Piscine reovirus One-Step RT-qPCR Kit, RUO

Segment S1

Catalogue number: MD04641, 150 reactions

Application

NZYTech Kit for Piscine reovirus (PRV) genomes is designed for the *in vitro* quantification of PRV genomes. The kit is designed to have a broad detection profile. Specifically, the primers represent 100% homology with over 95% of the NCBI database reference sequences available at the time of design. The dynamics of genetic variation means that new sequence information may become available after the initial design. If you require further information, or have a specific question about the detection profile of this kit, please send an e-mail to info@nzytech.com and our scientific team will answer your question.

Description

PRV belongs to the *Reoviridae* family and is the only known fish virus related to the *Orthoreovirus* genus. The virus is the causative agent of heart and skeletal muscle inflammation (HSMI), an emerging disease in farmed Atlantic salmon. PRV is ubiquitous in farmed Atlantic salmon and high loads of PRV in the heart are consistent findings in HSMI. PRV has a segmented, double-stranded RNA (dsRNA) genome and belongs to the *Reoviridae* family.

PRV has 10 genomic segments, as do the orthoreoviruses, but the overall amino acid identity between the homologous proteins is very low, particularly for the surface-exposed and non-structural proteins. However, several amino acid motifs central to protein function are conserved for orthoreoviruses and PRV.

Although high loads of PRV in the heart are a consistent finding in HSMI outbreaks, the virus can be detected at low levels in fish throughout the production cycle. PRV has also been detected in farmed Atlantic salmon in Canada and Chile, farmed steelhead trout (*Oncorhynchus mykiss*), wild chum salmon *(O. keta*) in Canada and in wild Atlantic salmon and brown trout (*Salmo trutta L*.) in Norway. In aquaculture farming, large numbers of animals are kept confined at high densities allowing for the rapid proliferation of the infectious agents. HSMI was first described in Norway in 1999. HSMI is mainly observed during the seawater grow-out phase of the fish, with morbidity close to 100% in affected cages, while cumulative mortality varies from negligible to 20%. Typical gross pathologic changes in affected fish include signs of circulatory disturbance, pale heart, yellow liver, swollen spleen and petechiae in perivascular fat. Diagnosis of HSMI is currently based on typical histopathological findings in heart and red skeletal muscle. Real-time PCR enables the non-veterinary pathologist to detect the virus and offer a reliable diagnosis without need for extensive post-mortem examinations. By explicitly targeting a conserved non-structural RNA protein gene, this NZYTech ensures high discrimination towards PRV and other, genetically similar samples.

Kit composition

The kit provides a comprehensive set of reagents sufficient to perform 150 *in vitro* One-step RT-qPCR reactions.

Component	Tubes	Cap colour
PRV specific primer/probe mix - FAM labelled	1	Brown
Internal extraction control primer/probe mix - VIC labelled	1	Brown
Endogenous control primer/probe mix - FAM labelled	1	Brown
RNase/DNase free water[†]	1	White
Template preparation buffer[¥]	3	Yellow
PRV positive control template - for Standard curve	1	Magenta
Internal extraction control RNA	1	Blue
Lyo NZYSupreme One-step RT-qPCR Probe Master Mix (2x) – 3x 50 reactions	3	Neutral
RT-qPCR master mix reconstitution buffer[Δ]	1	Yellow
Lyo ROX	1	Brown

[†] for resuspension of primer/probe mixes

[¥] for resuspension of positive control template, internal extraction control RNA and for standard curve preparation

[Δ] for resuspension of both Lyo NZYSupreme One-step RT-qPCR Probe Master Mix (2x) and Lyo ROX

Storage Conditions and Kit Stability

This Molecular Diagnostic Real-time PCR Kit is shipped at room temperature (RT). Although kit components are stable at RT, they should immediately be stored at -20°C upon arrival.

Once the lyophilized components have been resuspended, they should not be exposed to temperatures above -20°C for longer than 30 minutes at a time. Minimize the number of freeze-thaw cycles by storing in working aliquots. The kit is stable for six months from the date of resuspension under these circumstances. If standard curve dilutions are prepared, these can be stored frozen for an extended period. A fresh standard curve can be prepared from the positive control, if you see any degradation in the previous serial dilution. NZYTech does not recommend using the kit after the expiry date.

Required Reagents and Equipment

- Real-time PCR Instrument
- RNA extraction kit: we recommend using NZYTech's RNA extraction kits
- RNase/DNase free qPCR plasticware: PCR tubes, strips, caps, 96-well plates, adhesive films
- Pipettors and filter tips
- Vortex and centrifuge

Sample Material

All nucleic acid samples that are suitable for PCR amplification can be used with this kit. However, sample collection of biologic material, transport, storage and processing time are critical to achieve optimal results. Please ensure the samples are suitable in terms of purity, concentration and RNA integrity.

NZYTech provides an internal RNA extraction control that is co-purified and then co-amplified with the target nucleic acid. This is useful for the identification of template loss and/or inhibition during sample processing. In addition, we recommend running at least one negative control with the samples (see below). To prepare a negative control, replace the template RNA sample by RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions NZYTech's Molecular Diagnostic One-Step RT-qPCR Kits display very high priming efficiencies of >95%, and can detect less than 100 copies of target template from different samples.

Rational for the test

One-step RT-qPCR

One-step RT-qPCR combines the reverse transcription and amplification reactions in a simple closed tube protocol. This saves significant bench time but also reduces errors. A PRV specific primer and probe mix is provided and can be detected through the FAM channel in a real-time PCR experiment. The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the PRV cDNA. A fluorogenic probe, which consists of a DNA sequence labelled with a 5`-dye and a 3`-quencher, is included in the same reaction mixture to hybridize specifically in the cDNA target region between the two primers. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a wide range of real-time PCR platforms.

Positive control

The kit includes a positive control template that allows controlling the PCR set-up and is also useful for copy number determination. This can be used to generate a standard curve of PRV copy number / quantitation Cycle (Cq) value. Alternatively, the positive control can be used at a single dilution where full quantitative analysis of the samples is not required. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probe for detecting the target PRV gene worked properly in that particular experimental scenario. If a negative result is obtained, the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a post-PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To validate any positive findings, a negative control reaction should be included every time the kit is used. For this reaction the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run.

Internal extraction control RNA

When performing RNA extraction, it is often advantageous to have an exogenous source of RNA template that is spiked into the lysis buffer. This control RNA is then co-purified with the sample RNA and can be detected as a positive control for the

extraction process. Successful co-purification and real-time PCR for the control RNA also indicates that PCR inhibitors are not present at a high concentration. A separate mix of primers and probe is supplied with this kit to detect the exogenous RNA using real-time PCR. These primers are present at PCR limiting concentrations which allow multiplexing with the target sequence primers. Amplification of the control does not interfere with detection of the PRV target cDNA even when present at low-copy number. The Internal control is detected through the VIC channel and gives a Cq value of 28+/-3.

Endogenous control

To confirm extraction of a valid biological template, a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is NOT therefore possible to perform a multiplex with the PRV primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Kit Components Preparation

To help preventing any carry-over contamination, we recommend assigning independent areas for reaction set-up, PCR amplification and any post-PCR gel analysis. It is essential that any tubes containing amplified PCR product are not opened in the PCR set-up area. We also recommend the use of RNase and DNase-free plastic ware/reagents, filter tips (eventually of low-retention) for all pipetting steps and a clean area to work. Prepare the kit contents as described below:

1. Pulse-spin each tube in a centrifuge before opening. This will ensure that lyophilized One-Step RT-qPCR master mix (2x), primer/probe mixes, controls and ROX dye remain at the base of the tube, avoiding spilt upon opening the tubes.
2. Reconstitute the **Lyo NZYSupreme One-step RT-qPCR Probe Master Mix (2x)** with 525 µL of **RT-qPCR master mix reconstitution buffer**, as stated below. Flick and spin until complete resuspension. Do not replace the reconstitution buffer with water or any other buffer. The master mix is then ready to use as a 2x One-Step RT-qPCR master mix.

Component	Volume (µL/ per tube)
Lyo NZYSupreme One-step RT-qPCR Probe Master Mix (2x) (Neutral)	525

3. Optional: Lyo NZYSupreme One-step RT-qPCR Probe Master Mix (2x) is compatible with the majority of thermocyclers available in the market and can include ROX passive reference dye to normalize non-PCR-related fluctuations in fluorescence. If ROX addition is required for your qPCR platform, an optimal quantity of this dye should be included in your master mix. Reconstitute Lyo ROX (1x) with 100 µL of RT-qPCR master mix reconstitution buffer, as stated below. Flick and spin until complete resuspension.

Component	Volume (µL/ per tube)
Lyo ROX (1x) (Brown)	100

The recommended amount of ROX for the most common qPCR instruments is stated in the table below:

qPCR Equipments	Volume of ROX per 20 µL reaction	Volume of ROX per 525 µL of 2x master mix (2x)
Applied Biosystems: 7000/7300/7700/7900/7900HT/7900HT FAST/ StepOne™/StepOne™plus	0.57 µL	15 µL
Applied Biosystems: 7500/7500FAST/QuantStudio™ 6, 7, 12k Flex/ViiA7™	0.08 µL	2 µL
Bio-Rad®: CFX96™/CFX384™/iCycler®/iQ™5/Opticon™/ Opticon™ 2 **Qiagen:** Rotor-Gene™ 3000/6000/Q **Roche:** Lightcycler® 96/480/Nano	Not required	Not required

4. Reconstitute the following kit components in the RNase/DNase free water supplied, as follows:

Component	Volume (µL/ per tube)
PRV primer/probe mix (Brown)	165
Internal extraction control primer/probe mix (Brown)	165
Endogenous control primer/probe mix (Brown)	165

5. Reconstitute the positive control template and the internal extraction control in the template preparation buffer supplied, as follows:

Component	Volume (µL/ per tube)
Internal extraction control RNA (Blue)	600
PRV positive control template (Magenta) (*)	500

(*) Note: Beware that this component contains high-copy number template and is a HIGH contamination source. It must be opened and handled in a separate laboratory environment, away from the other components.

6. To ensure complete resuspension, vortex each tube thoroughly.

Nucleic Acids Extraction

The **Internal extraction control RNA** can be added either to the RNA lysis/extraction buffer or to the RNA sample once it has been resuspended in lysis buffer.

1. Add 4 µL of the **internal extraction control RNA** to each sample in RNA lysis/extraction buffer per sample.
2. Proceed to RNA extraction according to the manufacturer's protocols.

Note: Do not add the internal extraction control RNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

One-step RT-qPCR Detection Protocol

1. RNA sample

For each RNA sample, prepare a reaction mix according to the table below (final volume per reaction). Include sufficient reactions for positive and negative controls. We strongly recommend performing replicates of all reactions.

Component	Volume (µL)
Lyo NZYSupreme One-step RT-qPCR Probe Master Mix (2x) (Neutral)	10
PRV primer/probe mix (Brown)	1
Internal extraction control primer/probe mix (Brown)	1
RNase/DNase free water (White)	3
Final Volume	**15**

2. Optional: Endogenous control reaction

For each RNA sample, prepare an endogenous control reaction according to the table below. This control reaction will provide useful information regarding the quality of the biological sample.

Component	Volume (µL)
Lyo NZYSupreme One-step RT-qPCR Probe Master Mix (2x) (Neutral)	10
Endogenous control primer/probe mix (Brown)	1
RNase/DNase free water (White)	4
Final Volume	**15**

3. Reaction set-up

3.1. Pipette 15 µL of each mix into individual wells according to your real-time PCR experimental plate set-up.
3.2. Pipette 5 µL of RNA template into each well, according to your experimental plate set-up. The final volume in each well should be 20 µL.
3.3. For negative controls, use 5 µL of RNase/DNase free water instead of RNA template. The final volume in each well is 20 µL.

4. Standard Curve Preparation

If a standard curve is included for quantitative analysis, proceed according to described below.

4.1. Prepare a reaction mix as follows:

Component	Volume (µL)
Lyo NZYSupreme One-step RT-qPCR Probe Master Mix (2x) (Neutral)	10
PRV primer/probe mix (Brown)	1
RNase/DNase free water (White)	4
Final Volume	**15**

4.2. Standard curve dilution series & set-up:

4.2.1. Pipette 90 µL of template preparation buffer into 5 tubes and label 2-6.
4.2.2. Pipette 10 µL of Positive Control Template (Magenta) into tube 2.
4.2.3. Vortex thoroughly and spin.
4.2.4. Change tip and pipette 10 µL from tube 2 into tube 3.
4.2.5. Vortex thoroughly and spin.
4.2.6. Repeat steps 4.2.4. (from sequential tubes) and 4.2.5. to complete the dilution series.

Standard Curve	Copy number (per µL)
Tube 1 - Positive control template	2×10^5
Tube 2	2×10^4
Tube 3	2×10^3
Tube 4	2×10^2
Tube 5	20
Tube 6	2

4.3. Pipette 5 µL of each standard template dilution into each well containing the standard curve reaction mix, according to your experimental plate set-up. The final volume in each well should be 20 µL.

Suggested thermal cycling conditions

Lyo NZYSupreme One-step RT-qPCR Probe Master Mix (2x) is an optimized and highly efficient reaction mixture developed for One-step real-time PCR. The table below displays a standard protocol optimized on a number of platforms. However, these conditions may be adapted to suit different machine-specific protocols.

Cycles	Temperature	Time	Notes
1	50 °C	20 min	Reverse Transcription
1	95 °C	2 min	Polymerase activation
50	95 °C	5 s	Denaturation
	60 °C	30 s	Annealing/Extension*

*Fluorogenic data should be collected during this step through both FAM and VIC channels.

Data analysis

Target Cq (FAM)	Internal control Cq (VIC)	Positive control Cq	Negative control Cq	Result
≤ 30	+/-	+	-	**Positive quantitative result**: calculate copy number
> 30	+	+	-	**Positive quantitative result**: calculate copy number
> 30	-	+	-	**Positive qualitative result**: do not report copy number - possible poor sample extraction
-	+	+	-	**Negative result**
+/-	+/-	+	≤ 35	Experiment failed due to test contamination
+/-	+/-	+	> 35	♣
-	-	+	-	Sample preparation failed
+/-	+/-	-	+/-	Experiment failed

Positive Control: Positive control template is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

Internal Control: The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of RNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a PRV sample with a high genome copy number, the internal control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous control: The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.

♣ Sample Positive & Negative control with Cq >35: the sample must be reinterpreted based on the relative signal strength of the two results:

- **Sample Positive**: If the sample amplifies > 5 Cq earlier than the negative control, then the sample should be reinterpreted (via the table above) with the negative control verified as negative.
- **Inconclusive**: If the sample amplifies < 5 Cq earlier than the negative control, then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

Certificate of Analysis

Test	Result
Functional assay	Pass

Approved by:

Patrícia Ponte
Senior Manager, Quality Systems

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