

virotype[®] IHNV/VHSV RT-PCR Kit Handbook

For simultaneous detection of RNA from
Infectious Hematopoietic Necrosis Virus
(IHNV) and *Viral Hemorrhagic Septicemia*
Virus (VHSV)

Licensed in accordance with § 11 (2) of the German Animal Health Act
MA No.: FLI-C 133



100 reactions (Cat. no. VT290005)



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Kit contents

virotype IHN/VHSV RT-PCR Kit	(100)
Cat. no.	VT290005
Number of reactions	100

Master Mix (tube with orange cap) includes enzymes, primers, and probes	2 x 1000 µl
Positive Control (tube with red cap)	1 x 150 µl
Negative Control (tube with blue cap)	1 x 150 µl
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Intended use

The virotype IHN/VHSV RT-PCR Kit is intended for the simultaneous detection and differentiation of RNA from *Infectious Hematopoietic Necrosis Virus* (IHN) and *Viral Hemorrhagic Septicemia Virus* (VHSV) from tissue samples from salmonids and cell culture supernatant.

The kit is approved by the Friedrich-Loeffler-Institut and licensed in accordance with § 11 (2) of the German Animal Health Act (FLI-C 133) for use in Germany for veterinary diagnostic procedures.

For veterinary use only.

Symbols



Legal manufacturer



Lot number



Use by date



Temperature limitations for storage



Handbook



Catalog number



Material number



Protect from light



For samples from salmonids

Quality control

In accordance with INDICAL's ISO-certified Quality Management System, each lot of virotype IHNV/VHSV RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Storage

The components of the virotype IHNV/VHSV RT-PCR Kit should be stored at -30°C to -15°C and are stable until the expiration date stated on the label. Avoid repeated thawing and freezing (> 3x), as this may reduce assay sensitivity. Freeze the components in aliquots if they will only be used intermittently.

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available from your local sales representative or by Email request under **compliance@indical.com**.

All sample residues and objects that have come into contact with samples must be decontaminated or disposed of as potentially infectious material.

Introduction

The virotype IHNV/VHSV RT-PCR Kit is a multiplex PCR assay intended for the simultaneous detection and differentiation of RNA from *Infectious Hematopoietic Necrosis Virus* (IHNV) and *Viral Hemorrhagic Septicemia Virus* (VHSV) from tissue samples from salmonids and cell culture supernatant.

Infectious Hematopoietic Necrosis (IHN) and Viral Hemorrhagic Septicemia (VHS) are two of the most dangerous viral diseases to salmonid aquaculture. Both pathogens (IHNV and VHSV) belong to the family *Rhabdoviridae* – genus *Novirhabdovirus*. IHN and VHS are cold water diseases of salmonid species, in particular rainbow trout (*Oncorhynchus mykiss*) and occur in Canada, the United States of America, Asia and Europe.

Co-infection of salmonids with IHNV and VHSV have been described in the literature. IHNV is initially detected in gill epithelium skin, the oral region, pharynx, esophagus, stomach, and pyloric caeca before being detected in kidney, spleen, thymus, liver, muscle, and cartilage. It then spreads to the heart, pancreas, and brain. In rainbow trout, kidney and spleen endothelial cells are the first targets of VHSV. Afterwards, hematopoietic elements of kidney and spleen undergo necrosis and degeneration. Infections with IHNV and VHSV cause high economic losses in aquaculture as a direct consequence of mortality. Infection of fish with IHNV and VHSV is notifiable to the World Organization of Animal Health (WOAH), and various countries and trading areas, like the European Union, have particular legislation in place for the control of the disease (WOAH, 2017c; EU, 2006/88; EU, 2009/177). Furthermore, a differentiation of the two diseases based on clinical and pathological/anatomical symptoms is not possible. Therefore, monitoring and surveillance to detect and identify IHNV and VHSV are legally defined to protect fish cultures.

Principle

Polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time PCR, the amplified product is identified using fluorescent dyes. These are usually linked to oligonucleotide probes that bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e., in real-time) allows detection of the accumulating product without the need to re-open the reaction tubes afterward.

The virotype IHN/VHSV RT-PCR Kit contains all of the necessary reagents for the detection of IHN and VHSV RNA, including a Positive and Negative Control. With this kit, both, reverse transcription and PCR are performed in one reaction tube, reducing the risk of contamination. An internal control excludes the possibility of false-negative results.

The kit uses three specific primer/probe combinations:

- Cy[®]5 fluorescence for RNA from IHN
- FAM[™] fluorescence for RNA from VHSV
- HEX[™] fluorescence for the endogenous Internal Control (EC; β -actin present within the sample)

A Positive Control serves to verify the functionality of the reaction mix for the amplification of the pathogen RNA targets.

RNA extraction

The virotype IHN/VHSV RT-PCR Kit is intended for the simultaneous detection and differentiation of RNA from IHN and VHSV from tissue samples from salmonids and cell culture supernatant.

Prior to real-time RT-PCR, viral RNA must be extracted from the starting material.

INDICAL offers a range of validated kits for the extraction of RNA from animal samples.

Extraction based on magnetic beads:

- **IndiMag® Pathogen Kit** (SP947457)
- **IndiMag Pathogen Kit w/o plastics** (SP947257)
- **IndiMag Pathogen IM48 Cartridge** (SP947654P608, SP947654P224)
- **IndiMag Pathogen KF96 Cartridge** (SP947855P196)

Extraction based on spin columns:

- **IndiSpin® Pathogen Kit** (SP54104, SP54106)
- **IndiSpin QIAcube® HT Pathogen Kit** (SP54161)

INDICAL recommends **Pretreatment Protocol T1** for the extraction of viral RNA from salmonid tissue samples and tissue homogenates.

If real-time RT-PCR is not performed immediately after extraction, store the RNA at -20°C or at -80°C for longer storage.

For further information on automated and manual extraction of fish RNA from different sample types, refer to the respective handbook or contact INDICAL Support at support@indical.com.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Pipets
- Nuclease-free, aerosol-resistant pipet tips with filters
- Sterile 1.5 ml Eppendorf® tubes
- Nuclease-free (RNase/ DNase-free) consumables. Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive identification of viral nucleic acids
- Cooling device or ice
- Benchtop centrifuge with rotor for 1.5 ml tubes
- Real-time cycler with appropriate fluorescent channels
- Appropriate software for chosen real-time cycler
- Appropriate strip tubes and caps or 96-well optical microplate with optical sealing film or cover for chosen real-time cycler

Important notes

General precautions

The user should always pay attention to the following:

- Use nuclease-free pipet tips with filters.
- Store and extract positive materials (specimens, positive controls and amplicons) separately from all other reagents and add them to the reaction mix in a spatially separated facility.
- Thaw all components on ice before starting the assay.
- When thawed, mix the components by inverting and centrifuge briefly.
- Do not use components of the test kit past the expiration date.
- Keep samples and controls on ice or in a cooling block during the setup of reactions.

Negative control

At least one negative control reaction should be included in each PCR run, containing all the components of the reaction except for the pathogen template. This enables assessment of contamination in the reaction.

Positive control

When performing PCR on unknown samples, it is recommended to include a positive control reaction in the PCR run, containing a sample that is known to include the targeted viral RNA. A positive control serves to prove the functionality of the pathogen assay, e.g., the correct setup of the reaction mix. Use 5 µl of the Positive Control provided with

the virotype IHN/VHSV RT-PCR Kit to test for successful amplification of the target.

Extraction and amplification control

For increased process safety and convenience, one extraction and amplification control assay is included in the test kit.

An endogenous Internal Control (EC) detects the β -actin gene present within the sample. This allows extraction and amplification to be monitored.

Protocol: Real-time RT-PCR for detection of RNA from IHNV and VHSV

Important points before starting

- Please read „Important notes“ on page 10 before starting.
- Include at least one positive control (Positive Control) and one negative control (Negative Control) per PCR run.
- Before beginning the procedure, read through the protocol and ensure that you are familiar with the operation of the chosen real-time PCR cyclers.
- RNA is unstable. Perform the protocol without interruption.

Things to do before starting

- Thaw all reagents on ice and protect from light.
- Before use, spin the reagents briefly.
- Maintain reagents on ice or in a cooling block during PCR setup.

Procedure

1. Pipet 20 µl of the Master Mix into each reaction tube. Then add 5 µl of the sample RNA (Table 1).

Include positive and negative control reactions.

Positive Control: Use 5 µl of the positive control (Positive Control) instead of sample RNA.

Negative Control: Use 5 µl of the negative control (Negative Control) instead of sample RNA.

Table 1. Preparation of reaction mix

Component	Volume
Master Mix	20 µl
Sample	5 µl
Total volume	25 µl

2. Close the reaction tubes or seal the plate and invert 5 times or until mixed thoroughly. Then centrifuge briefly to collect the fluids.
3. Set the filters for the reporter dyes in the software of your thermal cycler according to Table 2.

Table 2. Filter settings for the reporter

Pathogen/ Internal Control	Reporter
IHNV	Cy [®] 5
VHSV	FAM [™]
Endogenous Internal Control (EC)	HEX [™] 1
Passive reference ²	ROX [™]

1 Use the option appropriate for your thermal cycler.

2 Internal reference for use with ABI PRISM[®] Sequence Detection Systems (Applied Biosystems[®])

4. Run the real-time PCR protocol according to Table 3.

Table 3. Real-time RT-PCR protocol for IHNV/VHSV

Step	Temperature	Time	Number of cycles
Reverse Transcription	50°C	10 min	1
Initial Activation	95°C	2 min	1
2-step cycling			
Denaturation	95°C	5 s	40
Annealing/ Extension*	60°C	30 s	

* Fluorescence data collection, approximate run time 67 min (Mx3005P®, Agilent Technologies, Inc.)

Data analysis and interpretation

Interpretation of results

For the assay to be valid the Positive Control must give a signal in the Cy5, FAM and HEX channels with a $C_T^1 < 35$. The Negative Control must give no signal.

The following results are possible if working with unknown samples. The possible sample results are also summarized in Table 4 on page 18.

The sample is positive for IHNV, and the assay is valid, if the following criteria are met:

- The sample yields a signal in both, the Cy5 and the HEX channels.
- The Positive Control yields a signal in the Cy5, FAM, and HEX channels.
- The Negative Control does not yield a signal in the Cy5, FAM, and HEX channels.

Note that very high concentrations of IHNV RNA in the sample may lead to a reduced HEX signal or no HEX signal due to competition with the Internal Control.

¹ Threshold cycle (C_T) — cycle at which the amplification plot crosses the threshold, i.e., there is the first clearly detectable increase in fluorescence

The sample is positive for VHSV, and the assay is valid, if the following criteria are met:

- The sample yields a signal in both, the FAM and the HEX channels.
- The Positive Control yields a signal in the Cy5, FAM, and HEX channels.
- The Negative Control does not yield a signal in the Cy5, FAM, and HEX channels.

Note that very high concentrations of VHSV RNA in the sample may lead to a reduced HEX signal or no HEX signal due to competition with the Internal Control.

The sample is positive for IHNV and VHSV, and the assay is valid, if the following criteria are met:

- The sample yields a signal in the Cy5, FAM and HEX channels.
- The Positive Control yields a signal in the Cy5, FAM, and HEX channels.
- The Negative Control does not yield a signal in the Cy5, FAM, and HEX channels.

Note that very high concentrations of IHNV and VHSV RNA in the sample may lead to a reduced HEX signal or no HEX signal due to competition with the Internal Control.

The sample is negative for IHNV and VHSV, and the assay is valid, if the following criteria are met:

- The sample yields a signal in the HEX channel only.
- The Positive Control yields a signal in the Cy5, FAM, and HEX channels.
- The Negative Control does not yield a signal in the Cy5, FAM, and HEX channels.

A positive HEX signal excludes the possibility of inhibition, as the endogenous Internal Control (EC) was successfully amplified.

The sample results are inconclusive, and the assay is invalid, if the following criteria are met:

- The sample yields no signal in the Cy5, FAM, and HEX channels.

If no signal is detected in the Cy5 (IHNV), FAM (VHSV), and the HEX (endogenous Internal Control, EC) channel, the result is inconclusive. The absence of a signal for the housekeeping gene indicates strong PCR inhibition and/or other malfunctions, e.g., during extraction.

To check for inhibition, we recommend 1:5 dilution of the sample RNA in nuclease-free water, to repeat the RNA extraction procedure, or repeat the whole test procedure starting with new sample material.

Check that there is a fluorescence signal in all the channels for the positive control reaction (Positive Control). Absence of a signal for the Positive Control indicates an error, which could be due to incorrect setup of the reaction mix or incorrect cycling conditions.

Table 4. Results interpretation table*

Sample result	Cy5 (IHNV)	FAM (VHSV)	HEX (EC)
IHNV positive	X		X
VHSV positive		X	X
IHNV and VHSV positive	X	X	X
IHNV and VHSV negative			X
Inconclusive			

* Interpretation of sample results can be determined provided positive and negative control reactions are performed. The Positive Control must yield a signal in the Cy5, FAM, and HEX channels. The Negative Control must yield no signal in any channel. For a complete explanation of possible sample results please refer to “Data analysis and interpretation” on page 15.

INDICAL offers a range of ELISA kits and real-time PCR and real-time RT-PCR kits for the detection of animal pathogens.

Visit **www.indical.com** for more information about afosa, bactotype, cador, cattletype, flocktype, pigtype, Svanovir, and virotype products.

For up-to-date licensing information and product-specific disclaimers, see the respective INDICAL kit handbook or user manual.

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Handbook	Version	Change
HB-2567-EN-001	April 2023	Product launch