NATIONAL TEST METHOD PROTOCOL

APPROVAL COVER PAGE

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Protocol Q-Pulse Location:	Protocols/National/PCR
Protocol Number:	NAT-PROT-RT-qPCR ISAV-1 ***(Draft #3 2011-02-18)***
Protocol Revision Number:	1
Protocol Title:	**RT-qPCR Diagnostic with TaqMan Universal PCR Master Mix for the Detection of Nucleic Acids from Infectious Salmon Anemia**

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This cover page serves as final approval of a document. Records of approval are also maintained in Q-Pulse. It provides you with all current coding information.

This Test Method Protocol has been approved for use by the following issuing authorities:

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Next Scheduled Review Date: 2012 - 02 - 18
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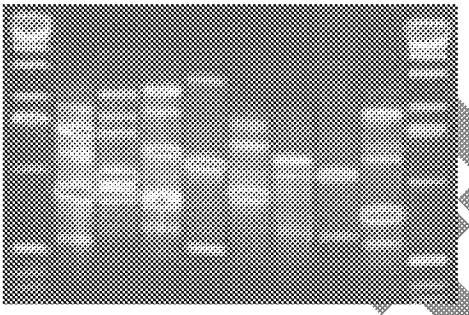
RT-qPCR Test Method Protocol using TaqMan Universal PCR Master Mix for the Detection of Nucleic Acids from Infectious Salmon Anemia Virus



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An Official Test Method Protocol of the
National Aquatic Animal Health Laboratory System,
Fisheries and Oceans Canada

Version 1.0
February, 2011
Test developed and protocol written by: Nellie Gagné and Mélanie Robichaud-Haché

This document was created as part of and/or to fulfill the requirements of the Quality Management System of the National Aquatic Animal Health Laboratory System, which was developed to comply with the requirements of ISO/IEC 17025:2005.

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Approved by: National Laboratory Manager Date

This signature constitutes an approval of the test method and related procedures described herein as fit for a specific, defined and/or potential purpose. The method may or may not be routinely in use for the production of diagnostic results to be used for regulatory purposes, depending on current customer/client needs.

REVISION PAGE

Previous Versions: Nil

Version no.	Author(s)	Date

Review Due: February 2012

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1.0 INTRODUCTION

1.1 The Principles of qPCR and RT-qPCR

Polymerase chain reaction (PCR) implies that there is an amplification reaction in the assay. The term "chain reaction" refers to several cycles of copying a specified stretch of deoxyribonucleic acid (DNA) from a target nucleic acid, in this case from the genome of an infectious agent. The amplified region is defined by two primers that are complementary to DNA regions flanking the target sequence. By repeating ~ 40 times a heat-cycling regime, the amount of copied target DNA gained is enough for further operations, such as detection, cloning or sequencing. The analytical sensitivity of the PCR is very high because several million copies of the selected target are produced. The specificity of the reaction may also be very high, as determined by the specific nucleotide sequences of the oligonucleotides (primers). The primers are designed to detect specific nucleotide sequences in the genomes of the selected target infectious agents.

The genomes of many infectious agents contain ribonucleic acid (RNA) that cannot be amplified directly by the PCR. For PCR amplification, a double-stranded DNA target is necessary, and this is not available in the case of RNA viruses. This problem can be solved by the addition of a step before the PCR is begun. Using reverse transcriptase, RNA is transcribed into complementary DNA (cDNA), which is double-stranded DNA and hence can be used in a PCR assay (the procedure is termed reverse transcriptase PCR: RT-PCR). The reverse transcription reaction is performed either in a separate reaction vessel and the cDNA produced is then transferred to a new tube for the PCR (two-step RT-PCR); alternatively, the PCR reaction proceeds directly after the reverse transcription in the same tube (one-step RT-PCR). After PCR amplification, the PCR product is submitted to gel electrophoresis and gel staining to determine if the sample is positive or negative for a specific target.

With real-time PCR (qPCR), the detection of PCR amplification products is done at each cycle with a laser. A fluorescent reporter is added to the PCR mixture for that purpose. There are several types of fluorescent molecules. SYBR green is a non-specific dye that attaches to DNA strands, hence it can be used with any PCR assay. Probes labeled with a reporter and quencher are specific to the assay as they complement the DNA strand amplified by the assay. There are several types of probe chemistry. Theoretically, there is a quantitative relationship between the amount of starting target in the sample and the amount of amplified product at any given cycle number.

"Conventional PCR" (or simply PCR) uses one pair of oligonucleotide primers to amplify a small part of the genome of the infectious agent. Analytical sensitivity is relatively high with a minimum number of 100 to 1000 copies of the genome detectable. Analytical specificity is also rather high, in comparison to virology or histology for example. However, the specificity is determined by selection of the primers. Primers can detect "universal" or conserved portions of DNA commonly found between pathogens. Primers can also be selected to recognize only specific DNA sequences that are unique to an organism (a pathogen in this case) or even to the species belonging to a genus.

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As RNA viruses are usually prone to mutation, primer design for their detection by RT-PCR is particularly crucial. Mutations, which may occur within the primer sites, can affect the established performance characteristics of the assay. It is also advisable to regularly sequence the selected genomic regions in recent isolates of these infectious agents. This is especially true for the primer sites, to ensure their specificity so that the validity of the RT-PCR assay cannot be questioned.

PCR can be used as a screening assay. It can also be used as a confirmatory assay when applied to a suspected pathogen detected by other methods. As well, it can be used as a preliminary step for further molecular assays such as DNA sequencing, restriction fragment length polymorphism (RFLP), cloning, etc. The experiences of the last decade indicate that PCR techniques will eventually supersede many of the classical direct methods of infectious agent detection. It is clear that in many laboratories, PCR is replacing virus isolation or bacteria cultivation for the detection of agents that are difficult or impossible to culture. Although PCR assays were initially expensive and cumbersome to use, they have now become relatively inexpensive, safe and user-friendly tools in diagnostic laboratories.

The technique is highly sensitive and requires rigorous controls in the workspace. The results are finite (yes-no) in end-point PCR, or can be quantitative as in real-time PCR or qPCR, which involves a quantitative laser detection in real-time of the PCR product as they are formed during the procedure.

The scope of this diagnostic test method protocol is limited to amplification by two-step RT-qPCR of viruses infecting fish.

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1.2 Synopsis of Viral Agent of Concern

Infectious Salmon Anemia Virus (ISAV)

Infectious salmon anemia (ISA) is an infectious disease of cultured Atlantic salmon caused by an orthomyxo-like virus. The genome of ISAV consists of eight single-stranded RNA segments with negative sense.

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European strains of ISAV are distinct from North American strains at the genomic level. Segment 6 codes for the hemagglutin-esterase (HE) gene and the hypervariable segment of that gene is useful in the characterization of different isolates and genotypes.

The disease has been reported in Norway, Scotland, Faroe Islands, Canada, Chile and the United States. Although there is experimental evidence that rainbow trout (*Oncorhynchus mykiss*) and sea trout (*Salmo trutta)* can act as asymptomatic carriers of the virus, Atlantic salmon is the only species known to develop the disease.

Comment [MSOffice1]: I thought disease has been seen in Coho in Chile and in the Faroe Islands?
(Nellie): the coho case reported in 1999 is unique and controversial, I would leave out.

ISA virus is shed in urine and feces resulting in fish to fish transmission of the disease. The gills appear to serve as a portal of entry for the virus. Spread of the virus has resulted from the introduction of subclinically infected smolts into cage culture sites, and from the discharge of non-disinfected fish offal and processing water in the vicinity of sea-cages. The spread and impact of the disease can be significantly reduced by restricting the movement of live fish, implementing sanitary slaughter procedures and strict sanitary

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procedures at sea-cage sites, disinfection of fish offal and processing water, and by the prompt removal and testing of mortalities.

1.3 Description of Test Method Development

The RT-PCR assay was designed to target as many isolates of ISAV as possible, based on current knowledge. ISAV RNA sequences publicly available through databases such as GenBank (National Center for Biotechnology Information) were gathered, aligned, and conserved regions of the segment 8 gene were examined carefully for the selection of primers appropriate for PCR. Segment 8 encodes a matrix protein that is expressed abundantly during virus replication and is well conserved across the various isolates of ISAV, hence constitutes a good candidate gene for the development of a sensitive assay. Several pairs of primers were tested and the pair with the best combination of sensitivity and specific amplification was retained.

As part of the optimization of the assay, the effect of homogenizing with various equipment and conditions was tested. Also, one-step vs two-step PCR assays were compared. Due to sensitivity losses, and the practicality of doing a two-step assay for the detection of multiple pathogens in the same sample, a two-step assay was chosen. Commercial reagent kits were tested, with criteria of sensitivity, and suitability with multiple targets (e.g. VHSV, IPN, etc.). Assay conditions were optimized by measuring the assay sensitivity with serial dilutions of tissue extracts, plasmids, and cell lysate extracts. Assay conditions such as primer and probe concentration, and annealing temperature were optimized.

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Robustness of the assay was partially evaluated by testing the effect of adding RNA several fold over the maximum recommended, and looking for inhibition.

1.4 Brief Description of the Validation of the qRT-PCR ISAV Assay

Several test characteristics can be measured. For the validation of ISA RT-PCR and RT-qPCR, a set of samples from populations of salmon with different apparent prevalences were used. Repeatability (within lab) and reproducibility (between lab) of the assay were measured with a set of 100 samples, with a prevalence of approximately 50%. Two external laboratories were involved for the reproducibility evaluation. A regular RT-PCR and the real-time version of this assay were compared. An additional set of 400 samples were analyzed by both methods, and by virology, to estimate the diagnostics sensitivity and specificity of the assays. A latent class model (LCM) using Maximum Likelihood estimation (TAGS program) and Bayesian approaches was used in absence of gold standard.

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1.5 Intended Use of the Protocol

This test method was developed in support of Canada's National Aquatic Animal Health Program (NAAHP). The Canadian Food Inspection Agency (CFIA) and Fisheries & Oceans Canada (DFO) are responsible for the co-delivery of the NAAHP. While CFIA has the overall responsibility for NAAHP programs and field operations, DFO provides diagnostic services, test method development, regulatory research, and expert scientific advice to its CFIA partners.

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The assay described in this protocol is primarily intended to be used as a screening assay. It can also be used as a confirmatory assay when applied to a suspect PCR result (if the confirmation is done with a second sample of the suspect animal). As well, it can be used in the preliminary characterization of virus isolates.

The protocol has been validated with Atlantic salmon infected and non-infected kidney tissues. Due to the nature of PCR, analytical assay characteristics should not vary significantly if the sample tissue differs, since most biological materials are amenable to RNA extraction. However, the Laboratory Manager should be consulted if the assay is applied to tissues other than kidney, spleen, gills, blood, brain, eye, muscle or heart. Body fluids (e.g. serum) or cell lysates are also potential samples.

Tissue preservation is very important; this should be discussed prior to sampling. Quality control measures can be used to determine the suitability of a tissue to detect ISAV. The choice of tissue to maximize the probability of detecting ISAV is important. Kidney and blood are both documented in the literature as appropriate.

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Note: The Laboratory Manager/Supervisor is responsible for ensuring that the test method and related procedures described in this protocol are performed in accordance with the International Standard: ISO/IEC 17025:2005 *General Requirements for the Competence of Testing and Calibration Laboratories*.

2.0 EQUIPMENT AND SUPPLIES

Note: Trade names used in this protocol are solely for the purpose of providing descriptive information. Mention of a trade name does not constitute a guarantee or warranty of the product by DFO or an endorsement over other products not mentioned.

2.1 Equipment

2.1.1 Real-time Thermocyclers with ROX and FAM detection (Stratagene Mx3000P, or equivalent real-time thermocycler with Peltier technology, and a ramping time of approx. 3°C/s (heating), approx. 2°C/s (cooling)).

2.1.2 Pipettors, calibrated, of appropriate volume capacity.

2.1.3 UV cabinet or clean space protected and isolated from any DNA and RNA work area.

2.1.4 Vortex

2.1.5 Mini-centrifuge

2.1.6 Liquid Handling robot (optional)

2.1.7 Freezers, -70±10°C and -20±5°C

2.1.8 Refrigerator, 4±3°C

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2.1.9 Timer (time is not critical i.e. timers do not need calibration to ISO standard).

2.1.10 Biosecurity cabinet (class II highly recommended)

2.2 Supplies

2.2.1 Filter tips with low retention, polypropylene e.g. Progene, Eppendorf, etc. The tips must be of good quality. Be careful when changing the brand; verify that the tips fit well with the pipettors.

2.2.2 PCR tubes, either 0.2 mL, strips of tubes and covers, or PCR plates with plastic film, aluminum seal or plastic cover (optically clear). Some real-time thermocyclers require specific types of plates. A plate sealer may be necessary to use with some qPCR plates.

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2.2.3 Rack for tubes, cooled racks for PCR tubes.

2.2.4 Permanent markers, fine tip

2.2.5 Powder free disposable gloves (e.g. nitrile)

3.0 REAGENTS

3.1 Chemicals

Notes: For all chemicals refer to the appropriate Material Safety Data Sheets (MSDS).

If chemicals or reagents other than those listed herein are to be used (either commercial or prepared in-house), it is the responsibility of the Laboratory Manager/Supervisor to ensure equivalency.

3.1.1 Cleaning supplies appropriate for the type of work (e.g. Viralex, ~ 0.525% bleach, ~ 70% ethanol, 1% Sparkleen, 1% Tergazyme, 1% Alconox, etc.).

3.1.2 TaqMan Universal PCR master mix kit from ABI, cat. # 4318157 (if using other supplier, the method may need to be validated again, in full or partially).

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3.1.3 Primers and probe specific to the assay (order primers and probes from a reputable supplier e.g. Invitrogen, Sigma Genosys, ABI) – see section 5.1.2 for primers and probes.

3.1.4 DMPC (dimethyl pyrocarbonate) or DEPC (diethyl pyrocarbonate) H_2O, or molecular grade water, or sterile type II H_2O.

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3.2 Biologicals

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3.2.1 cDNA prepared from standard RNA extracts during the cDNA step (see SOP GFC-DIA-MBU-18 *Reverse Transcription using High Capacity cDNA kit with RNA denaturation* or equivalent site-specific SOP). It is highly desirable to avoid manipulating a true pathogen and increase the risk of cross-contamination. Whenever possible, a positive control that is reacting to the assay like a true sample but that can be distinguished from the true pathogen is used. For ISAV, we use RNA transcripts produced from plasmids, with an insert of 26bp. They are introduced in the Reverse transcription step.

If there is no other choice than to use true pathogen material for the test, the RNA extracts to use as positive controls are prepared from tissue homogenates from an infected animal. Two positive RNA samples; one classified as low (e.g. 10^{-4} standard, or an RNA extract giving a Ct in the middle 30's) and high (e.g. 10^{-2} standard, or an RNA extract giving a Ct in the high 20's). For more details, see the lab policy on positive controls (GFC-DIA-MBU-55 *Preparation and storage of controls for PCR-type assays*) or equivalent site-specific SOP).

3.2.2 Standards to include in the assay may vary, e.g. a positive tissue sample can be included at the RNA extraction step. Negative controls (blank, or water) should be included at all steps, and positive control needs to be included in some way, so it can be demonstrated that all steps of the process (extraction, RT, PCR) are working, and a positive result is not obtained by accidental contamination with RNA or DNA. A reference gene (housekeeping gene) can be amplified for QA/QC purposes, see SOP GFC-DIA-MBU-15 Reference Gene Assays or equivalent laboratory specific SOP.

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4.0 PREPARATION FOR THE TEST

4.1 Preparation of Laboratory Personnel

4.1.1 It is the responsibility of the Supervisor/Manager to ensure each analyst has:

- read, understood and agreed to abide by the contents of this protocol
- successfully completed training on all aspects of the protocol including sample reception and tracking
- successfully completed training on all pertinent instrumentation and SOPs related to this protocol
- passed all pertinent proficiency panels (as required)
- completed Workplace Hazard Management Information System (WHMIS) training and has been instructed on all hazards associated with the test (biological and chemical) and must be familiar with the pertinent MSDSs located in the laboratory
- documented all the training done.

4.2 Preparation of Equipment

4.2.1 Refer to lab specific SOPs (Appendix 2) for preparation of equipment used in this protocol. It is the responsibility of each laboratory to ensure that all equipment used

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are properly calibrated (if required), stabilized and maintained within specified requirements.

4.2.2 The PCR workstation in the clean area is decontaminated before use. Follow the SOP on PCR workstation. If using a laminar flow hood or another type of enclosure with UV, follow the appropriate SOP.

Before and after each use, wipe the interior space with ~ 70% ethanol and 1% Sparkleen (or appropriate disinfectant). Wipe dry with a paper towel. Whenever necessary, all components may be washed gently with water and a non-abrasive detergent, and rinsed and dried with a paper towel.

4.2.3 The Stratagene Mx3000P real-time thermocycler is turned on 20 minutes prior to running the machine to make sure the lamp is warm before adding the samples to the machine. If using another thermocycler, refer to the appropriate SOP (Appendix 2).

4.3 Preparation of Reagents

4.3.1 H_2O used for the preparation of PCR mixes is aliquoted from sterile type II H_2O in the clean area. Aliquots are for single use.

4.3.2 Primers and probes are aliquoted in the clean area, as described in the SOP on primers (Appendix 2).

4.3.4 TaqMan Universal PCR master mix kit from ABI is prepared in the clean area, as described in the SOP on Molecular Biology practices (Appendix 2).

4.4 Preparation of Test Sample

Note: All submissions to the laboratory for testing must arrive in good condition. Submissions must have been collected as per the CFIA standard operating procedures for field collection and be packed in a manner such that they remain cold but not frozen during shipment, e.g. on wet ice or with ice packs. Necropsy is done in another area according to SOP. Samples for PCR are usually preserved in RNAlater according to the SOP on necropsy. SOP on RNA extraction provides information on the type of sample, etc. This protocol describes the RT-qPCR assay only.

Note: Continuity of the identification of the original specimen and all subsequent samples and aliquots from the time of arrival in the laboratory to the time of reporting of the test results is of critical importance. Specimens arriving at the laboratory must be accompanied by an Official Submission Form. The laboratory will assign a unique laboratory case number to each submission which is written on the Official Submission Form, tubes, worksheets etc. maintaining full traceability from the time of arrival to disposal.

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Comment [D3]: Would freezing affect results of PCR test?

Comment [NG4]: (Nellie) I would simplify this note to simply say that collection and submission of sample are done according to established SOP

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4.4.1 RNA extraction is done according the GFC-DIA-MBU-13 (*RNA extraction with TRI or TRI-LS reagent*) or comparable SOP.

4.4.2 The RNA is normalized according to GFC-DIA-MBU-54 (*RNA or DNA normalization*) or comparable SOP.

4.4.3 Reverse transcription is described in GFC-DIA-MBU-18 (*Reverse Transcription using High Capacity cDNA kit with RNA denaturation* or equivalent). A maximum of 1000ng of RNA is used for reverse transcription. For example, with 5ul of RNA normalized at 200 ng/ul, the amount of RNA used is 1000ng. RT reactions are diluted 50:50 with H_2O before PCR, so the final concentration is approximately a maximum of 1000ng/ 40ul or 25ng/ul.

4.4.4 qPCR is done using 2ul of the cDNA, which is approximately 50ng of template.

4.4.5 Once a RT-qPCR protocol is validated, the accompanying RNA extraction SOP and reverse transcription SOP are considered a crucial part of the protocol. It is possible to use a different RNA extraction SOP, i.e. a different RNA extraction procedure such as column-based methods versus phase separation methods. However, the laboratory must demonstrate their proficiency using this SOP and the RT-qPCR protocol before proceeding with further analysis. The reverse transcription SOP should be used as described. Note that a cDNA extract can be used for different PCR assays. It is therefore usual to use a common RNA extraction, and reverse transcription SOP, followed by specific PCR assays to detect the desired target / pathogen.

4.4.6 Samples for this protocol are cDNA (prepared from total RNA).

5.0 PERFORMANCE OF THE TEST

Note: Specific laboratory configurations may vary between laboratories. A critical requirement for qPCR work is to have the following areas: (1) a main area (or main lab), where samples are processed and RNA and DNA are extracted; (2) a clean area, where no DNA or RNA containing materials are allowed, for reagents and mix preparations; (3) an inoculation area, where the qPCR mixes are inoculated with nucleic acids; and (4) a PCR area where amplification is done.

5.1 Preparation of Sample Worksheet

5.1.1 For a set of qPCR reactions, prepare a worksheet with all the necessary details (see Appendix 1 for an example, and the SOP on Molecular Laboratory Practices). If necessary, adjust volumes depending on stock concentrations.

5.1.2 For the amplification of ISAV, the specific details for the worksheet are:

- Name of the assay: RT-qPCR ISAV 404F / RA3
- Expected product length: 179 bp (for information)
- Mix to prepare for one sample (multiply by the number of tubes needed):

8.8 ul sterile H_2O (or to make up to 25 ul)
12.5 ul 2x TaqMan Universal PCR master mix

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0.6 ul of primer F (20 uM stock), 5' tgg gca atg gtg tat ggt atg a
0.6 ul of primer R (20 uM stock), 5' gaa gtc gat gaa ctg cag cga
0.5 ul of probe (10 uM stock), 491_ISA8-FamMGB:
(6-Fam) cag gat gca gat gta tgc-MGB
2 ul of cDNA (maximum of ~25ng/ul, see 4.4.3)
Total: 25 ul

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5.2 Preparation of the Master Mix

5.2.1 If using an automated liquid handling system, refer to the SOP of this equipment. The master mix and aliquoting in qPCR plates will be handled by the liquid handling system. Vortex each reagent (thaw if necessary), and follow the SOP of the liquid handling system.

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5.2.2 The master mix is prepared in the clean area. A clean lab coat should be assigned for clean work only.

5.2.3 If using a PCR workstation (or laminar flow hood), follow the SOP of the equipment.

5.2.4 Wipe the interior space with ~ 70% ethanol or appropriate disinfectant. Wipe dry with a paper towel.

5.2.5 Prepare your PCR tubes (strip, individual, plate), place them on a clean cold rack, label appropriately (i.e. the top with numbers and label tube number 1 with the virus being tested).

5.2.6 Get all reagents from the freezer and place them on a cold rack:

- 2X Taq Man Universal Enzyme
- Primer F
- Primer R
- Probe

5.2.7 Note the reagent lot number, stock identification of primers and probe, expiry dates and pipette numbers (to update the worksheet).

5.2.8 In a clean ~1.5 mL microtube (or 5.0ml tube if using liquid handling robot), on the cold rack, prepare the master mix by pipetting the quantities of reagents as calculated initially. Start with H_2O (if appropriate) and TaqMan Universal Enzyme, and finish with the probe. Work cautiously, avoiding movements over open tubes, etc.

5.2.8 When the master mix is ready, vortex briefly and distribute in the qPCR tubes or wells or follow Liquid Handling Robot SOP. Close the tubes or cover the plate.

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5.2.9 Store reagents, leave the clean lab coat in the clean area, clean the workstation as indicated in Section 4.2, and bring the rack of qPCR mixes, and the aliquots of water (you will use it as your negative control) to the inoculation area.

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5.2.10 If using a liquid handling robot, bring the master mix to the main lab and let the robot aliquot the master mix in the selected wells.

5.3 Addition of Templates

5.3.1 In the inoculation area, transfer the qPCR tubes on a cold rack, or over ice, depending on your setup.

5.3.2 If using a liquid handling robot, open the qPCR protocol on the computer, put the qPCR master mix tube in the robot and let it distribute the master mix in the plate.

5.3.3 Get the cDNA extracts from the fridge or freezer (let them thaw in the inoculation zone).

5.3.4 Use clean pipettors to work with the cDNA extracts (see SOP on general PCR laboratory practices). A dedicated pipette is used for this.

Note: Cross contamination and false positives can occur at the inoculation step. It is important to observe the lab policy on avoidance of contamination sources. Avoid air circulation during inoculation, and if using a laminar flow hood, turn off the air flow, as it will contribute to aerosol formation.

5.3.5 Mix well and spin down each tube before pipetting, work carefully, and inoculate the PCR mixes with cDNA in the following order:

1) samples
2) two RNA positive controls (low and high)
3) negative control

5.3.6 Cap each tube after adding the template (if possible). If working with qPCR plates, use a plate sealing compressing mat and press each well individually to make sure it is well sealed. If using a film, it is recommended to use a heat sealer to make sure the plate is properly sealed.

5.3.7 Securely holding the rack of tubes or the qPCR plate, vortex lightly and then spin down in a mini centrifuge or tap it on the counter a few times to collect any drops from the sides.

5.3.8 Return the nucleic acid extracts to storage. Discard the tube of H_2O. Leave your lab coat in this area.

5.4 qPCR Amplification of qPCR Mixes

5.4.1 Bring the rack of tubes to the qPCR area.

Note: The PCR area is considered a contaminated zone. Do not take anything out of this room. If you need to put something on a counter, it has to remain there or it will need to be decontaminated before it can re-enter the main lab. Hold racks of tubes in your hands if you want to bring them back. This rack will be

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decontaminated with bleach immediately afterward as a precaution, but not brought to the clean area. Hands are washed before leaving this area.

5.4.2 In the PCR area, open the lid of a pre-warmed real-time thermocycler, and put the tubes or plate in place, making sure that the tubes or plate are well closed.

5.4.3 Close the real-time thermocycler cover, and start the run. Note: a qPCR run can be started at the end of the day.

5.4.4 Verify the program details, see the SOP on Mx3000 Q-PCR system for more details. For ISAV 404F / RA3:

(Program name: ANN60Elongation7240cycles)
Quantitative PCR (Multiple Standards)

Hold (initial): 50°C 2 min, then at 95°C 10 min
Cycles: 40 cycles of 95°C, 30 s // 60°C, 30s // 72°C, 30s
Fluorescence read at end of each cycle.

5.4.5 Click on the start run button on the computer screen to start the run, you may be prompted to save the run, if it was not saved when the machine was first turned on previously (see SOP on real-time thermocyclers).

5.4.6 Wash hands, leave lab coat in PCR room, take the rack back with you for decontamination.

5.5 Analysis and Documentation of Results

5.5.1 When the qPCR run is complete, proceed with saving the run of the computer.

5.5.2 Turn off the real-time thermocycler and turn off the real-time software.

5.5.3 Wash your hands before leaving the area.

5.5.4 PCR plates and tubes are discarded after the run is completed and preliminary results have been viewed. If additional analyses are needed, such as sequencing, RFLP, etc, samples can be saved.

5.5.5 To analyse samples, follow the SOP on *Real-time PCR analysis*, GFC-DIA-MBU-21 or equivalent (Appendix 2). Report Ct in the PCR worksheet, and report control values in the appropriate databases.

6.0 INTERPRETATION OF THE TEST

6.1 Interpretation of the RT-qPCR Assay

6.1.1 Check the results of the RT-qPCR assay:

6.1.1.1 PCR positive controls (cDNA introduced at the PCR step): low positive control should show a Ct similar between runs (in the 30's), and the high

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Comment [NG7]: Not sure if we should specify the Mx3000 details here (remove moving avg etc). Some place might use other types of machine.

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positive control should show a Ct around the mid 20's, or as per characterized material used for controls and determined through trend analysis. The difference in Ct between the high and low control should be consistent. Control values are captured and monitored closely for obvious signs of problems with the assay. For more details, see SOP on preparation and storage of controls for PCR-type assays.

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6.1.1.2 PCR negative control: should be clean. If showing a Ct value, there is a contamination in the qPCR mixture and all positive results are invalid. This is not normal and the problem should be solved before any other test is performed with samples.

6.1.1.3 RNA positive controls (RNA low and high standard introduced at the cDNA step): should yield values similar to the PCR positive controls.

6.1.1.4 RNA negative control (water introduced at the cDNA step): idem PCR negative control. If showing a Ct value, but the PCR negative control is clean, there is a contamination in the cDNA mixture and positive result in samples are invalid. This should be resolved before any other test is performed.

6.1.1.5 Extraction blank: they should be negative. A Ct value in these samples can mean that contamination occurred at the extraction step (especially if the RNA and PCR negative controls are negative). A Ct value is not normal and the problem should be solved before performing another assay with samples.

6.1.1.6 Samples: check for Ct values in the samples.

Note: If all controls performed correctly, the results are considered valid, and results are entered in the RT-qPCR worksheet (or according to the lab policy). When all the work is completed on the case submission, the results are also summarized in the sample database, or according to the lab policy.

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- **If one of the controls appears to be erroneous, or in case of doubt e.g. strange curve and fluorescence during the RT-qPCR, the Technical Manager is notified immediately and results are not reported until a decision is made. Note that due to the numerous levels of controls, a case by case decision should be made. These events are recorded in a database for future reference, and a corrective action is initiated if necessary.**

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6.2 Presumptive positive Results, Confirmatory Assays

Note: The choice of method for confirmation is laboratory dependent and may include virus isolation or additional PCR testing, possibly with other primers, and preferably starting from the original sample (e.g. a backup piece of tissue, or a homogenate).

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6.2.1 If a viral agent is presumptively identified, it must be confirmed by the appropriate DFO Reference Laboratory under the following circumstances:

a) the first report of disease/pathogen occurrence or re-occurrence in Canada,
b) the first report of an endemic disease/pathogen in an area previously considered to be free,
c) the first report of an endemic disease/pathogen in a new host species, or
d) the first report of a new strain or new disease manifestation of an endemic pathogen.

If none of the above circumstances apply, it is at the discretion of the laboratory to confirm the positive on site or send it to the Reference Laboratory.

Note: Minimally 1-5 (maximum) presumptive positive samples per population/case should be confirmed (depending on the size and type of population).

6.2.2 An aliquot of the RNA and the corresponding original tissue/homogenate sample must be sent to the Reference Laboratory for confirmation and characterization purposes.

6.2.3 Methods of confirming the presumptive identification are determined by the Reference Laboratory following TMA guidelines.

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6.2.4 If a presumptive positive result is not confirmed, or if only weak and one of two duplicates give a positive result, the decision to call the sample will be taken based on a decision tree (see ……

Comment [NG8]: We should decide on when something is not confirmed. We have several options for confirmation depending on tests. Should the ultimate confirmation be the possibility to sequence or not a fragment of the pathogen?

7.0 RETENTION OF SAMPLES

7.1 cDNA extract are kept according to the sample retention policy. For cDNA, they are stored at 4 ±3°C for a few days and at - 20 ±5°C after the analysis is completed. They are transferred for long term storage in the ultralow freezer -80±10°C during the semi-annual freezer clean-up.

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Comment [NG9]: That's local policy, should be reworded.

7.2 Samples (backup tissue in RNAlater) testing negative remain at -20±5 °C for a minimum of 6 months following the issuance of a test report. After this time, the negative samples can be discarded.

7.3 Samples (backup tissue in RNAlater) testing positive should be stored in a designated freezer at -70±10 °C for a minimum of 3 years following the issuance of a test report.

Comment [NG10]: To check

7.4 All specimens and test samples (tissues, homogenates, filtrates and cell culture supernatants) must be decontaminated prior to disposal in accordance with bio-containment standards.

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8.0 REFERENCES

Literature cited:

8.1 Byrne, P. J., MacPhee D.D., Ostland V.E., Johnson G. & Ferguson H.W. (1998). Hemorrhagic kidney syndrome of Atlantic salmon, *Salmo salar* L. *Jounal of Fish Diseases*, **21**, 81-91.

8.2 OIE 2007. Office International des Epizooties, Aquatic Animal Health Code. Tenth Edition. Chapter 1.1.1., 3-17.

8.3 OIE 2006. Office International des Epizooties, Manual of Diagnostic Tests for Aquatic Animals. Fifth Edition. Chapter 2.1., 67-70, 82-104.

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APPENDIX 1

Example of a RT-qPCR Worksheet

GFC-MBU- SOP # MBU-xx-000-00 PCR ISAV 404F / RA3, **179 bp**

404F: 5' tgg gca atg gtg tat ggt atg a — RA3 (583R): 5' gaa gtc gat gaa ctg cag cga

491_ISA8-FamMGB (6-Fam) cag gat gca gat gta tgc-MGB

DATE (YY-mm-dd):	
Technician:	Verified By:

Enter tubes tot (incl. neg and pos ctrls):		154
	Master MIX x ul	Pipette Vol (ul)
sterile H2O	8.8	1373 GFC-MBU-196
2X Taq Man Universal Enzyme Mix	12.5	1950 GFC-MBU-108
Primer F (20uM)	0.6	93.6 GFC-MBU-051
Primer R (20uM)	0.6	93.6 GFC-MBU-103
Probe (10uM)	0.50	78.0 GFC-MBU-188
cDNA (≤25 ng/ul)	2	GFC-MBU-256
total	25	

Corbett Robotics	
Plaque:	

PcR prog name:	TaqMan-FAM-ANN60-40c
PcR machine used:	*GFC-MBU-*
Program:	Quantitative PCR (Multiple Standards)

	Hold init.	40 cycles at	
	50 C	95 C, 30s	
	2 min	60 C, 30s	
	95 C	72 C, 30s	Fluorescence read at end (read 3 times
	10 min		

2X Taq Man Universal Enzyme Mix
2X Taq Man Universal Enzyme Mix lot no
Probe ISAV (10 uM)
H2O dMPC
Primer F stock id:
Primer R stock id:

Reason for testing:

baseline:

Plate ID

NO	PCR no	case no, ctrl pos or H20	Result (Ct)	Note	NO	PCR no	case no, ctrl pos or H20	Result (Ct)
1 - A1					25 - A2			
2 - B1					26 - B2			
3 - C1					27 - C2			
4 - D1					28 - D2			
5 - E1					29 - E2			
6 - F1					30 - F2			
7 - G1					31 - G2			
8 - H1					32 - H2			
9 - A3					33 - A4			
10 - B3					34 - B4			
11 - C3					35 - C4			
12 - D3					36 - D4			
13 - E3					37 - E4			
14 - F3					38 - F4			
15 - G3					39 - G4			
16 - H3					40 - H4			
17 - A5					41 - A6			
18 - B5					42 - B6			
19 - C5					43 - C6			
20 - D5					44 - D6			
21 - E5					45 - E6			
22 - F5					46 - F6			
23 - G5					47 - G6			
24 - H5					48 - H6			

Comment [FaOC11]: The worksheet just says PCR ISAV, shouldn't it say qRT-PCR or RT-QPCR instead? Would need to make the changes on the worksheet model. Same thing for PcR prog name, should changed it for qRT-PCR or RT-qPCR.

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APPENDIX 2

NAAHLS Supporting Standard Operating Procedures (SOPs):

Gulf Fisheries Centre, Moncton, New Brunswick

- GFC-GEN-9 General molecular laboratory practices
- GFC-DIA-57 Preparation of Reagents and Media
- GFC-DIA-FHU-8 Necropsy of Finfish Samples
- GFC-DIA-MBU-13 RNA extraction with TRI or TRI-LS reagent
- GFC-DIA-MBU-18 Reverse Transcription using High Capacity cDNA kit with RNA denaturation
- GFC-DIA-MBU-54 RNA or DNA normalization
- GFC-DIA-MBU-55 Preparation and storage of controls for PCR-type assays
- GFC-DIA-MBU-19 Primers and probes design and usage
- GFC-DIA-MBU-15 Reference Gene Assays
- GFC-EQ-42 Mx3000P QPCR system
- GFC-DIA-MBU-21 Real-time PCR analysis
- GFC-EQ-32 CAS-1200N Robotic Liquid Handling System
- GFC-EQ-24 Qiagility Robotic Liquid Handling System
- GFC-EQ-28 PCR Workstation
-

Pacific Biological Station, Nanaimo, British Columbia

Freshwater Institute, Winnipeg, Manitoba

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