# Validation Pathway for NAAHLS Diagnostic Test Methods*

# Molecular Analysis for Infectious Salmon Anemia Virus

* NOTE on TERMINOLOGY: The nouns **Test, Assay**, and **Test Method** are used synonymously in this document. Subtle or preferred distinctions between these terms are not implied nor should they be assumed. These terms refer to the principles, systematic procedures, and processes required for detection of an analyte.

## Advisory Note:

Before embarking on the validation pathway described herein, it is advised that NAAHLS scientific and technical staff read the current introductory chapter found in either web version of the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* or the OIE *Manual of Diagnostic Tests for Aquatic Animals*:

1) Principles of validation of diagnostic assays for infectious diseases, Chpt. 1.1.4/5 (*Terrestrial Manual*) or Chpt. 1.1.2 (*Aquatic Manual*)

Either of these chapters may be downloaded from the website (www.oie.int) under the heading *OIE Expertise – Specialist Commissions* and should be used as companion guidelines for this validation pathway.

# Table of Contents

# Section 1. Guide for applicants

## 1.1. General information

The first version of the DFO dossier was based on the original template found in the 2006 version of the Standard Operating Procedure (SOP) for OIE Validation and Certification of Diagnostic Assays. The OIE template is currently undergoing revision and this next DFO version incorporates the modifications to the original OIE version which are yet to be unpublished.

A series of specific guidelines to support the new OIE template are also currently under development. Once finalized, they too will be appended to the DFO version as appropriate.

As with the previous DFO version, the new DFO version reflects minor modifications to the new OIE template validation of tests used by the National Aquatic Animal Health Laboratory System (NAAHLS). The original validation principles have not been altered.

## 1.2. User guide on filling in this form

1. In some fields where you are required to select one or more options, double click on a box to switch the option on or off (select “Checked” to answer yes).

2. Type or paste your information inside the yellow box under each field. To keep the yellow background, apply the “Body Text” style for the text (*press Ctrl-Shift-B*). For text consistency, use only “Time New Roman” font, size 10-11 points for normal text.

3. The “Table of Contents” is generated automatically. To update the “Table of Content”, place the cursor on the Table of Content area, press F9, select “Update entire table”.

# Section 2. General information

## 2.1. Information about the laboratory

### 2.1.1. Name of the laboratory responsible for the dossier

| | |
|---|---|
| Laboratory | Molecular Biology Unit, Aquatic Animal Health Section, Gulf Region |
| Organization | Department of Fisheries and Oceans Canada |
| Address | Gulf Fisheries Center, 343 Université Avenue, Moncton , NB, E1C 9B6 |

### 2.1.2. Type of organisation

*Double click on a check box to switch the option on or off (to answer Yes, select "Checked").*

| | | | |
|---|---|---|---|
| ☒ Federal | ☐ Provincial | ☐ Institutional | ☐ Commercial |
| ☐ Other: (specify) | | | |

### 2.1.3. Name and contact details of the person responsible for this validation dossier

| | |
|---|---|
| Contact person | Nellie Gagné |
| Job title | Research Scientist |
| Laboratory *(If different from 2.1.1)* | |
| Organization *(If different from 2.1.1)* | |
| Address *(If different from 2.1.1)* | |
| Phone | (506) 851-7478 |
| Fax | (506) 851-2079 |
| E-mail | Nellie.gagne@dfo-mpo.gc.ca |

### 2.1.4. Accreditation or certification status of laboratory

*Double click on a check box to switch the option on or off (to answer Yes, select "Checked").*

| | | |
|---|---|---|
| ☐ OIE Quality Standard | ☐ ISO/IEC 17025 | ☐ ISO/IEC 9000 series |
| ☐ Other: (Specify) | Preparation for ISO 17025 started in 2005, no accreditation as of November 2011. Tentative date for accreditation: early 2017. | |

## 2.2. Name and purpose of the diagnostic test

### 2.2.1. Type of method

*Indirect or competitive ELISA, conventional or real-time PCR, etc.*

Real-Time RT-PCR (RT-qPCR)

### 2.2.2. Commercial name *(if applicable)*

n.a.

### 2.2.3. Intended purpose(s) of the test

*Check the specific purpose(s) of the test from the list below.*
***Note**: Specific details on the intended use of the test will be prompted in Section 3.1. below.*

*Double click on a check box to switch the option on or off (to answer Yes, select "Checked").*

| | | **Purpose or Application** |
|---|---|---|
| | | **National Aquatic Animal Health Program (CFIA/DFO)** |
| ☒ | 1 | Health profile (Facility recognition) |
| ☒ | 2 | Export |
| ☒ | 3 | Import |
| ☒ | 4 | Morbidity/mortality investigation |
| ☒ | 5 | Survey |
| ☒ | 6 | Surveillance |
| ☒ | 7 | Laboratory referral (confirmatory) |
| ☐ | 8 | Other: (specify) |
| | | |
| | | **Aquatic Animal Health Science (DFO)** |
| ☒ | 1 | Health profile (FHPR) |
| ☒ | 2 | Export |
| ☒ | 3 | Domestic (I&T) |
| ☒ | 4 | Morbidity/mortality investigation |
| ☒ | 5 | Survey |
| ☒ | 6 | Surveillance |
| ☒ | 7 | Laboratory referral (confirmatory) |
| ☐ | 8 | Other: (specify) |

## 2.3. Test description and requirements

### 2.3.1. Protocol of the test

*Reference current version of test method protocol. Include the full title and current version number below.*

Document Number: NAT-PROT-qRT-PCR-ISAV-1
Document Title: qRT-PCR Diagnostic with TaqMan Universal PCR Master Mix for the Detection of Nucleic Acids from Infectious Salmon Anemia
Revision 1
Submission Date: 2011-02-18 14:37

### 2.3.2. Disease/analyte target

*State infectious agent and analytical targets (e.g. name of viral/bacterial/parasitic pathogen, strains, lineages etc.; whole organism, antibody, antigen, gene targets, etc.)*

Virus: Infectious salmon anemia
Target: portion of the viral RNA segment 8 *
Strains targeted: all known strains based on available segment 8 sequences (65, as of October, 2011)

* Note: ISAV possesses 8 RNA segments. Segment 8 codes for a structural protein.

### 2.3.3. Species and specimens

*Species and specimens that can be examined (e.g. salmonids, oyster, lobster, etc.; whole animal, kidney, reproductive fluids, gill, etc.). List only those that have been validated sufficiently.*

Validation was done on head region of kidney samples.

Literature reports that ISAV is mainly detected in heart, kidney, blood (Giray et al 2005).

A non-virulent form of ISAV, herein referred as **HPR0,** is supposed to cause a transient infection and is detected preferentially in gills ( Christiansen et al 2011).

### 2.3.4. Sampling procedures

*Describe briefly the recommended procedures for acquiring, preserving and shipping specimens for the test. The specimen collection protocol may be referenced or appended in Section 5 but include the full title and current version number below.*

Specimens can be whole fish; healthy, moribund or fresh mortalities. If necropsy is not done in our facility, and not done by trained and authorized persons, samples can be analyzed with a disclaimer regarding chain of custody and quality of necropsy.

Samples must be collected in a manner that preserves sample integrity. If collected from different facilites, specimens or samples must be placed in separate coolers, etc.

Fish must be necropsied within 48-72 h of collection i.e. tissue must be ready for cell culture or preserved for RT-qPCR within 72h of sampling the fish, and all assay procedures should be initiated within this time, therefore whole fish samples must be delivered to the laboratory allowing sufficient time for the necropsy to be performed within the time limit.

Necropsy is done in a manner that prevents cross contamination between cases. Within a case, precautions are taken to ensure that fish-to-fish contaminations are avoided.

Measures must be followed to avoid contamination between lots; individual lots of fish are collected separately in properly labeled plastic bags. Particular care is attributed to fish of small size in order to avoid them being crushed in transit. See the necropsy procedure for full details.

Shipping procedures require sufficient amount of ice in coolers, and proper disinfection and packaging.

Refer to these SOPs:

| | | | |
|---|---|---|---|
| GFC-DIA-FHU-8 | Necropsy of Finfish Samples | v2 | 2011-10-24 |
| GFC-GEN-23 | Collection of finfish samples- FHPR | v1 | 2009-11-17 |

### 2.3.5. Controls included

*If not fully described in the test method protocol, describe positive and negative control materials used in the test, including source, biosafety/biosecurity considerations and test activity.*

Details are provided as guidelines in the protocol.
In summary, the current controls to use in the detection of ISAV by RT-qPCR are:

Negative extraction controls : blank tubes added every 15 samples and/or after the last sample of each extraction batch.
Negative RT control: a water sample introduced in the Reverse Transcription.

Low Positive RT control: an RNA transcript diluted to produce a product with a high Ct range (approx 30)*
High Positive RT control: an RNA transcript diluted to produce a product with a low Ct range (approx 24)*

Negative PCR control: water in the qPCR run.
Low Positive PCR control: a cDNA (from a prior run) that is producing a high Ct (approx 30), added at the qPCR step.
High Positive PCR control: a cDNA (from a prior run) that is producing a low Ct (approx 24), added at the qPCR step.

*The RNA transcript is produced by *in vitro* transcription from a plasmid, and is distinct from true ISA as it contains an insert of 26bp in its sequence. It is transcribed into cDNA, and detected by qPCR assay.
*These RNA controls were adopted in the DFO laboratory in August 2011. Prior to this date, we were using low and high controls made from standard homogenized tissues, i.e. positive kidney serially diluted in negative kidney.

#### 2.3.6. Laboratory requirements

*If not specified in the test method protocol, describe minimum laboratory requirements for optimal test performance; include environmental, equipment, chemical and/or biological requisites.*

Details are in the protocol.

#### 2.3.7. General precautions/ safety aspects/ disposal of reagents

*List potential health hazards and the safety precautions; refer to Material or Biological Safety Data Sheets if necessary.*

All associated SOPs linked to the protocol refer to the safety precautions surrounding the use of some chemicals and equipment.

# Section 3. Development and Validation of the Assay

## 3.1. Assay Development Pathway

#### 3.1.1 Fitness of assay for its intended purpose

*The design of the test must be consistent with its intended purpose and the population for which it is intended. Please refer to the discussion of this topic in Chapter 1.1.4. for an overview of 'reasons for test'. Give a brief description of how you see the test being applied in support of a specific type(s) of testing programmes. Test applications may be relatively broad or highly specific depending on the diagnostic application being targeted.*

This assay was developed to detect the nucleic acids of all possible strains of ISAV. As PCR assays are known to be sensitive and specific, this assay is fit for various purposes:

- Screening in carrier and diseased fish: this assay has the sensitivity needed for carrier detection
- Surveillance, survey
- Confirmation of CPE (cytopathic effect) or cell culture supernatant
- Detection of ISAV in samples other than fish tissues, e.g. water filtrate. Although the assay was not validated with such types of samples, PCR can be applied to many matrices with an appropriate disclaimer.

The detection of ISAV by RT-qPCR does not imply that a live virus is present in the sample, so positive results cannot be interpreted as presence of infection, and the final diagnosis depends on additional testing, and case history.

**Note: Design, development, optimization and standardization of the assay**

*Assay design, development, optimization and standardization, as well as, validation must be based on sound scientific principles and carried out using best practices, leading to a validated assay that is publishable in peer reviewed journals. Reviewers, as part of the dossier review process, may ask for documents on, for instance, the statistical methods and conclusions reached in drawing*

*inferences relative to reagent optimization/standardization or result interpretations (any factors which may affect data acceptance and interpretation of the test result) – or any other data deemed essential to drawing conclusions about the validity of the test.*

## 3.2.Validation Pathway Stage 1 - Analytical characteristics

### 3.2.1. Stage 1. Repeatability data

*Repeatability is level of agreement between replicates of a sample both within and between runs of the same test method in a given laboratory.*

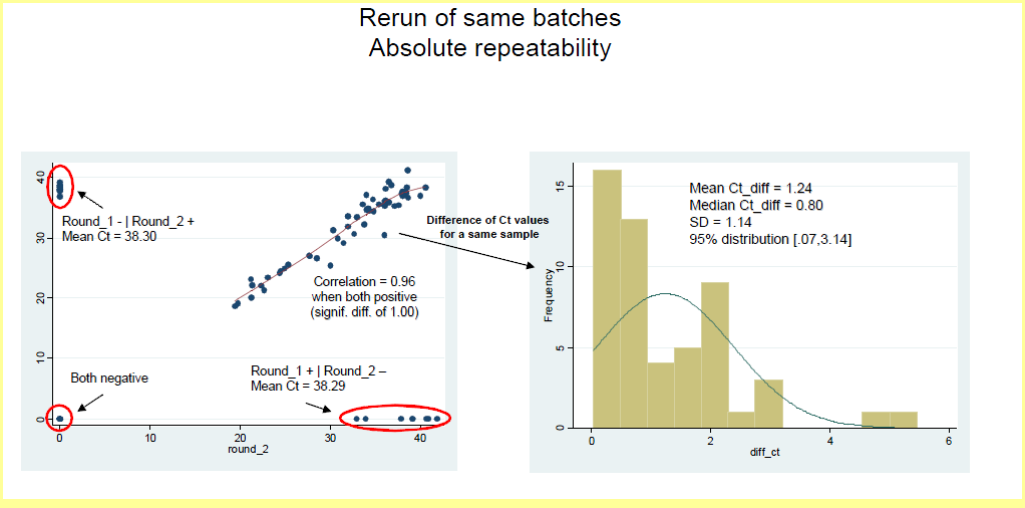
**Within run repeatability**:
qPCR reactions are usually done in duplicate, i.e. two repeated wells for one sample. The repeatability for wells is high for samples up to ~ 35 Ct. Between 35 and 40 Ct (end of cycling), the repeatability of duplicate wells decreases.
We have various data regarding within run repeatability. Over the years, we have accumulated the duplicate values of the positive controls. They are run as duplicates, and in several runs, so a trend analysis can be done. For the year of 2009, and for a single batch of controls ($10^{-2}$ and $10^{-4}$), we obtained the following values, after averaging approximately 35 runs:

| test | ctrl | AVG | STDev |
|---|---|---|---|
| ISAV | 10-2 | 26,15 | 1,38 |
| | 10-4 | 33,10 | 1,04 |

**Between runs repeatability**:
During Stage 2 validation, 100 samples were provided in duplicate sets (200 samples of 100 animals), and processed blindly in our laboratory. The samples were coming from different populations with different prevalences: moribund and fresh mortalities from infected cages, healthy fish from infected cages, or healthy fish from non-infected cages.

We obtained the following repeatability for the end-point PCR ISAv assay (this assay is a one-step version, end-point PCR, using the same primers as in the real-time version of the assay) (ref Caraguel et al 2009): the observed agreement for 100 kidney tissues was 0.84 (C.I. 0.75-0.90); using another set of the same tissues but provided as homogenates, the observed agreement decreased to 0.80 (C.I. 0.71-0.87) (C.I. = confidence interval). The prevalence of ISA in the set of samples was close to 50%, which was ideal, but also increased the cross-contamination pressure during testing. Similar values were obtained in two other laboratories that participated in the validation. As was explained in the report, the samples that did not repeat were usually found to be lightly infected.

Using the real-time version of the ISAV assay, we analyzed the effect on repeatability versus the amount of target in the samples. As can be seen in the figure below, when samples are lightly infected, the repeatability decreases. On average, at Ct 38, samples are less likely to repeat, and the difference in Ct values for samples that are repeatedly detected will increase.



### 3.2.2. Stage 1. Analytical specificity data (as appropriate for the test type and disease)

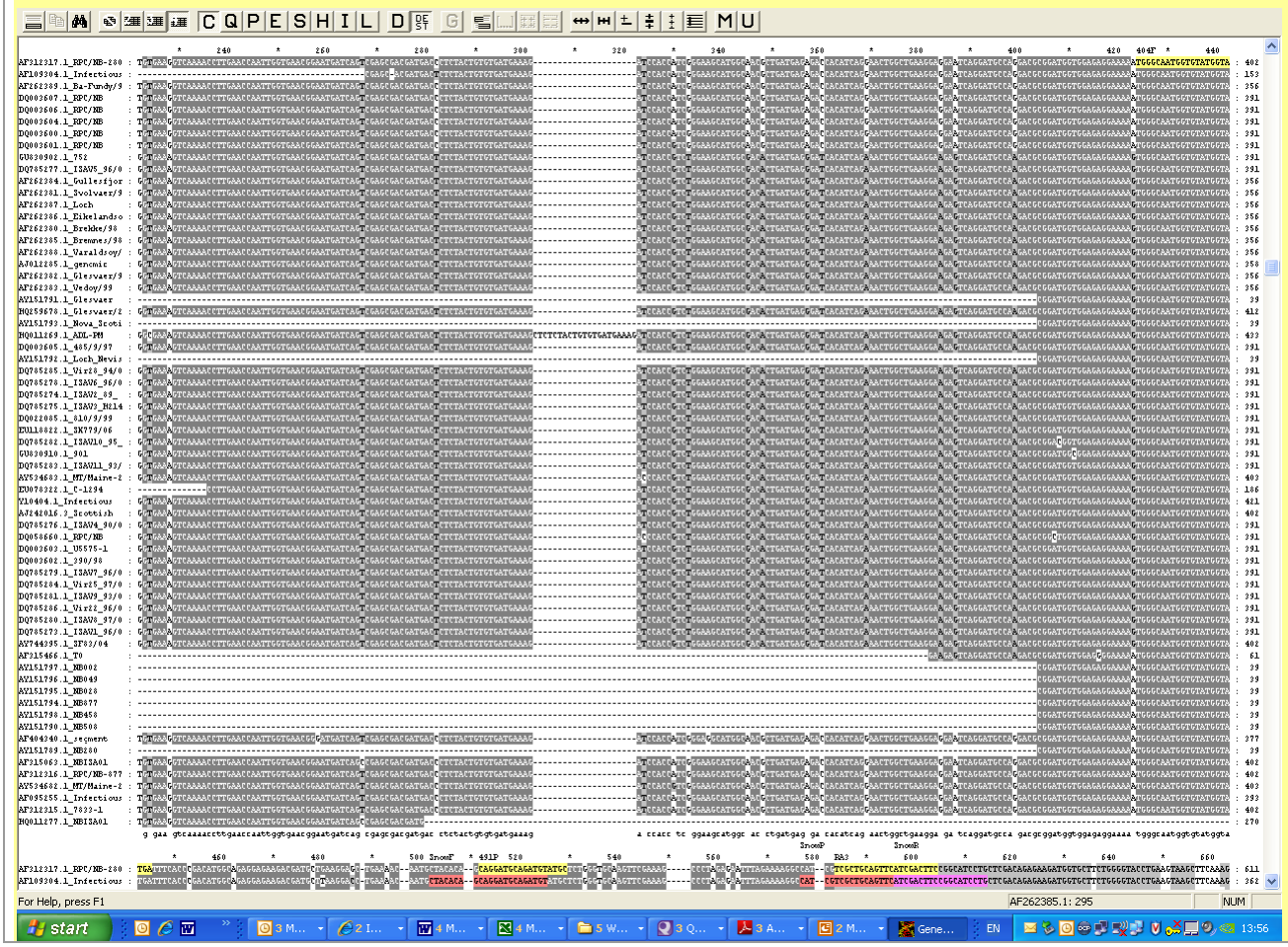
*Analytical specificity is the degree to which the assay distinguishes between the target analyte and other components in the sample matrix; the higher the analytical specificity, the lower the level of false-positives.*

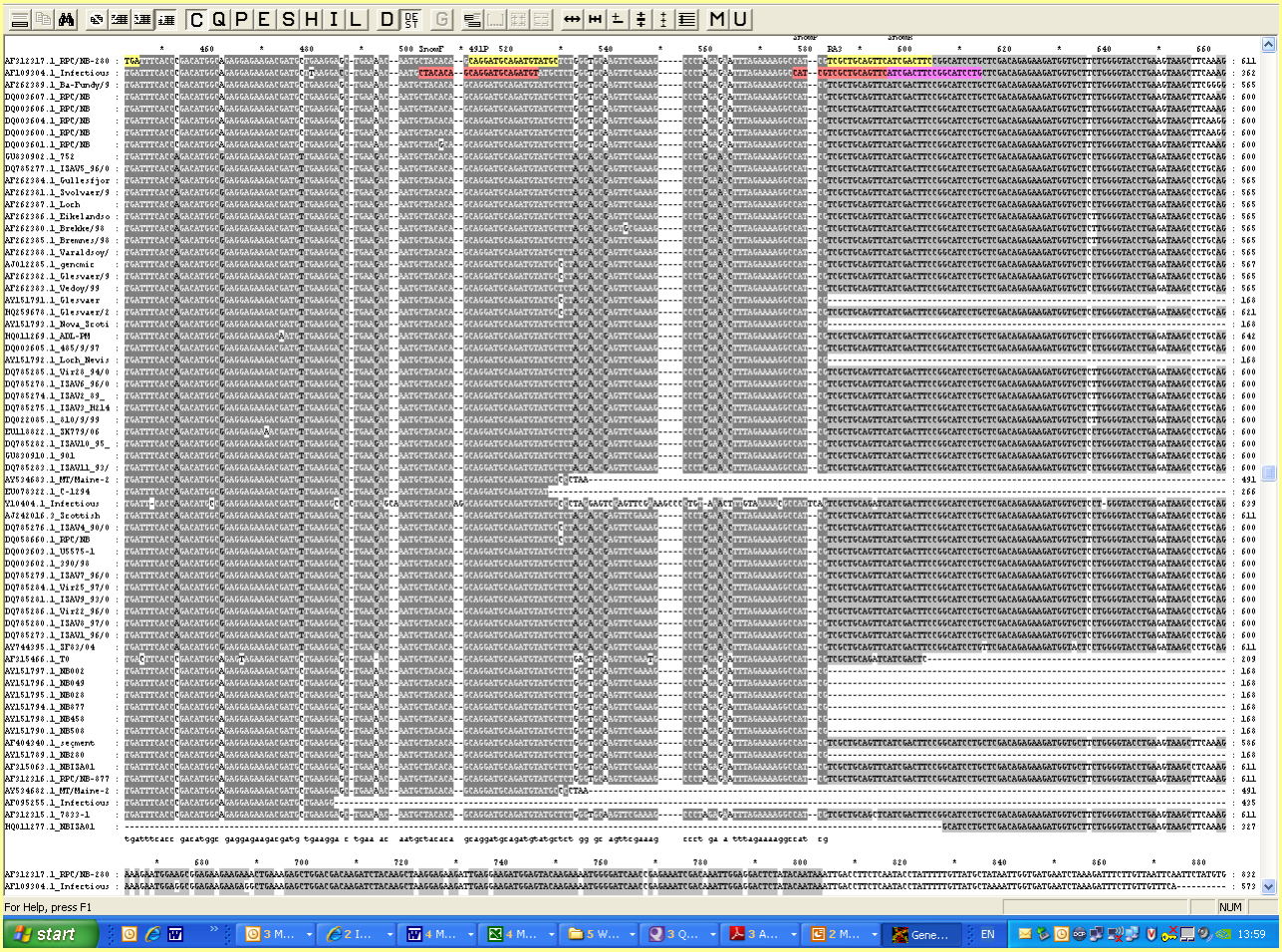
Specificity was determined initially *in silico*: primers and probe were selected based on alignments of ISAV sequences from public sources (Genbank, NCBI). Each pair of primers selected initially was screened (BLAST analysis) to verify that matches to other sequences would not affect the assay. We tested several pairs of primers with similar specificity, but our priority was to develop a sensitive assay able to detect low amounts of ISAV. Primers were tested usually by comparing the limit of detection on a set of serial dilutions, and the pair selected was showing a good sensitivity based on initial testing. Note that at this initial stage, general PCR conditions were used, and optimization of the chosen primers and chemistry were done later.

Specificity was also tested using other virus extract from our regions, e.g. IPN, VHS, NNV, etc and as predicted, they were not detected with our assay. We also tested the assay on RNA extract from various fish species, i.e. cod, hake, Atl tomcod, white hake, red hake, striped bass, landlocked salmon, haddock, halibut, brook trout, gaspereau, brown bullhead, yellow perch, herring, bluegill, freshwater drum, smallmouth bass, gizzard shad, white bass, pumkin seed, rock bass and verified that no host gene or non specific reaction would be detected or produced.

We do not have a very large collection of ISAV isolates, but we are able to detect European and North American strains available in our laboratory, including the HPR0 (avirulent) form of the virus.

A revised version of the ISAV segment 8 alignment is shown here, and we can see that our primers and probe do match all publicly available sequences of ISAV (from various genotypes) as of October 2011.
The first figure shows the position of our forward primer (yellow top sequence), and second figure shows the probe and reverse primer (yellow, top sequence). Primers from the OIE ref assay (Snow et al, 2006) are also shown on this alignment (red and pink). Our primer F and probe have 100% homology to the known sequences; the reverse primer shows one mismatch in the middle of the sequence with a few ISAV isolates. The regions of segment 8 used for primer and probe design are the most conserved on this segment of ISAV.





### 3.2.3. Stage 1. Analytical sensitivity data

*Analytical sensitivity is synonymous with 'Limit of Detection', smallest detectable amount of analyte that can be measured with a defined certainty; analyte may include antibodies, antigens, nucleic acids or live organisms.*

During the analytical part of the validation, we tested **various kits with our primers and probe** in order to get the best sensitivity. Each of these tests involved serial dilutions of ISAV, which were used as guidelines for the selection of reagents and conditions, and guided us regarding the next steps. Four suppliers and 5 kits were evaluated, those initially tested were:

1) Quantitech probe PCR master mix (Qiagen)
2) Quantitech probe RT-PCR master mix (one-step) (Qiagen)
3) 2X Brilliant III qPCR Master Mix (Stratagene)
4) 2X Platinum PCR mix (Invitrogen)

Probe was initially 6'FAM –C+AG+AGG+AG+AA+GA+CG+AT– BHQ1 labeled. (+ = Locked Nucleic Acids).

Based on the sensitivity, availability, compatibility with other assays, and cost, we selected a two-step assay with reagents from Applied Biosystems, i.e.
RT: High capacity cDNA RT kit
qPCR: Taqman universal PCR mix
Probe: FAM and MGB (minor grove binding quencher), and the probe was moved to the current position.

We also tested some variables related to the **extraction protocol**:
Homogeneization: Fast Prep (MpBio) versus Tissue Lyser (Qiagen), and the use of bead versus no beads in the Fast Prep. Results showed Fast Prep without beads to provide the same sensitivity, and more easy to use.
Extraction chemistry: TRI reagent, RNA pellet washes were initially increased but upon testing (post-validation), we followed the recommended 1 wash.

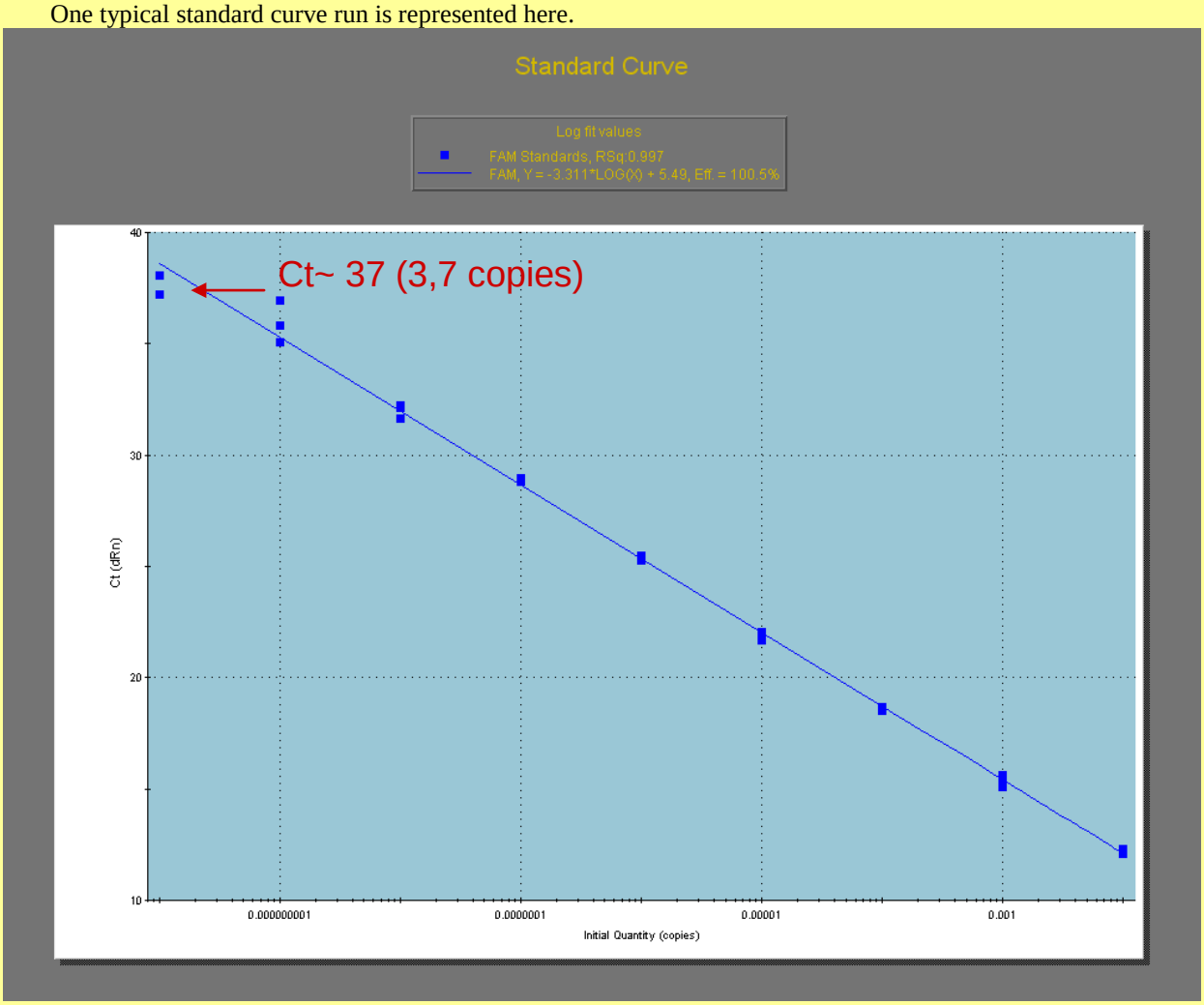
We tested the effect of **denaturing the RNA** prior to reverse transcription, and found that a denaturation at 95ºC for 5min produced a significant difference corresponding to approximately 1 log of increased sensitivity. This had been seen in the end-point PCR assay also. Note that this denaturation was revised in 2011, and although we still observed the increased sensitivity in the detection of ISAV after RNA denaturation, we reduced the denaturation temperature and duration to 80ºC for 1 min., in order to accommodate other validated assays that do not require denaturation and may have been impacted by this additional step.

We optimized the following reagents and thermocycling conditions for qPCR:

- Trials of 0,32µM to 0,9 µM primers per reaction:
  - 0,48 µM optimum
- 0,2 or 0,4 µM probe per reaction:
  - 0,2 µM probe sufficient
- Annealing temp= 60 ºC optimum
- Inhibition of amplification by RNA input = comparison with standard protocol which prescribes a maximum of 1000ng of RNA. Increased RNA 2 fold and 5 fold :
  - Difference of 1 Ct max at the higher RNA concentration (so the test is robust to extreme deviation from recommended RNA input).

To test the **limit of detection**, we used:

1) A plasmid, serially diluted (10 folds), in a matrix of cDNA negative for ISAV. This allowed to calculate the efficiency of the assay (close to 100%) and the limit of detection based on the number of copies estimated for the plasmid molecular weight and concentration. This limit was around a Ct of 37, representing 3.7 copies. One typical standard curve run is represented here.




2) We produced another set of standard material. These were positive tissue homogenates (slurry), serially diluted (10 folds) in a matrix of negative tissue homogenates. These we analyzed 9 times over a period of 5 months, and results were averaged (+/- std dev). We determined that the limit of detection was as follows:

At a Ct of 37.5 we can detect a std sample representing 5,7 ± 2 copies of ISA RNA targets, 7 times out of 9.
At a Ct of 35, we can detect a std sample representing 17 ± 7 copies of ISA RNA targets, 9 times out of 9.

### 3.2.4. Stage 1. Standard(s) of comparison

*At a minimum, the standard method(s) of comparison (reference standard) should be run in parallel on small but select group of highly characterized test samples representing the linear operating range of the new method(s). Identify and cite the reference method(s) and protocol(s) used in the study.*

The standard of comparison in this case would be cell culture. However, during the analytical part of the validation, there was no cell culture done in parallel with the samples used for assay development. The samples provided to us, e.g. infected kidney used for the production of homogenates, and the few sample used for testing during the assay development had been confirmed positive for ISAV previously. Some were from ISAV detection by cell culture, confirmed with PCR, some were confirmed by an external laboratory and verified by our laboratory through PCR and sequencing.

Since sequencing is an absolute verification of the sample identification, we often consider this analytical method as sufficient.
Also, since viral culture detect lives viruses, and PCR detects the presence of specific viral RNA, live or not, it is often difficult to compare the results of both methods. Through validation, we demonstrated that test agreement between PCR assays and viral culture decreases when qPCR Ct values above 20 are obtained (will be discussed further in the diagnostic validation section).

### 3.2.5. Stage 1. Accuracy of analytical methods

*Test methods used solely for the characterization or identification of pathogens are not diagnostic tests per se as they are applied only after the presence of the pathogen has been detected. Never-the-less, these analytical methods need to be verified in terms of their accuracy in correctly characterizing a particular trait or identifying a pathogen type, sub-type, lineage, etc.*

Sequencing provides an absolute identification of a positive PCR signal, and is often the method of choice for the characterization and identification of pathogens.
In the case of a positive finding by cell culture or by RT-qPCR, we would sequence the PCR partial segment 8 obtained directly with the diagnostic assay, or preferably, sequence a fragment obtained after a secondary PCR targeting the segment 6 hypervariable region (HPR), which is more informative and provide a tool to classify the virus in genogroups.
We are not further validating the sequencing of PCR products.

## 3.3. Stage 2 – Diagnostic characteristics

### 3.3.1. Study design(s)

*(Note: Several approaches may be taken in the determination of diagnostic sensitivity and specificity estimates. The most suitable and/or feasible approach for any given disease agent and host should be considered. The availability of reference animals or reference populations will have the greatest impact on the approach.* ***Therefore, once decided, only those applicable sections below need be completed.****)*

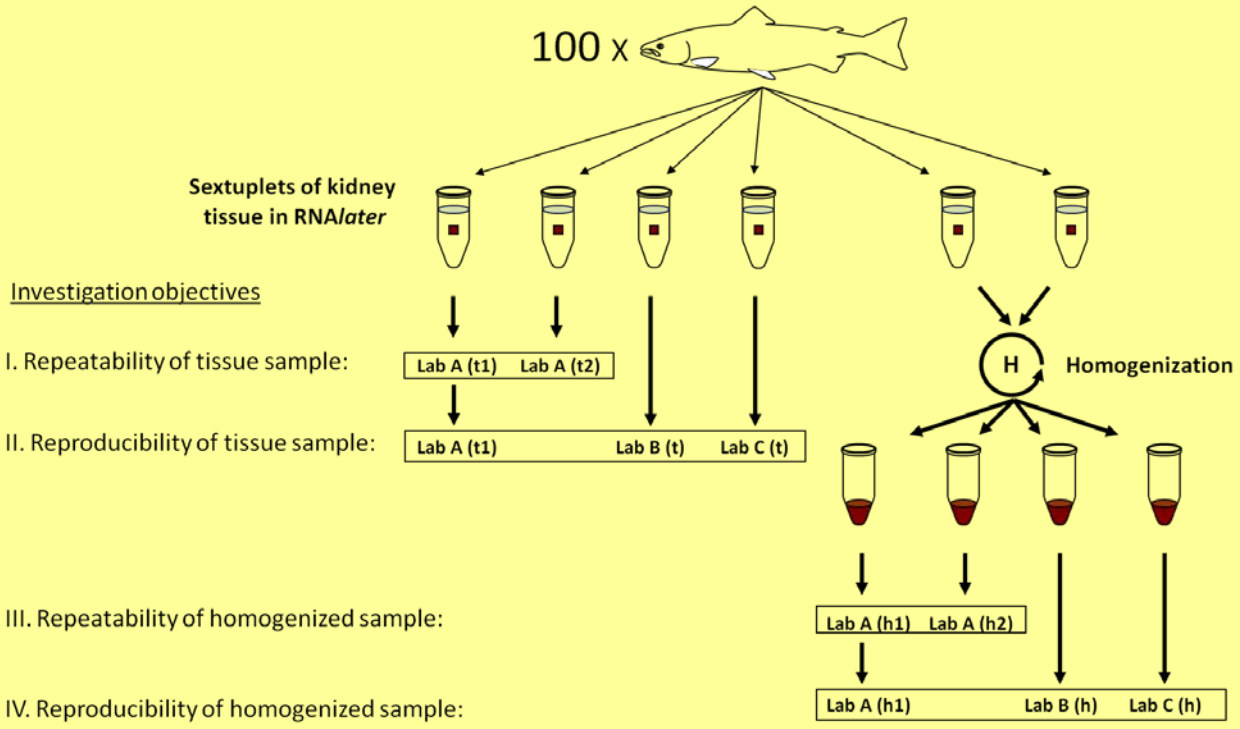
*Reference samples may be obtained from the field or from experimentally infected animals as appropriate to the nature of the disease. Their key characteristic is that their true status (positive/negative etc) should be independently verified by a different technique.*

*Give an overview of the chosen approach used for determination of diagnostic specificity and sensitivity estimates. Include rationale for statistical design, choice of populations, animals or animal models, numbers of animals used to generate confidence intervals for sensitivity and specificity etc.).*

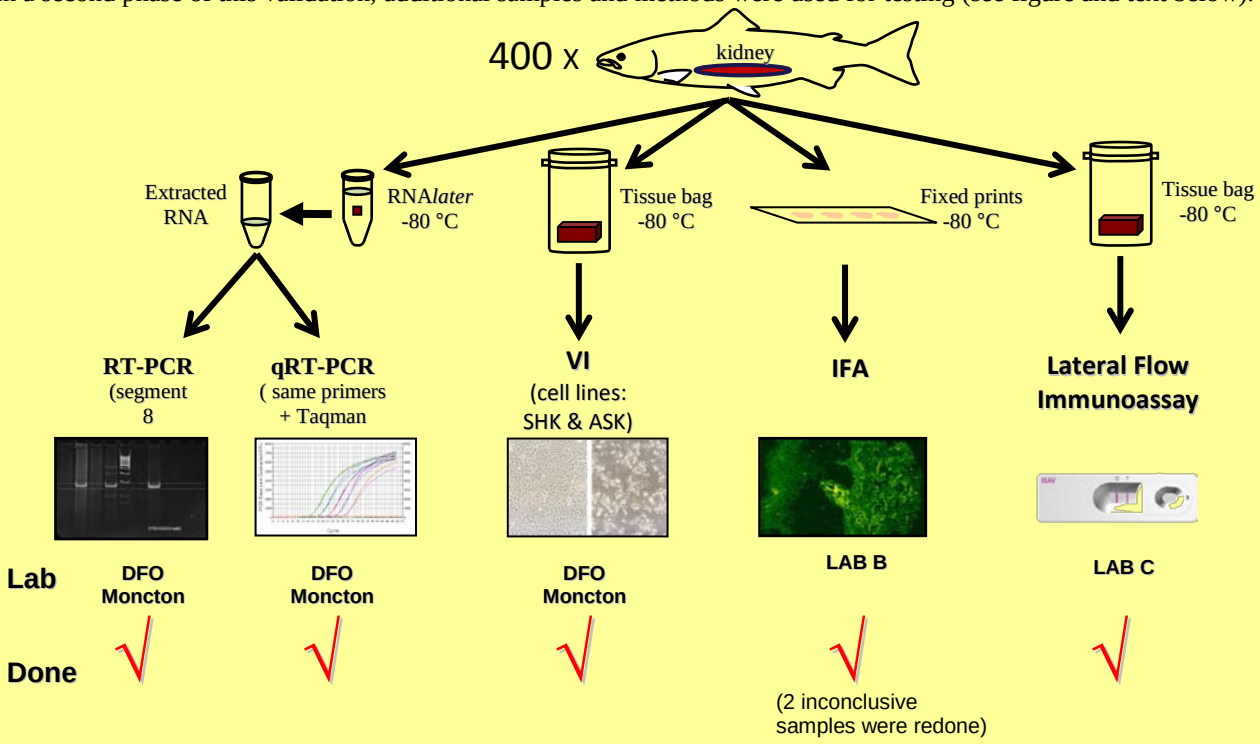
In the absence of a reference method, a latent class model (LCM) was used in this study to assess diagnostic sensitivity (DSe) and specificity (DSp) of our end-point (RT-PCR) for infectious salmon anaemia virus (ISAV). The study included 4 populations of Atlantic salmon, and to ensure the identifiability of the LCM, four additional detection methods were used in parallel including the qRT-PCR version of our assay, virus isolation (VI), indirect fluorescent antibody test (IFAT), and a lateral flow immunoassay (LFI).

Since we had access to naturally infected samples from outbreak cages, Atlantic salmon from farm were collected.

An initial evaluation of the test repeatability and reproducibility was done as follows:
A total of 100 Atlantic salmon, Salmo salar, were selected from archives by combining different origins to target a prevalence of approximately 50% according to McClure et al. (2004); in detail, 45 apparently healthy fish were from three exposed cages (15 from each infected sites) (expected prevalence of 28.1% according McClure et al. (2004), 35 apparently healthy fish were from an infected cage (expected prevalence of 41.5% according McClure et al. (2004), and 20 mort and moribund fish were collected from infected cages (10 from two different sites) (expected prevalence of 100% according McClure et al. (2004). Kidney samples were collected in six replicates (sextuplets) and stored in RNAlater (Ambion Houston, TX, USA) at -80 °C after a 24 hour period at 4 °C according to manufacturer indications.



In a second phase of this validation, additional samples and methods were used for testing (see figure and text below).




In a second phase of the validation, additional samples were provided:
A total of 400 farmed Atlantic salmon were collected from 4 distinct populations with different infection prevalences representing a range of infection stages according to clinical and historical information (McClure et al., 2004). The 4 study populations included: (i) 100 apparently healthy fish from 2 non-exposed cages (50 fish each) at distinct non-infected sites but from a region historically infected (Pop I: near-zero prevalence population); (ii) 130 apparently healthy fish from 3 exposed cages (20, 50 and 60 fish, respectively) at distinct sites declared infected (Pop II: low prevalence population); (iii) 70 apparently healthy fish from 2 infected cages (20 and 50 fish, respectively) after an ISA outbreak at distinct sites (Pop III: moderate prevalence population); and (iv) 100 fish including a mixture of apparently healthy, dead and moribund fish from 4 infected cages (10, 14, 31 and 45 fish, respectively) during an ISA outbreak at distinct sites (Pop IV: high prevalence population). Test results from 100 of these fish were repeated to assess the precision of RT-PCR (i.e. repeatability and reproducibility) in a previous study (Caraguel et al., 2009, as described above in this section).

All samples were submitted blindly to participating laboratories, including our lab (A).

### 3.3.2. Stage 2. Negative reference animals/samples

*(Note: Negative refers to lack of exposure to, or infection with, the agent in question).*

*Complete description: age, sex, breed, etc. Relatedness to intended target population. Selection criteria including historical, epidemiological and/or clinical data. Pathognomonic and/or surrogate tests used to define status of animals or prevalence within population. Sampling plan and procedures.*

As explained in section 3.3.1, we used 100 apparently healthy fish from 2 non-exposed cages (50 fish each) at distinct non-infected sites but from a region historically infected with ISA, and considered this population (Pop I) to have a near-zero prevalence of ISAV.

These Atlantic salmon were thus selected based on historical data, and their status was not defined prior to validation testing. According to latent class modeling, their true status is determined by the modeling.

### 3.3.3. Stage 2. Positive reference animals/samples

*(Note: Positive refers to known exposure to, or infection with, the agent in question).*

*Complete description: age, sex, breed, etc. Relatedness to intended target population. Selection criteria including historical, epidemiological and/or clinical data. Pathognomonic and/or surrogate tests used to define status of animals or prevalence within population. Sampling plan and procedures.*

As explained in section 3.3.1, an expected prevalence of ISAV in the selected samples had been predetermined. This expected prevalence based on the farm status (outbreak, adjacent cages to infected cages, etc) was used to collect a number of samples. The farms were diagnosed with ISAV by the province of New Brunswick, through their surveillance program. The number of samples collected from the different populations aimed to achieve an overall prevalence of 50%.

### 3.3.4. Stage 2. Experimental animals (where used)

*(Note; Experimental animals maybe be used when it is not possible to define or obtain sufficient positive reference animals from the field.).*

*Complete description: age, sex, breed, etc. Immunological status, if applicable. Relatedness to intended target population. Challenge material, source, dose, etc. Type of exposure – inoculation, aerosol, contact, etc. Sampling plan and procedures.*

n.a.

### 3.3.5. Stage 2. Threshold determination

*Complete description of method used to determine thresholds (cut-off(s)) used to classify animals as test positive, negative or indeterminate (if relevant). Include statistical calculations, frequency distributions, etc., as applicable.*

Cutoffs, depending on the intended purpose of the test, can be selected graphically to minimize the probability of either false-positive or false-negative results by using two-graph receiver operating characteristics (ROC) curves. The assay's diagnostic sensitivity and specificity may vary with the tested population, thus, the estimated two-graph receiver operating characteristics curve is population dependent and should be established for the targeted population. Although the selection of a cutoff based on misclassification cost depends on infection prevalence, the selection based on predictive values does not. To optimize the test average diagnostic performance, the Ct cutoff should be selected when diagnostic odds ratio is maximal. Nonetheless, one may want to justify a cutoff based on the probability or the cost of having a false test result.

As we reported (Caraguel et al 2011a), in theory and using the RT-qPCR data produced with validation samples, there is no advantage of adding a cutoff, apart from the one imposed by our limit of 40 cycles. Indeed, we could set the thermocycler to continue cycling beyond the $40^{th}$ cycle, but experience showed us that results at this late stage of the PCR process are difficult to interpret and confirm.

We rely instead on confirmatory assays, e.g. second extraction from back-up tissue to confirm the initial result, viral culture, sequencing of a PCR product, amplification of an alternate segment of ISA, and case history, to make a diagnosis.

### 3.3.6. Stage 2. Diagnostic sensitivity and specificity estimates – ***with*** defined reference animals

*Complete either 3.3.6 or 3.3.7 as appropriate.*

*Diagnostic sensitivity is the proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false-negative results. Diagnostic specificity is the proportion of known uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results.*

n.a.

### 3.3.7. Stage 2. Diagnostic sensitivity and specificity estimates – ***without*** defined reference animals

*Complete either 3.3.6 or 3.3.7 as appropriate.*

*Complete description of latent class model used (Bayesian or maximum likelihood). Describe rationale for use of this approach, and sources of priors (e.g. experts and published papers) for Bayesian models providing relevant, supporting data. Population selection criteria should be presented, including prevalence estimates. Other test methods evaluated should also include the standard method of comparison. The source data tables with cross-classified test results should be presented for each test population. Using best available priors, choose test populations with appropriate prevalences and select animals in sufficient numbers to generate estimates of sensitivity and specificity with an allowable error of ± 5% at a level of 95% confidence. If multiple laboratories are involved in the study design, data on reproducibility should be presented in Section 3.4.3.*

For a complete description of the LCM, see Caraguel et al (2011b) for details.
This is a summary of the modeling and some results. The population selection based on estimates of prevalence was described in section 3.3.1.

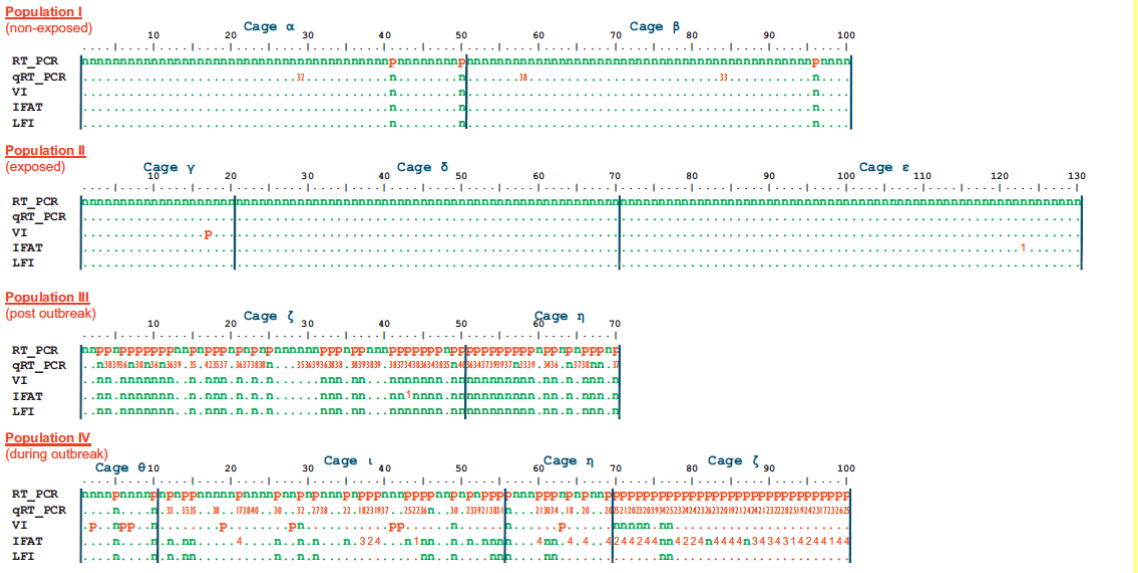
While a conventional LCM assumes DSe and DSp to be constant across the populations, Nérette et al. (2008) previously reported concerns about non-constant DSp of RT-PCR, which detects viral RNA from both active and inactive viral particles. It was suspected that some ISAV recovered fish may carry residual RNA and may be more likely to test positive compared to naïve fish. The various mixture distributions of the two sub-classes of non-infected fish would lead to a non-constant combined DSp estimate across populations.

After many discussions with Charles Caraguel who was doing the LCM analysis, and our own experience with ISA diagnostic using PCR versus cell culture, it was decided to explore the introduction of a third class of samples:

Within a **Bayesian** framework, the conventional two-class LCM was extended to three classes of infection stages (naïve non-infected, **recovered non-infected carrying RNA**, and infected). The resulting analysis confirmed the existence of three classes of fish with substantially different test performances for ISAV. For infected fish, DSe of RT-PCRs (both conventional and the real-time version) and VI approximated 90%, and antibody based assays were the least sensitive (DSe around 65%). Regardless of the test, the DSp estimates on naïve fish were all above 98% with LFI being in average the most specific. Only RT-PCR and qRT-PCR tested positive with the additional class of recovered fish (DSp around 30%). The true infectious status of this sub-class (i.e. viral RNA carriers) is debatable and requires further knowledge about ISAV infection dynamics at the fish level.

In the next figure, test result are aligned: sampled fish (in column) were clustered by cage origin and prevalence level populations (Population I: 100 apparently healthy fish from non-exposed cages with an anticipated near-zero prevalence; Population II: 130 apparently healthy fish from exposed cages with an anticipated low prevalence; Population III: 70 apparently healthy fish from post outbreak cages with an anticipated moderate prevalence; Population IV: mixture of apparently healthy, mortality and moribund fish from outbreak cages with an anticipated high prevalence). “n” codes for negative; “p” codes for positive; and by column a dot indicates same result as the first row (RT-PCR). When positive, cycle threshold values were reported for qRT-PCR, and intensity score (0–4) for IFAT. Greek letters are arbitrary cage numbers. Note that the same cages were sampled for moderate and high level prevalence populations.

Note in this alignment that in the post-outbreak cages, positive samples were found only with RT-PCR and the RT-qPCR, hence the introduction of a third class of samples (fish recovering from ISAV carrying RNA but non-infected).




In summary, for all tests, the probability to test negative in class A (uninfected fish) (DSpA) was substantial, exceeding 98%. LFI (lateral flow immunoassay) had the highest DSpA with the narrowest distribution, while the DSpA of VI was comparable to the PCRs (referred as NAAT in the paper) but showed the widest distribution (i.e. uncertainty) in the estimation. In class B fish (recovering fish), only PCRs (or NAATs) tested positive resulting in substantially lower DSpB while the other assays had almost perfect DSpB. In class C (infected), the probability for the antibody based assays (ABAs) to test positive (DSe) was substantially lower compared to the three other tests (RT-PCR, RT-qPCR and VI).

The results of the 3 class model are presented here:

**Table 3**
Posterior estimates (median, mean, and mode) and corresponding 95% credibility posterior interval (CPI) of probabilities of testing positive and negative in the three class of fish for each of the five ISAV diagnostic assays and class prevalences for each of the 4 populations from the final three-class LCM including conditional dependence and thinning. Three classes of fish (A, B and C) where A and B are non-infected fish.

| Estimates (%) | RT-PCR | | | | qRT-PCR | | | | VI | | | | IFAT | | | | LFI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Median | Mean | Mode | 95% CPI | Median | Mean | Mode | 95% CPI | Median | Mean | Mode | 95% CPI | Median | Mean | Mode | 95% CPI | Median | Mean | Mode | 95%CPI |
| Class A | | | | | | | | | | | | | | | | | | | | |
| Prob (negative) DSp | 98.82 | 98.67 | 98.98 | 96.5–99.9 | 98.76 | 98.58 | 98.96 | 96.2–99.9 | 98.58 | 98.27 | 99.24 | 95.2–99.8 | 99.36 | 99.25 | 99.59 | 97.9–99.9 | 99.73 | 99.62 | 99.98 | 98.6–99.9 |
| Class B | | | | | | | | | | | | | | | | | | | | |
| Prob (negative) DSp | 31.32 | 31.42 | 32.09 | 19.3–44.2 | 26.63 | 26.84 | 26.16 | 15.3–39.6 | 98.82 | 98.33 | 98.82 | 94.0–99.9 | 97.85 | 97.43 | 98.63 | 92.9–99.7 | 99.10 | 98.72 | 98.95 | 95.3–99.9 |
| Class C | | | | | | | | | | | | | | | | | | | | |
| Prob (positive) DSe | 89.54 | 89.55 | 90.92 | 78.2–99.4 | 88.15 | 88.09 | 86.98 | 76.6–98.4 | 88.56 | 88.11 | 89.18 | 78.2–95.4 | 61.02 | 61.05 | 61.94 | 47.7–74.5 | 70.81 | 70.78 | 70.15 | 57.3–83.9 |
| Covariance[a] | 7.38 | 7.61 | 10.54 | 0.14–15.1 | 7.38 | 7.61 | 10.54 | 0.14–15.1 | | | | | 15.93 | 16.19 | 17.37 | 9.78–20.7 | 15.93 | 16.19 | 17.37 | 9.78–20.7 |

| Prevalence (%) | Population I | | | | Population II | | | | Population III | | | | Population IV | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Median | Mean | Mode | 95% CPI | Median | Mean | Mode | 95% CPI | Median | Mean | Mode | 95% CPI | Median | Mean | Mode | 95% CPI |
| Class A | 96.1 | 95.6 | 96.3 | 88.8–99.5 | 98.6 | 98.4 | 97.7 | 95.6–99.8 | 12.2 | 12.4 | 5.23 | 2.09–24.2 | 28.7 | 29.0 | 28.0 | 18.8–40.7 |
| Class B | 2.90 | 3.46 | 1.67 | 0.12–9.86 | 0.58 | 0.83 | 1.09 | 0.02–3.00 | 86.4 | 86.2 | 87.6 | 74.1–96.8 | 12.3 | 12.5 | 11.9 | 5.37–21.1 |
| Class C | 0.67 | 0.98 | 1.11 | 0.03–3.63 | 0.54 | 0.78 | 1.07 | 0.02–2.81 | 1.00 | 1.44 | 1.01 | 0.04–5.24 | 58.6 | 58.5 | 58.7 | 46.9–69.2 |

[a] Conditional dependence between the 2 RT-PCRs and between the 2 antibody-based tests.

### 3.3.8. Stage 2. Comparison of performance between tests

*For standard method(s) of comparison (reference methods) used in full field studies, indicate diagnostic sensitivity and specificity estimates as determined in either Section 3.3.6 or 3.3.7. Provide statistical measures of agreement between the reference methods and the new test being validated and suggest explanations for results <u>not</u> in agreement.*

n.a.

## 3.4. Stage 3 - Reproducibility

Reproducibility is the ability of a test method to provide consistent results when applied to aliquots of the same sample tested by the same method in different laboratories. This is the same definition found in Section 3.2.5; however, Stage 3 is more international in scope and is a better indicator of the ruggedness of the test method. Ruggedness is a measure of an assay's capacity to remain unaffected by substantial changes or substitutions in test conditions anticipated in multi-laboratory utilization, part of fitness studies and reproducibility assessments (e.g. shipping conditions, technology transfer, reagents batches, equipment, testing platforms and/or environments).

### 3.4.1. Stage 3. Laboratory identification

*Selection criteria for laboratories involved in the reproducibility study. Location, i.e. country. Status, i.e. regional, national, provincial/state. Level of expertise, familiarity with technology. Accreditation status. State the number of laboratories included (minimum of three) which should also include OIE Reference Laboratories where they exist.*

Three laboratories were involved in the reproducibility study. The two external laboratories were selected based on the on-going collaborations with them, their availability and interest in the exercise.
Note that for the reproducibility study, the end-point RT-PCR ISAV assay (same primers as the real-time version) was tested. At the time, real-time was relatively new and not introduced in some laboratories.

Lab A: Department of Fisheries and Oceans Canada (DFO), our laboratory.
Lab B: Research Productivity Council (RPC), New Brunswick, Canada. http://www.rpc.ca/english/about.html
Lab C: Department of Agriculture and Aquaculture (DAA), New Brunswick, Canada.

Lab B is a private laboratory. RPC is accredited by various organizations including the Standards Council of Canada (SCC) and is ISO 9001:2008 certified. They are offering ISAV testing as a fee-for-service using their own method at the time. We reviewed with them the documents related to our methodology prior to the beginning of the validation. N.Gagné had seen their facility in previous visits and had been involved with their lab in prior inter-laboratory comparison (ILC). Note that our laboratory had participated in a few ILC prior to the validation of the assay reported here. Assays used in the ILCs were different and each laboratory used their own assays.

In the case of lab C, the provincial laboratory of the DAA performs diagnostic for the surveillance program on Atlantic salmon fish farms in the province of NB. At the time of the validation, they had introduced molecular testing relatively recently, with help and guidance from our laboratory. We visited them on site to review the procedures thoroughly with them, visit their facilities, and insure they felt comfortable with the methods.

### 3.4.2. Stage 3. Evaluation panel

*Description of test panel used for independent reproducibility study (interlaboratory comparisons).*

n.a.

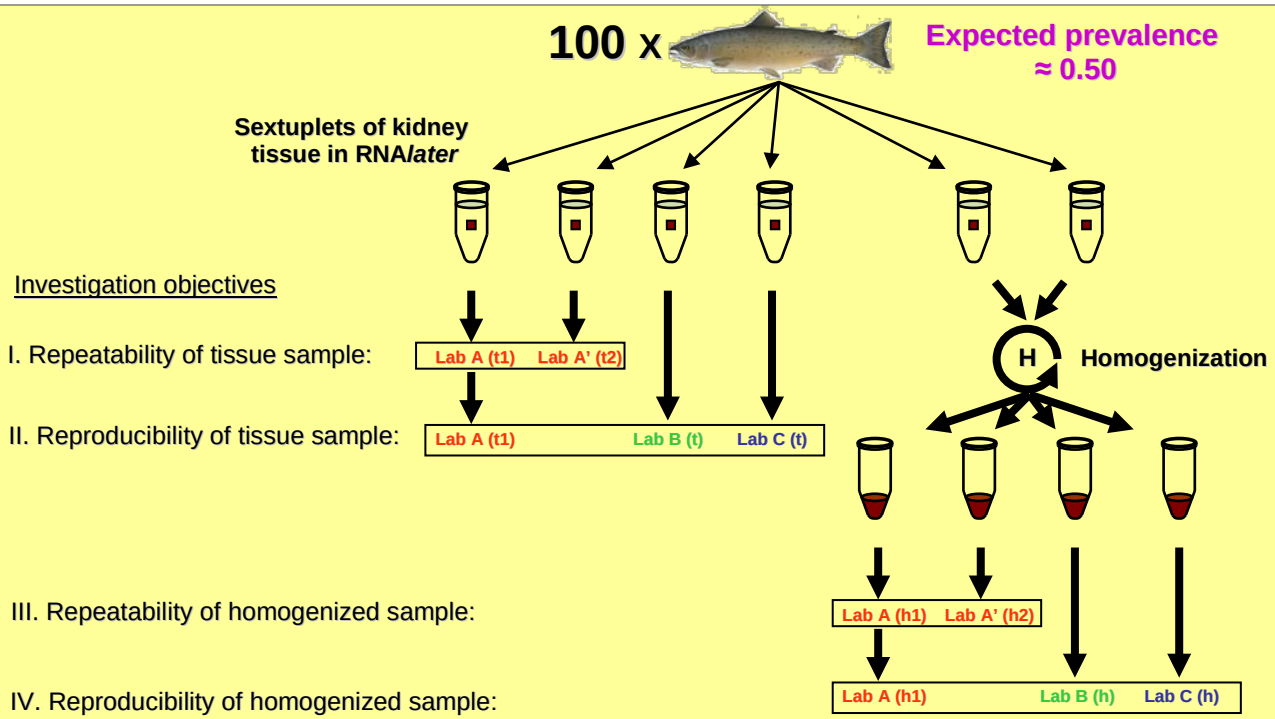
### 3.4.3. Stage 3. Analysis of reproducibility

*Description of reproducibility study and interpretation of results.*

The reproducibility aspect of the validation is described in Caraguel *et al* 2009. In summary:

The samples used for testing were pieces of kidneys from Atlantic salmons from various populations (see next figure): A total of 100 Atlantic salmon, Salmo salar, were selected from archives by combining different origins to target a prevalence of approximately 50% according to McClure et al. (2004); in detail, 45 apparently healthy fish were from three exposed cages (15 from each infected sites) (expected prevalence of 28.1% according McClure et al. (2004), 35 apparently healthy fish were from an infected cage (expected prevalence of 41.5% according McClure et al. (2004), and 20 mort and moribund fish were collected from infected cages (10 from two different sites) (expected prevalence of 100% according McClure et al. (2004). Kidney samples were collected in six replicates (sextuplets) and stored in RNAlater (Ambion Houston, TX, USA) at -80 °C after a 24 hour period at 4 °C according to manufacturer indications.

To compensate for the lack of material, part of the samples were pooled and homogeneized. Lab A (DFO) received more samples than lab B and C, so we were able to evaluate the repeatability of ISA detection in a set of 100 fish ( (2 x 100 tissues and 2 x 100 homogenates).




The results obtained by the 3 laboratories are shown here:



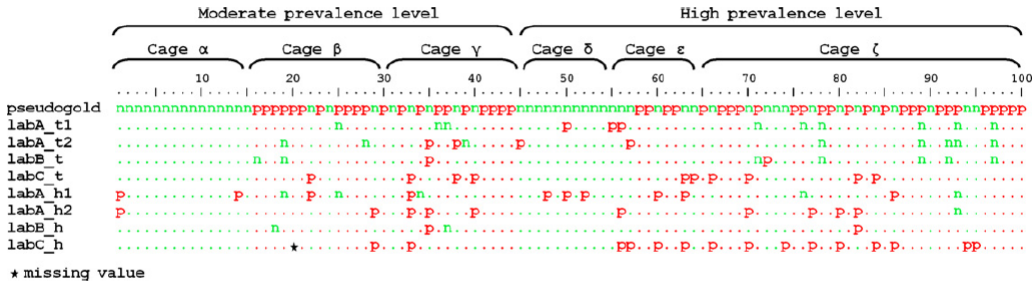



Fig. 2. Test result alignment: sampled salmon (in column) were clustered by cage origin and expected prevalence level population grouping (moderate level: apparently healthy fish from exposed cage; high level: mix of apparently healthy, mortality and moribund fish from infected cage). Negative was recoded as "n"; positive as "p"; and by column a dot (.) indicates same result as the first row. Greek letters: arbitrary cage number. (t1): Non-homogenized sample, duplicate 1; (t2): non-homogenized sample, duplicate 2; (t): non-homogenized sample; (h1): homogenized sample, duplicate 1; (h2): homogenized sample, duplicate 2; (h): homogenized sample.

Among the 100 non-homogenized samples, duplicates 1 and 2 of lab A detected respectively 42 and 44 positives, lab B detected 43 and lab C detected 58. Among the 100 homogenized samples, duplicates 1 and 2 of lab A detected respectively 53 and 57 positives, lab B detected 48 and lab C detected 62 (out of 99 results). Agreement statistics of interest (Pa and k) and CIs are summarized in the tables below.

Since the true status of the samples was not know, a pseudogold standard was calculated based on the combined testing results of the same fish (n=8 test per fish). The pseudogold value is showing on the top row of the figure above.

**Table 2**
Summary of ISAV diagnostic test descriptive agreement statistics, proportions and Kappa values, according to sample type and laboratories comparison. A1: reference laboratory A, duplicate 1; A2: reference laboratory A, duplicate 2; B: laboratory B; C: laboratory C.

| Agreement level | Repeatability | | Reproducibility | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample type | Non-homogenized | Homogenate | Non-homogenized | | | Homogenate | | |
| Lab comparison | A1/A2 | A1/A2 | A1/B | A1/C | B/C | A1/B | A1/C | B/C |
| 0–0 | 49 | 35 | 51 | 39 | 40 | 40 | 31 | 35 |
| 1–1 | 35 | 45 | 36 | 39 | 41 | 41 | 46 | 45 |
| 1–0 | 7 | 8 | 6 | 3 | 2 | 12 | 6 | 2 |
| 0–1 | 9 | 12 | 7 | 19 | 17 | 7 | 16 | 17 |
| Total (count) | 100 | 100 | 100 | 100 | 100 | 100 | 99 | 99 |
| Pa (CI) | 0.84 (0.75–0.90) | 0.80 (0.71–0.87) | 0.87 (0.79–0.93) | 0.78 (0.69–0.86) | 0.81 (0.72–0.88) | 0.81 (0.72–0.88) | 0.78 (0.68–0.85) | 0.81 (0.72–0.88) |
| Pa average[a] (CI) | 0.81 (0.75–0.86) | | | 0.82 (0.76–0.88) | | | 0.80 (0.74–0.86) | |
| | | | | | 0.82 (0.77–0.86) | | | |
| McNemar's test (*P*-value) | 0.610 | 0.370 | 0.780 | 0.000* | 0.000* | 0.250 | 0.033* | 0.000* |
| Kappa (Cohen's) | 0.674 | 0.597 | 0.734 | 0.571 | 0.621 | 0.621 | 0.550 | 0.628 |
| CI | 0.53–0.82 | 0.44–0.75 | 0.60–0.87 | 0.42–0.72 | 0.47–0.77 | 0.47–0.77 | 0.40–0.71 | 0.48–0.77 |
| 3-Rater Kappa | na | na | | 0.639 | | | 0.595 | |
| CI (bootstrap = 1000) | na | na | | 0.52–0.76 | | | 0.47–0.71 | |

na: non-applicable; Pa: observed proportion of agreement; CI: confidence interval.
[a] Computed as the mean of all possible Pa estimates between runs within lab A or among the three laboratories.
* Significant McNemar's test ($P < 0.05$): significant difference of proportion of positive results between the two test runs; thus corresponding Kappa value is less relevant.

C. Cunguel et al. / Preventive Veterinary Medicine 92 (2009) 9–19

**Table 3**
Agreement matrix with proportion of agreement (lower left corner) and proportion of disagreement or distance (top right corner in bold) between runs; (t1): non-homogenized sample, duplicate 1; (t2): non-homogenized sample, duplicate 2; (t): non-homogenized sample; (h1): homogenized sample, duplicate 1; (h2): homogenized sample, duplicate 2; (h): homogenized sample.

| Runs | Lab A(t1) | Lab A(t2) | Lab B(t) | Lab C(t) | Lab A(h1) | Lab A(h2) | Lab B(h) | Lab C(h) |
|---|---|---|---|---|---|---|---|---|
| Lab A(t1) | – | **0.16** | **0.13** | **0.22*** | **0.19*** | **0.19*** | **0.14** | **0.25*$_{Max}$** |
| Lab A(t2) | 0.84 | – | **0.09$_{Min}$** | **0.20*** | **0.23*** | **0.19*** | **0.14** | **0.25*$_{Max}$** |
| Lab B(t) | 0.87 | 0.91$_{Max}$ | – | **0.19*** | **0.22*** | **0.18*** | **0.11** | **0.24*** |
| Lab C(t) | 0.78* | 0.80* | 0.81* | – | **0.19** | **0.13** | **0.12*** | **0.15** |
| Lab A(h1) | 0.81* | 0.77* | 0.78* | 0.81 | – | **0.20** | **0.19** | **0.22*** |
| Lab A(h2) | 0.81* | 0.81* | 0.82* | 0.87 | 0.80 | – | **0.11*** | **0.14** |
| Lab B(h) | 0.86 | 0.86 | 0.89 | 0.88* | 0.81 | 0.89* | – | **0.19*** |
| Lab C(h) | 0.75*$_{Min}$ | 0.75*$_{Min}$ | 0.76* | 0.85 | 0.78* | 0.86 | 0.81* | – |

Min: minimum; Max: maximum.
* Significant McNemar's test ($P < 0.05$): significant difference of proportion of positive results between the two runs; thus serious disagreement.

Overall repeatability revealed slightly lower Pa than overall reproducibility (0.81 and 0.82, respectively), although the overlapping of CIs provided little evidence for significant difference.

On average, homogenized samples had a higher proportion of positive results than non-homogenized samples (56.4% vs. 46.8%), which implies that homogenization impacted the test performances with either increased analytical sensitivity and DSe, decreased DSp, or both. Homogenized samples revealed slightly lower repeatability and reproducibility compared to non-homogenized samples. It was expected that homogenization would improve reproducibility since distinct pieces of kidney tissues could potentially have different viral loads. However, the difference observed here could be due to a release of more viral RNA in samples homogenized, or to cross contamination during the preparation of homogenates.

Note that the since this study was using RT-PCR (end-point) detection, single runs were done, and gel interpretation was necessary. The use of RT-qPCR (real-time) introduces advantages such as duplication of wells and easier interpretation, which could have impacted the values obtained here. Further, Ct values in RT-qPCR provides an estimate of viral load which can improve the interpretation of discrepancies between runs of the same samples, e.g. correlation of high Ct values with lower reproducibility.

## 3.5. Stage 4 - Applications

Stage 4 validation is recognised as an ongoing process that continues for the lifetime of the assay. Although this section gives important information regarding the validation of the diagnostic test, it is not a compulsory requirement for the OIE evaluation. Please complete where the information is available.

### 3.5.1. Stage 4. Test applications

*(Note: This Section applies to tests that have been incorporated into routine diagnostic regimens.)*

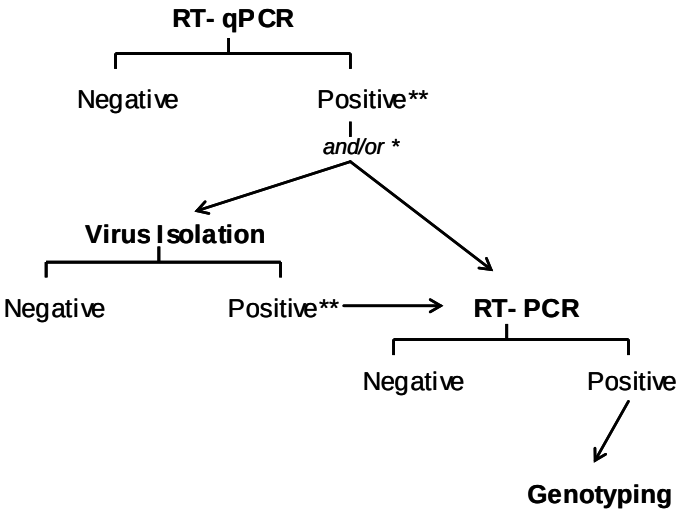
*Describe functional test applications (i.e. screening, confirmatory, supplemental applications) and integration with other tests into*

*diagnostic regimen. Include flowcharts and decision trees where applicable.*

ISAV RT-qPCR is used for screening in the National Aquatic Animal Health Program. The test is part of other diagnostic assays, but used mainly following this scheme, as defined in our Test Method Agreement with our client (CFIA):

| Section D | Testing Requisites | | | |
|---|---|---|---|---|
| **Test Method Type** | **RT-qPCR** | **Virus Isolation** | **RT-PCR** | **Genotyping** |
| **Anticipated Submissions per year:** | > 30 | > 30 | < 30 | < 30 |

**ISAV Diagnostic Flowchart 1 - *Apparently Healthy Populations***

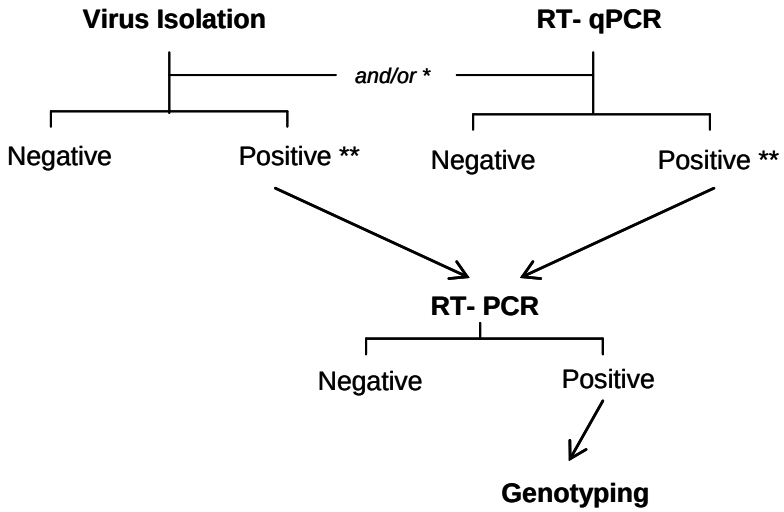



*Wherever possible, both tests will be performed, but in some cases the specimen/sample may be of insufficient quantity or unsuitable quality (unfit) to attempt Virus Isolation.

** Only a portion of positive specimens from any one submission need be genotyped (i.e. all positive specimens up to a maximum of 5 for any given submission).

| Section D | Testing Requisites |
|---|---|

**ISAV Diagnostic Flowchart 2 - *Populations Showing Clinical Signs***




*Wherever possible, both tests will be performed, but in some cases the specimen/sample may be of insufficient quantity or unsuitable quality (unfit) to attempt Virus Isolation.

** Only a portion of positive specimens from any one submission need be genotyped (i.e. all positive specimens up to a maximum of 5 for any given submission).

### 3.5.2. Stage 4. Laboratories

*List laboratories where this test method is in current use. Location, i.e. Country. Status, i.e. regional, national, provincial/state. Accreditation status.*

*For each laboratory, indicate purpose of test, integration with other tests and status of test, i.e. official test, supplementary, etc.*

As of November 2011, the molecular virology laboratory of DFO, Pacific Biological Station, Nanaimo, British Columbia (Canada) is using this assay as specified. The PBS lab is part of the DFO network of laboratories. They are also using the test as a screening tool. This laboratory is also seeking ISO 17025 accreditation in the near future.

### 3.5.3. Stage 4. International reference standards

*List type and availability of international reference reagents. Source. Negative, weak/strong positive reference reagents. Other key biologicals, e.g. antigens, antibodies, etc.*

n.a.

### 3.5.4. Stage 4. Inter-laboratory testing programmes

*Describe programmes involving inter-laboratory comparisons using this test method. National, international. Describe eligibility and number of laboratories participating.*

n.a. The only program we have in place is the provision of proficiency panel samples to external labs who test for ISAV by RT-qPCR or RT-PCR. According to our program, technicians need to analyze panel samples twice per year if they perform ISAV testing.

### 3.5.5. Stage 4. International recognition

*List internationally recognised reference laboratory responsible for this test method and/or biologicals. Listed international standards containing this test method. Listed international programmes employing this test method.*

n.a.

## Section 4. Performance summary

### 4.1. Summary of validation data

| SECTION | ELEMENT | SUMMARY DATA |
|---|---|---|
| **2..2 General** | **Type & purpose** | |
| 2.2.1 | Type of test | Real-time RT-PCR (RT-qPCR) |
| 2.2.2 | Intended purpose | Diagnostic, surveillance, confirmation, etc |
| **2.3 General** | **Test description** | |
| 2.3.2 | Disease/analyte | Viral segment of Infectious salmon anemia virus |
| 2.3.3 | Species/specimen | Atlantic salmon. Other species can be tested; validation done on A.salmon. |

| | | |
|---|---|---|
| **3.2 Validation** | **Stage 1 - Analytical Characteristics** | |
| 3.2.1 | Repeatability | For RT-PCR: Overall proportion agreement: 0.84 (2 x 100 tissues); 0.80 (2 x 100 homogenates). Prevalence around 50%.<br>For RT-qPCR: evaluated on repeated runs of serially diluted standards. Repeatability high (~100%) when Ct are below 35 |
| 3.2.2 | Analytical specificity | 100%, based on *in silico* evaluation and testing. There is no known closely related disease in fish. |
| 3.2.3 | Analytical sensitivity | 9/9 detection of 17± 7 copies of target (plasmid). |
| 3.2.4 | Std of comparison (analytical correlation) | n.a. |
| 3.2.5 | Reproducibility (national) | Overall proportion agreement: 0,82 |
| **3.3 Validation** | **Stage 2 - Diagnostic characteristics** **(Note: complete only 3.3.6 or 3.3.7)** | |
| 3.3.5 | Threshold (diagnostic) | None applied beside the program ending cycling at 40 cycles. |
| 3.3.6 (i) | Diagnostic specificity | n.a. |
| 3.3.6 (ii) | Diagnostic sensitivity | n.a. |
| 3.3.7 (i) | Diagnostic specificity | LCM in Bayesian mode, using 400 fish from various populations (aiming 50% prevalence) and 5 methods.<br>RT-qPCR DSp in :<br>Uninfected fish: 98.8<br>Recovering fish*: 26.63<br>* see Caraguel et al (2011b) |
| 3.3.7 (ii) | Diagnostic sensitivity | RT-qPCR DSe in:<br>Infected fish: 88.15 |
| 3.3.8 (i) | Std of comparison (diagnostic specificity) | n.a. |
| 3.3.8 (ii) | Std of comparison (diagnostic sensitivity) | n.a. |
| **3.4 Validation** | **Stage 3 - Reproducibility** | |
| 3.4.3 | International (if applicable) | National : 0.82 Proportion of agreement between 3 laboratories using end-point RT-PCR |
| **3.5 Validation** | **Stage 4 - Applications** | |
| 3.5.1 | Diagnostic integration | As of November 2011, the ISAV RT-qPCR assay is used in another DFO laboratory (British Columbia) as developed. |
| 3.5.2 | Laboratories (in scope) | None yet. |

## Section 5. Additional data

*Tables of raw data or other supporting information can be provided at the discretion of the applicant. Such material can be helpful to the expert panel in completing their evaluation. If you choose not to provide such information, reviewers may request it in order to clarify their decisions.*

*The raw data can be provided as separate Microsoft Excel files. You may also provide other documents as PDF files. Please specify the name and purpose for each file and use a meaningful title. If a file is providing data for more than one claim then indicate each link to the headings in submission form in "what does the file show?" column.*

| No. | File Name | Links to submission numbers | What does file show? Describe very briefly the intention of the data in the file. |
|---|---|---|---|
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Add more rows if you need them.

## Section 6. References cited in the dossier

*List the scientific literature related to the diagnostic test described in this application and cited in this dossier. Use a consistent reference style throughout.*

Caraguel C, Stryhn H, Gagné N, Dohoo I, Hammell L. (2009) Traditional descriptive analysis and novel visual representation of diagnostic repeatability and reproducibility: application to an infectious salmon anaemia virus RT-PCR assay. *Prev Vet Med.* Nov 1;92(1-2):9-19. Epub 2009 Sep 11.

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Caraguel C, Stryhn H, Gagné N, Dohoo I, Hammell I (2011b) Use of a third class in latent class modelling for the diagnostic evaluation of five infectious salmon anaemia virus detection tests. *Prev. Vet. Med.* , article in press

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Giray C, Opitz HM, MacLean S, Bouchard D (2005). Comparison of lethal versus non-lethal sample sources for the detection of infectious salmon anemia virus (ISAV) DAO 66:181-185

McClure CA, Hammell KL, Dohoo IR, Nerette P, Hawkins LJ. (2004) Assessment of infectious salmon anaemia virus prevalence for different groups of farmed Atlantic salmon, Salmo salar L., in New Brunswick.
J Fish Dis. 27(7):375-83.