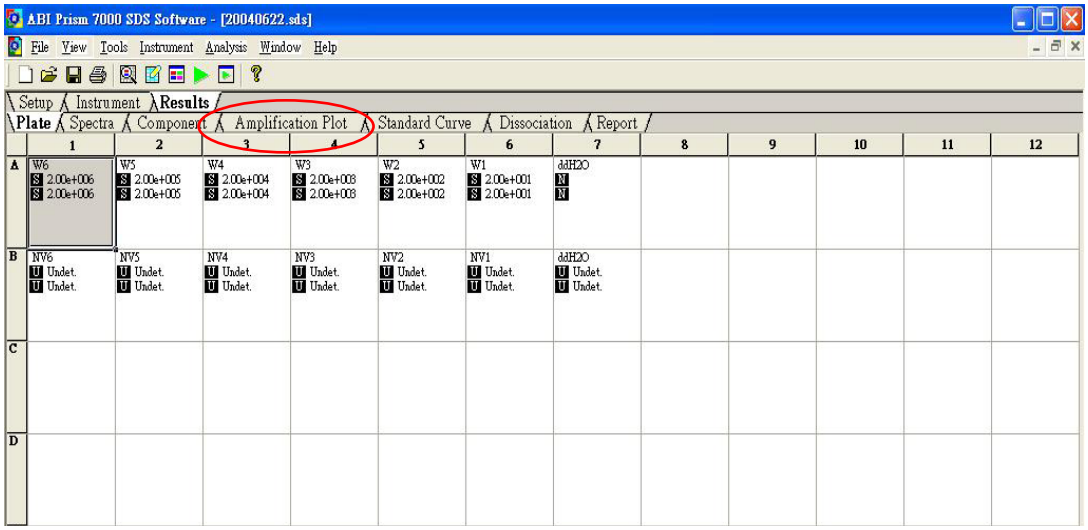
2. Q_{ab} vs. Q_{cp} (absolute quantitation and comparative quantitation)

	Q_{ab}	Q_{cp}
Unit	viral copies/ul sample	viral copies/10^4 shrimp cells
Quantitate from	Calibration curve	$\Delta\Delta C_t$
Sample range	All	Shrimp sample
Same sample under different dilution	Different results	Same result
Standard	At least 4 standards	No need or only one
Meaning	Number of template	Infectious level

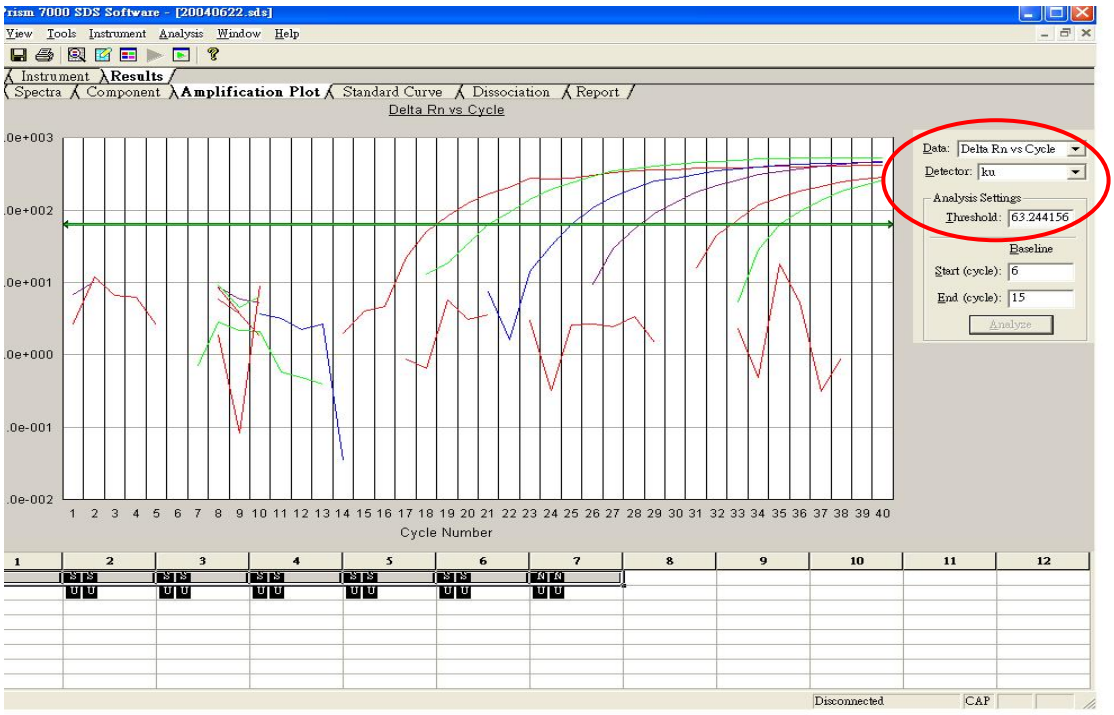
3. Define C_t, ΔC_t, and $\Delta\Delta C_t$, an example by ABI serial instrument.

a. Threshold determination:

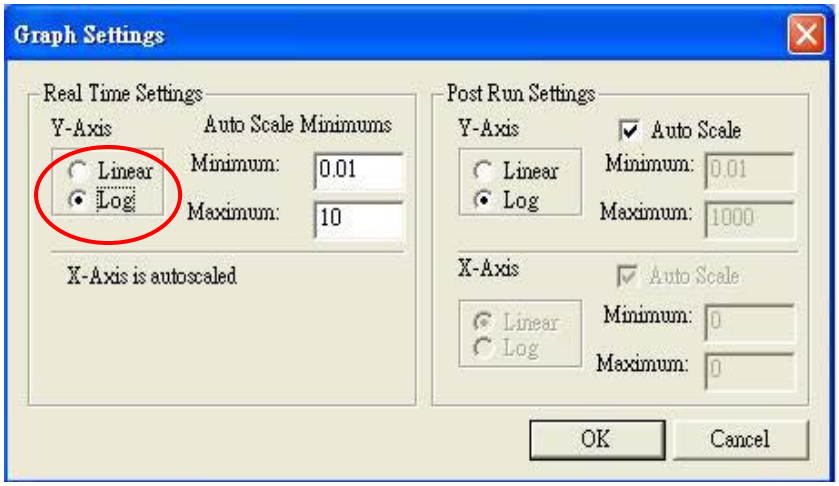
i. After amplification is complete, click the “Amplification Plot”.



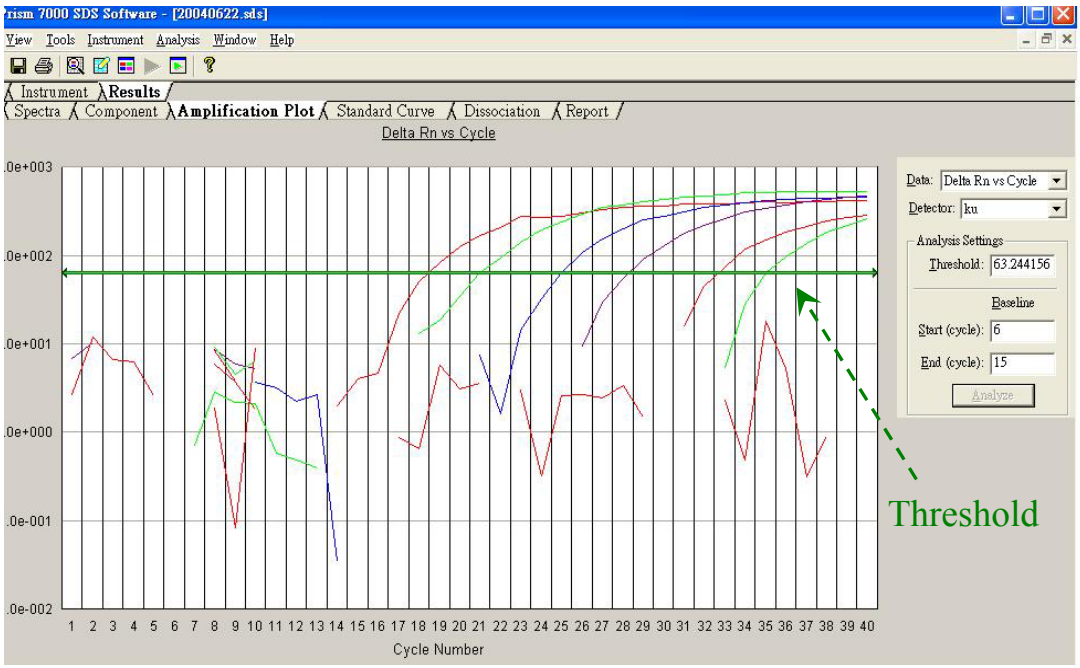
ii. Choose “Delta Rn vs. Cycle” in “Data” and fluorophore (FAM for virus and VIC for internal control) in “Detector”.



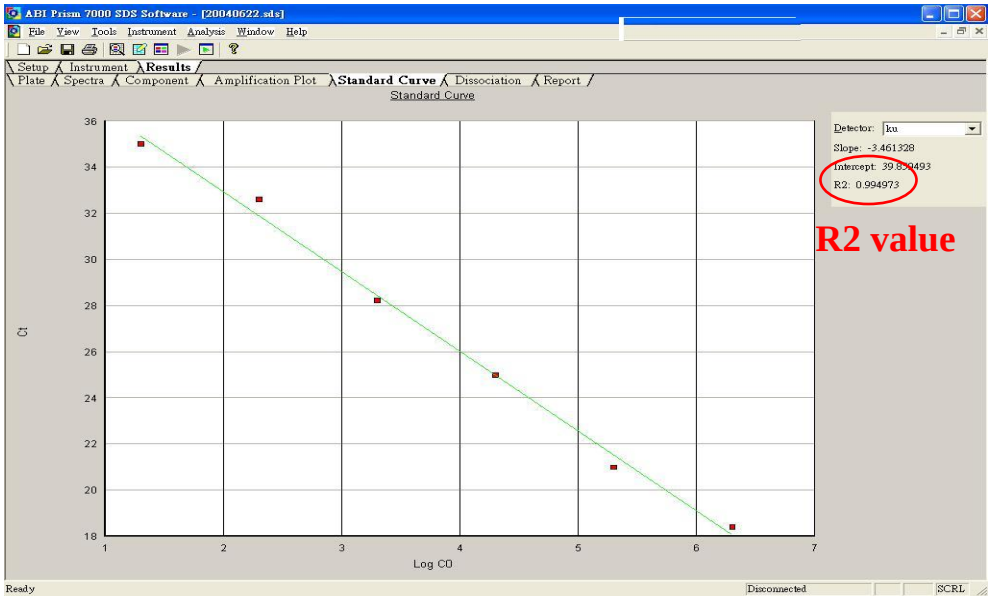
iii. Click on the Y-axis to open “Graph Settings” window. Choose “Y-Axis” as “Log”.



iv. Then drag the Threshold (bar) to the middle of the geometric phase and press the Analyze. The Threshold is green, when the analysis is completed.

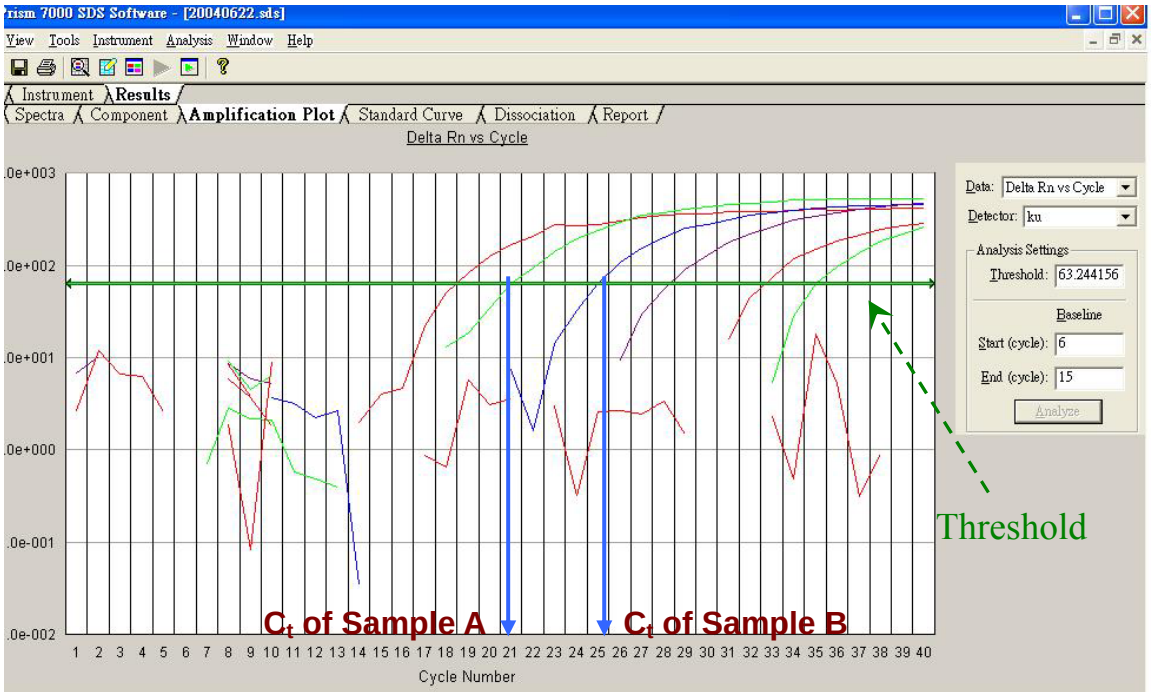


v. For Q_{ab} method, click the "Standard Curve" to check R2 (R square) of the calibration curve.



The R square should be within 0.95 to 1.

b. C_t (threshold cycle): The C_t is the fractional cycle number at which the fluorescence passes the fixed threshold.



c. ΔC_t: The ΔC_t for a specific sample (Sn) is the difference of C_t between FAM and VIC.

For sample Sn: $\Delta C_{tSn} = C_{tFSn} - C_{tVSn}$

The ΔC_t of the **Dual P(+) standard** (10^6) and its dilutions (from 10^5 to 10^2) are between –2 to 1. Basically, ΔC_t reflects the original template ratio between virus and genomic nucleic acid. Independently of the nucleic acid concentration (more or less ddH2O when dissolving the pellet), the ratio of virus/shrimp cell and the ΔC_t will always remain the same for a specific infected sample. In another word, if a positive shrimp nucleic acid sample is dissolved by 100ul and 1000ul ddH_2O, after Q_{ab} strategy, the viral amount is 10X difference between these two dilutions. But these two dilutions will have a same ΔC_t because the virus/cell ratio is the same.

The smaller the ΔC_t is, the earlier the FAM signal passes the threshold than VIC, and the higher the virus/shrimp cell ratio is. That is, the more severe of the infectious status.

Please note the ΔC_t here may be different from other definitions.

d. $\Delta\Delta C_t$: The $\Delta\Delta C_t$ for a specific sample (Sn) is the difference of its ΔC_t to a standard ΔC_{t0}.

$$\Delta\Delta C_{tSn} = \Delta C_{t0} - \Delta C_{tSn}$$

Here, the 10^4 standard is recommended for the ΔC_{t0} definition, but the diluted standards from 10^5 to 10^2 are all qualified for this purpose. Generally speaking, the ΔC_{t0} should be from –2 to 1.

As described above, the more severe the infectious level of Sn is, the smaller the ΔC_{tSn} will be. Thus, the larger the $\Delta\Delta C_{tSn}$ is.

ΔC_{t0} means the virus/shrimp cell ratio = 1. For 10^4 standard, it equals to 10,000 virus in 10,000 cells. Consequently, the comparative quantitative data of sample Sn (Q_{cpSn}) is:

$$Q_{cpSn} = 2^{\Delta\Delta CtSn} \text{ X } 10{,}000 \text{ copies}/10{,}000 \text{ shrimp cells}$$

For example, if the $\Delta\Delta C_{tSn} = 5$,

$Q_{cpSn} = 2^5$ X 10,000/10,000 = 3.2 X 10^5 copies/10^4 shrimp cells.