Canadian Food Inspection Agency | Agence canadienne d'inspection des aliments

# DRAFT DOCUMENT

# Infectious Salmon Anaemia (ISA) Laboratory Assessment:

# ISA OIE Reference Laboratory Atlantic Veterinary College

December 14, 2011



Canada

# EXECUTIVE SUMMARY

The recent reports from the ISA OIE Reference Laboratory at the Atlantic Veterinary College (AVC) stating that the Infectious Salmon Anaemia virus (ISAV) has been found in British Columbia (BC) salmon have not yet been corroborated by federal officials through established processes. After initial investigations, concerns have been raised regarding the standard operating procedures for sample management and diagnostic testing at the laboratory and subsequent reporting of these findings.

The Canadian Food Inspection Agency (CFIA), which has the regulatory mandate in this issue and Fisheries and Oceans Canada (DFO) are currently working together to assess the results based on scientifically sound and internationally recognized standards and procedures. As part of this process, the CFIA is leading an assessment of diagnostic laboratories involved in testing samples submitted by a third party that led to the original claim that Infectious Salmon Anaemia (ISA) infection has been detected in BC salmon.

The focus of this report is an evaluation of the quality system in place in the ISA OIE Reference Laboratory where the ISA tests were carried out. In parallel, an assessment of the DFO-Gulf Fisheries Center (GFC), the reference center for ISAV testing for the National Aquatic Animal Health Program, was also carried out and is documented in a separate report.

The general objective was to assess the functional laboratory capability for the diagnosis of ISAV, an OIE reportable aquatic animal health disease. The specific objectives were to assess laboratory capability in: a) bio containment, b) quality assurance program, and c) validation of ISA test methods performed and to assess conformity of ISA testing with acceptable practices (e.g. OIE standards). This assessment will be used by the NERT team to interpret the test results on samples submitted for ISA testing during the month of October 2011 from two sources in BC.

Three people with technical expertise in diagnostic laboratory testing procedures and methodologies were identified to carry out the actual on-site laboratory assessment. These included two individuals from CFIA's Science Branch with extensive experience in laboratory quality assurance programs and one person from the Animal Health Laboratory, Laboratory Services Division, University of Guelph who acted as an independent third-party expert.

The process included an assessment of both real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) and cell culture methodologies and consisted of a review of documentation on laboratory procedures and methodologies, followed by an on-site evaluation visit.

The team recognized that the ISA OIE Reference Laboratory in Canada has a solid foundation of knowledge and expertise in ISA disease. Multiple scientific publications and communications are available.

The laboratory has started implementation of a quality assurance system which includes documents detailing methodologies and supporting SOPs. However, the team noted a number of weaknesses and gaps in the area of quality assurance and the validation of the ISA real-time RT-PCR and cell culture methodologies. These were not in line with the OIE standards.

Major areas of concern included possible cross-contamination, possible cross-reaction and inconsistencies in test procedures. Furthermore, there was no record of the various analytical steps or materials and equipment used which meant that no verification was possible.

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## LABORATORY ASSESSEMENT

# 1. OVERVIEW

## 1.1. BACKGROUND

The recent reports from the ISA OIE Reference Laboratory at the Atlantic Veterinary College (AVC) stating that the Infectious Salmon Anaemia virus (ISAV) has been found in British Columbia (BC) salmon have not yet been corroborated by federal officials through established processes. After initial investigations, concerns have been raised regarding the quality management procedures which govern the laboratory's operating environment as well as the diagnostic protocols used in the testing at the laboratory.

The Canadian Food Inspection Agency, which has the regulatory mandate in this situation, is taking actions to investigate claims concerning the presence of the ISA disease in BC. As part of this process, the CFIA is leading an assessment of diagnostic laboratories involved in testing samples submitted by a third party.

The focus of this report is the assessment of the ISA OIE Reference Laboratory which performed the ISA testing. An investigation into the collection, handling, transportation and storage of samples arriving at this laboratory has also been undertaken and is described in a separate report.

In parallel, an assessment of the DFO-Gulf Fisheries Center, the reference center for ISAV testing for the National Aquatic Animal Health Program, was also carried out and is documented in a separate report.

## 1.2. OBJECTIVE

To assess the functional laboratory capability for the diagnosis of the ISAV, an OIE reportable aquatic animal health disease:

**Specific Objectives**

- To assess laboratory capability in: a) bio containment, b) quality assurance program, and c) validation of ISA test methods performed.

- To assess conformity of ISA testing with acceptable practices (e.g. OIE standards).

## 1.3. APPROACH

As part of the NERT, a working group (WG) was established to carry out the laboratory assessment. The Laboratory Assessment WG, chaired by a representative from CFIA's Science Branch, consisted of members from CFIA's Science, Operations and Policy and Programs Branches as well as a representative from DFO. Details about the WG membership, objectives, roles and responsibilities as well as a more detailed description of the assessment process are found in Appendix 1.

Three people with technical expertise in diagnostic laboratory testing procedures and methodologies were identified to carry out the laboratory assessment. These included two individuals from CFIA's Science Branch and one person from the Animal Health Laboratory, Laboratory Services Division, University of Guelph who acted as an independent third-party expert. (See Appendix 1).

The laboratory assessment included an assessment of both real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) and cell culture methodologies. The process consisted of a review of the documentation of laboratory procedures and methodologies (Appendix 1 Annex 2), which was then followed by an on-site visit.

# 2. LABORATORY ASSESSMENT INFORMATION

Included below is a description of areas where issues of concern were identified in the laboratory assessment process.

Detailed information assembled during the assessment process is documented in Appendix 2.

## 2.1. LABORATORY ENVIRONMENT

### 2.1.1. Laboratory Space and Organization

The laboratory operates in six rooms in the AVC building. They also use space and equipment in a Central Service area for incubation and storage of viruses and cell lines. Viruses and cell lines are stored in separate liquid nitrogen canisters. Central Services also provides glassware cleaning service.

Both diagnostic testing and research activities occur in the same rooms. There is some separation of incompatible activities, but there were places where separation was not adequate.

Several of these rooms are shared by other users. At the time the samples in question were analyzed, there were two other people working in this space, one of which was working on an ISAV project, but not related to the testing of the BC samples in question.

### 2.1.2. Bio-Containment

The laboratory has met CFIA's Office of Biohazard, Safety and Containment requirements of the Containment Standard for Veterinary Facilities and the Containment Standard for Facilities Handling Aquatic Animal Pathogens (Animal Pathogen Containment Level 2 (CL2)).

### 2.1.3. Quality Management

In documentation provided, the laboratory indicated they have started development of a quality manual using ISO/IEC 17025 as a guide. The laboratory has some elements of quality management but they are not yet part of a comprehensive system. Some elements are missing, for example, the Standard Operating Procedures (SOPs) for Sample Receiving and Tracking does not include a requirement to document the condition of the sample upon receipt, nor is a process for cleaning included.

There were SOPs for real time RT-PCR and the ancillary activities such as RNA extraction, and setting up standard curves. There were also SOPs for cell culture work.

### 2.1.4. Training and Qualification of Personnel

The laboratory has implemented training of laboratory personnel but it does not define or document what is included in the training.

Curricula vitae for Dr. F. Kibenge and Dr. M. Kibenge were provided. Additional training information for Dr. M. Kibenge (who carried out the analysis of the samples in question) included the certification for real-time RT-PCR assays that are carried out as part of the Canadian Animal Health Surveillance Network.

### 2.1.5. Proficiency Testing

In accordance with recognized laboratory best practices, when diagnostic testing is carried out, analysts should have demonstrated their proficiency through proficiency panels. The laboratory provided information on a ring test for ISAV with laboratories in Chile in 2009 (Kibenge *et al*., 2011) in response to a request for proficiency testing (PT) data. They organized this ring test in their role as an ISAV OIE Reference Laboratory. The values obtained by Dr. M. Kibenge were the reference values. No other PT information was provided. The information provided was insufficient to assess the PT of the laboratory for ISAV testing.

#### 2.1.6. Documents

The documents provided contained detailed information on the analytic testing; however, detailed information for reporting results in accordance with OIE requirements was missing.

#### 2.1.7. Records

The laboratory tracks samples received using an Excel spreadsheet. This sheet contains basic information about what type of analysis is done on the samples.

There was no record of the various analytical steps or materials and equipment used.

## 2.2. SAMPLE INFORMATION

### 2.2.1. Sample Receipt

The room where the fish samples are initially received is also used for inoculating fish cell lines with ISAV. In addition, RT-PCR associated with cloning and sequencing of ISAV has also been carried out in the room used for sample receipt. The samples were not opened in the room where they were received.

An additional concern identified for the sample receipt room is the potentially high levels of complementary DNA (cDNA) that can be generated as a result of conventional RT-PCR, agarose gel electrophoresis and associated cloning steps involving bacteria. This cDNA can easily contaminate the environment if stringent clean up measures are not carried out on a regular basis. Bleach is the only disinfectant that is effective is completely removing contaminating DNA from the environment.

In the absence of documented procedures for cleaning and disinfection as part of the standard operating procedures, and the absence of records of cleaning and disinfection prior to these samples arriving, it is difficult to know how much of a risk for cross contamination may have existed.

### 2.2.2. Processing – RNA Extraction

The room and biosafety cabinet where RNA extraction takes place (which includes extraction of strong positive control material) is the same room and biosafety cabinet where RNA template is added to the RT-PCR master mix. This leads to the potential for cross contamination. Positive controls are extracted in a separate extraction batch, apart from diagnostic samples.

The same set of pipettors used for RNA extraction is used for loading the RNA template. Although barrier tips were used, which should help to prevent the pipettors from becoming contaminated, this is not considered good practice. Since relatively high tittered

ISAV stocks were used for RNA extraction and these in turn used as the positive controls in the real-time RT-PCR runs, the potential exists for residual RNA to be in the environment and hence a source of cross-contamination. This can be mitigated by cleaning steps, but since there was no documentation present, it is difficult to evaluate the cross-contamination risk.

### 2.2.3. Internal Sample Control

A protocol for an internal control of samples is described in the SOP; however, it was not used to test any of the sample submissions. RNA quantification by Nanodrop or by other spectrophotometric means was also not used.

## 2.3. REAL-TIME RT-PCR TEST METHODOLOGY

### 2.3.1. Primer and Probe Concentration

Primer and probe concentrations in combination with the chemistries used can affect the analytic sensitivity of RT-PCR assays. Inconsistencies with RT-PCR master mix prep were noted. These involved the concentrations of various components of the master mix. The significance of these inconsistencies with respect to the results obtained could not be determined.

### 2.3.2. Test Validation

For the real-time RT-PCR for ISAV Segment 8, the methodology used is one that is referenced in the OIE's Manual of Diagnostic Tests for Aquatic Animals (Snow *et al*., 2006), however, OIE Reference Laboratory is different from the one described by Snow in that OIE Reference laboratory method is performed in a closed system in a 1-step real-time RT-PCR test, which minimizes risk of contamination (Workenhe *et al.* 2008).

For the real-time RT-PCR for Segment 6 which is used to distinguish between European and North American Genotypes, the reference supplied for validating the methodology is a poster which was presented at an International Conference on Diseases of Fish and Shellfish, Prague, Czech Republic, September 14-19, 2009 (Kibenge *et al*., 2009). Further assessment of the validation was not possible.

Although several publications were provided in support of method validation, the laboratory did not provide a complete package of validation data (i.e. one that includes at least the initial elements required by the OIE validation protocol) for the method used to analyse the samples in question.

### 2.3.3. Positive Control

The Ct (cycle threshold), also know as the Cp (crossing point), is defined as the number cycles required for the florescent signal to cross the threshold (i.e. exceed the background level).

The positive control used is ISAV RNA extracted from TO cell culture lysates. The Ct values of the positive controls are low (<20) indicating a strong positive control is being used. Because ISAV RNA is used, and in high concentration, there is a risk of cross contamination. Furthermore, because genomic RNA is used, it makes distinguishing between true positives and contamination with the positive control difficult and dependent on sequencing the real time RT-PCR products.

### 2.3.4. Interpretation of Test Data

Interpretation of Ct by the OIE Reference Laboratory are as follows: Cts ≤ 30 are considered strong positive; Cts ≥ 30.1 and ≤35 are considered weak positive; Cts ≥ 35.1 and ≤40 are considered very weak positive; Cts ≥ 40.1 and ≤ 45 are considered suspicious; No Ct is interpreted as negative. This is consistent with general practice however; interpretation of Ct values varies from lab to lab.

Ct cut-off is test/instrument dependent; it is not only the Ct that matters, but also the shape of the curve, it should be sigmoidal. Flat curves may be indicative of software artefacts and/or primer/probe-template mismatches should be interpreted cautiously and further evaluated.

There are different ways that the data can be interpreted, the Fit Points Method and the 2$^{nd}$ Derivative Maximum method. The 2$^{nd}$ Derivative Maximum method used by the OIE Reference Laboratory classifies samples as positive, negative or suspicious. The advantage of this method is there is minimal user input. The disadvantage is that it can assign a Ct to a sample by an instrument error and does not allow baseline adjustments.

The ISA OIE Reference laboratory has stated that the Fit Point method is a better method for analysing PCR data, but they did not use it for analysing the BC samples data. Page 5 of OIE Reference Laboratory SOP #ISAV-Seg-8-cRNA-Standards–02-08 states: “The Second Derivative Maximum Method looks promising in theory but generally in practice the accuracy of quantitative RT-PCR is greater when the data are analyzed by the threshold crossing method, e.g., Fit Point Method.”

The on-site assessment team saw curves produced by the instrument software, and noted that some were atypical, and that these were identified as positive. The laboratory sent the data files to the team and subsequent analysis confirmed atypical curves.

**Discussion:**

A number of issues were noted that explain the difference in interpretation between the ISA OIE Reference Laboratory and the on-site assessment team:

- Three (3) of the 5 samples were classified as positive when only one of two duplicates tested positive in at least one of the RT-PCR segment 8 and 6 testing[1]. These samples should have been re-tested.
- Indiscriminate use of the 2nd Derivative Maximum method for data analysis (See Section 2.3.4).
- A lack of consideration for the shape of the curve (See Section 2.3.4). This was particularly noted for Sample Set #1 Segment 6 (EU) genotyping run, where the runs were declared positive in duplicate, however all curves were "flat" and therefore not indicative of true positives. The recommendation would have been to re-test [1]. This was also evident for a number of test Runs for Sample Set #2.
- While the Cp values may fall within a range identified as positive, a visual examination of the curves should have be carried out. Had this been carried out, the conclusion would be that a re-test was necessary[1].

Analysis of the data revealed instances of test abnormalities which were not reported:

- Abnormal results for the controls were noted for the Segment 6 (NA) genotyping test run for Sample Set #1. The NTC (no template control) was reactive in one of two duplicates. The samples were classified as negative based on the run results; however, when NTC is reactive, a test is typically considered invalid.
- The same issue was again noted for Sample Set #2, Segment 6 (NA). The NTC was reactive, in both duplicates for this Run.
- For Sample Set #2, test Run for Segment 6 (NA) genotype, the positive control for European (EU) genotype which should have been negative, was also positive in duplicate.

## 2.4. CELL CULTURE

The laboratory provided an SOP for cell culture (SOP #Cell culture-Virus Propagation-02-06) and reported inoculating the specified cell lines with the sample material for virus isolation. No worksheets or other records showing details of the inoculation were available for review on site to verify that the procedure outline in the SOP had been followed.

[1] As identified by the expert on-site assessment team, under accepted laboratory protocol practices these results would have bee subjected to a re-test for verification.

# 3. TECHNICAL AND TEST RESULT INFORMATION ON BC SAMPLES

Four sets of samples from BC were tested for ISAV by the ISA OIE Reference Laboratory at the AVC in October and November 2011.

Information concerning these sample sets was extracted from the records provided from the ISA OIE Reference Laboratory. These reports can be found in Appendices 3, 5, 6 and 7. Information varied from sample set to sample set. The following are common elements although, as stated above, the same amount of detail cannot be found in the reports for each sample set.

## 3.1. Information on Sample Sets

**Description:**

Sample Set #1 consisted of forty-eight (48) samples made up of heart tissue from wild Sockeye salmon smolts. They were received at the OIE Reference Laboratory on October 4, 2011 and immediately processed and tested.

These samples received by the OIE Reference Laboratory were described as extremely small.

Sample Set #2 consisted of twenty (20) (10 heart and gill sets) from wild caught pre-spawning Coho, Chinook, Chum and Sockeye salmon, collected in October 2011. They were submitted for testing on October 13, 2011.

Sample Set #3 consisted of five (5) sockeye smolt heart and gill sets (10 samples) collected in June 2011and kept in a household freezer. They were submitted for ISAV testing on Oct 27th, 2011.

Sample Set #4 consisted of fourteen (14) hearts and gills collected from dead Sockeye, Coho, and Pink salmon on November 4, 2011 and kept on ice until they were submitted for ISAV testing.

**Testing**

Sample Integrity

For all Sample Sets there was no testing carried out for sample integrity and there were no records describing the condition of the samples upon receipt.

Real-time RT-PCR

For all Sample Sets, heart tissue samples were tested for ISAV using real-time RT-PCR with TaqMan probe for segment 8. Samples that were positive were then further tested using a real-time RT-PCR with TaqMan probes for ISAV segment 6 for genotyping.

Cell Culture

In Sample Set #1 all samples were inoculated on CHSE-214 cell monolayers in 24-well plates, and incubated at 16C for 21 days. The two samples positive by real-time RT-PCR were inoculated on ASK-2 cell and incubated at 16C for 6 days (Passage 1 (P1)). Samples from P1 were blind-passaged on ASK-2, SHK-1 and TO cell lines at 16C for 10 days.

Cell lysates from the CHSE-214 cultures and the ASK-2, SHK-1, and TO cultures were used to extract RNA for real-time RT-PCR testing.

In Sample Set #2, Passage 1 (P1): Samples positive on RT-PCR were inoculated in ASK-2 cell line in T-25 cm flasks and incubated at 16C for 10 days. The cell lysates were used to extract RNA for real-time RT-PCR testing.

Further passages in CHSE-214 and ASK-2, SHK-1 and T0 cell lines have either not yet been carried out or are awaiting real-time RT-PCR testing.

No cell culture was carried out in Sample Sets # 3 and 4.

**Results reported by the ISA OIE Reference Laboratory**

Real-time RT-PCR

In Sample Set #1, two (2) of the 48 samples were reported to have tested positive for ISAV of the European genotype. The report did not state that the positive results were not positive in duplicate samples.

In Sample Set #2, three (3) of the 20 tissues samples tested positive by real-time RT-PCR with TaqMan probe for ISAV segment 8. These 3 positives were further tested using real-time RT-PCR with TaqMan probes for ISAV segment 6 for genotyping. One (1) of the 3 tissues tested positive for ISAV of the European genotype. Explanatory notes include statements that the ISAV sequences detected from the samples could be from viable or non-viable virus and that the virus could be pathogenic or non-pathogenic. The report did not state that the positive results were not positive in duplicate samples.

The notes also include the statement that the presence of ISAV sequences in the tissue samples does not imply that the subject fish had ISA or that ISA is present in the area where the subject fish were collected.

In Sample Sets # 3 and 4 all tissue samples provided to the laboratory were real-time RT-PCR negative.

Cell Culture

In Sample Set #1 the report of preliminary findings states that in 13 of the samples inoculated for cultures, a cytopathic effect (CPE) was observed 14-17 days post-inoculation; however, the CPE effects observed were noted to be not characteristic of ISAV.

P1 and P2 were CPE negative for the two samples positive on real-time RT-PCR Further passages in CHSE-214 and ASK-2, SHK-1 and T0 cell lines have either not yet been carried out or are awaiting RT-PCR testing.

Final results still pending - A sample is considered negative for virus isolation after three blind passages without CPE.

In Sample Set #2 the report of preliminary findings states that all the P1 cultures were cytopathic effects (CPE) negative. Using real-time RT-PCR with TaqMan probes for ISAV segment 8, all the three P1 cultures were negative.

Final results still pending - A sample is considered negative for virus isolation after three blind passages without CPE.

**Reporting to CFIA:**

Sample Set #1
October 9, 2011 – Preliminary notification to the sample submitter in BC that some samples tested positive for ISAV.

October 15, 2011 – Results reported to Dr. Brian Evans, OIE delegate and CFIA Chief Veterinary Office. The report stated that two samples from Sockeye salmon tested positive for the European genotype of ISAV by real-time RT-PCR.

November 30, 2011 – ISA OIE Reference Laboratory sent report on preliminary cell culture test work. Work is ongoing; therefore final results are not available.

Sample Set #2
October 17, 2011 – ISA OIE Reference Laboratory notified CFIA of additional samples received and being tested for ISAV.

October 20, 2011 – ISA OIE Reference Laboratory notified CFIA of one positive test result.

October 27, 2011 – ISA OIE Reference Laboratory submitted the final laboratory analysis of these samples which include three positive test results and the explanatory notes described above.

November 30, 2011 – ISA OIE Reference Laboratory sent report on preliminary cell culture test work. Work is ongoing; therefore final results are not available.

Sample Set #3
November 3, 2011 – ISA OIE Reference Laboratory notified CFIA of negative test results.

Sample Set #4
November 25, 2011 – ISA OIE Reference Laboratory notified CFIA of negative test results.

# 4. ANALYSIS OF TEST RESULTS BASED ON THE OIE REFERENCE LABORATORY ASSESSMENT FINDINGS

There are a number of factors that can lead to either false positive or negative test results including cross-contamination, cross-reactions and errors in test procedures and/or interpretation.

The areas of concern identified below relate to real-time RT-PCR testing. Very few records were available to assess the cell culture methodology.

## 4.1. Risk of Cross-Contamination

A number of issues were identified that create a risk for cross-contamination including:

- Physical location where samples are received. Risk associated with other activities (inoculation and cloning) being carried out in the same space;
- Physical location of RNA extraction;
- Use of non-dedicated equipment (pipettors).

## 4.2. Risk of Cross-Reaction

References were provided as validation of the RT-PCT methodology (Section 2.3.2). No package of validation data was provided. As a result, it was not possible to evaluate whether cross-reactions could pose a problem.

## 4.3. Risk of Error in Test Procedure and/or Interpretation

A number of issues were identified with the test procedures and test interpretation which could lead to errors in the test results reported:

- Inconsistencies with concentration of the real-time RT-PCR master mix preparations were noted (See section 2.3.1);
- No testing was carried out to determine the quality of the RNA in the samples. The effect of sample degradation on the test is not known;
- Aberrant control results in some of the real-time RT-PCR test runs which could affect the validity of the test, were not explained or reported;
- An analysis and interpretation of the real-time RT-PCR data by the on-site assessment team was inconsistent with that of the OIE Reference Laboratory interpretation.

An overall deficiency of records and documentation was noted. Procedures for cell culture were documented; however, there were no records of the steps being carried for the BC samples and it was difficult to fully assess cell culture methodology due to this lack of records. Limited records make it difficult to trace back what was done on any given sample, and without an audit trail it is difficult to determine if, or where a problem may have occurred.

# 5. REPORTING OF TESTING RESULTS

ISA is reportable under the National Aquatic Animal Health Program (NAAHP) under the authority of the *Health of Animal Act*, as well as reportable to the OIE.

## 5.1. Reporting to CFIA

ISAV is a reportable disease under the National Aquatic Animal Health Program (NAAHP) under the authority of the *Health of Animal Act*. This requires that anyone who owns or works with aquatic animals and knows of or suspects a reportable disease is required by law to notify the CFIA. Furthermore, when test results indicate the presence of a reportable disease in a region of the world where it has never previously been confirmed, a greater level of care is required before reporting these results.

> ***Health of Animal Act*, Control of Disease and Toxic Substances, Notification and Samples, Section 5 (2):**
>
> "Immediately after a person who is a veterinarian or who analyses animal specimens suspects that an animal is affected or contaminated by a reportable disease or toxic substance, the person shall so notify a veterinary inspector."

The ISA OIE Reference Laboratory informed the sample submitter of test results four days before notifying CFIA.

## 5.2. OIE Reporting

The OIE's Manual of Diagnostic Tests for Aquatic Animals, has identified corroborative diagnostic criteria for confirmed cases.

> **OIE Manual of Diagnostic Tests for Aquatic Animals**
>
> **7.2 Definition of confirmed case**
>
> The following criteria in i) should be met for confirmation of ISA. The criteria given in ii) and iii) should be met for the confirmation of ISAV infection.
>
> i) Mortality, clinical signs and pathological changes consistent with ISA (Section 4.2), and detection of ISAV in tissue preparations by means of specific antibodies against ISAV (IFAT on tissue imprints [Section 4.3.1.1.2] or fixed sections as described in Section 4.3.1.1.3) in addition to either:

a) isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm, as described in Section 4.3.1.2.1
or
b) detection of ISAV by RT-PCR by the methods described in Section 4.3.1.2.3;

ii) Isolation and identification of ISAV in cell culture from at least two independent samples (targeted or routine) from any fish on the farm tested on separate occasions as described in Section 4.3.1.2.1;

iii) Isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm with corroborating evidence of ISAV in tissue preparations using either RT-PCR (Section 4.3.1.2.3) or IFAT (Sections 4.3.1.1.2 and 4.3.1.1.3).

The ISA OIE Reference Laboratory classified samples as positive for IASV based only on RT-PCR test results. This does meet OIE requirements.

# 6. CONCLUSIONS

The laboratory expert team did an assessment of the functional laboratory capability for the diagnosis of the infectious salmon anaemia (ISA) virus, an OIE reportable aquatic animal health disease, at the ISA OIE Reference laboratory, Atlantic Veterinary College, Charlottetown, PEI.

The team recognized that the ISA OIE Reference laboratory in Canada has a solid foundation of knowledge and expertise in ISA disease. Multiple scientific publications and communications are available.

The laboratory has started implementation of a quality assurance system which includes documents detailing methodologies and supporting SOPs. However, the team noted a number of weaknesses and gaps in the area of quality assurance and the validation of the ISA real-time RT-PCR and cell culture methodologies. These were not in line with the OIE standards.

Major areas of concern included possible cross-contamination, possible cross-reaction and inconsistencies in test procedures. Furthermore, there was no record of the various analytical steps or materials and equipment used which meant that no verification was possible.

# References

Kibenge F., Kibenge M., Simard S., Riveroll A., Pallapothu M., and Salonius K. 2009. Development of a DIVA system for an infectious salmon anaemia (ISA) virus vaccine using a qRT-RT-PCR test based on segment 6 of the ISA virus. 14th European Association of Fish Pathologists International Conference on Diseases of Fish and Shellfish, Prague, Czech Republic, September 14-19, 2009.

Kibenge F., Kibenge M, and Masaoud E. 2011. Infectious Salmon Anaemia Virus (ISAV) Ringtest: Validation of the ISAV Diagnostic Process using Virus-spiked Fish Tissues and ISAV TaqMan® Real-time RT-PCR. Journal of Aquaculture Research and Development 2011, 2:2.

Snow M., McKay P., McBeath A., Black J., Doig F., Kerr R., Cunningham C., Nylund A. and Devold M. 2006. Development, application and validation of a Taqman® real-time RT-RT-PCR assay for the detection of infectious salmon anemia virus. In New Diagnostic Technology: Applications in Animal Health and Biologics Controls. Developments in Biologicals (Basel, Switzerland) Karger. 126, 133-145.

Workenhe T., Kibenge M, Iwamoto T. and Kibenge F.. 2008. Absolute quantification of infectious salmon anaemia virus using different real-time reverse transcription PCR Chemistries. Journal of Virological Methods, 154, 128-134.]

# Appendices

Appendix 1 - Lab Assessment WG Process Document

Appendix 2 - Laboratory Assessment Detailed Data Table

Appendix 3 – Report Sample Set #1

Appendix 4 – Report Norway Results

Appendix 5 – Report Sample Set #2

Appendix 6 – Report Sample Set #3

Appendix 7 – Report Sample Set #4

Appendix 8 – Reference - Kibenge et al 2009

Appendix 9 – Reference - Kibenge at al 2011

Appendix 10 – Reference –Snow et al 2006

Appendix 11 – Reference –Wokenhe et al 2008

# Appendix 1 - Lab Assessment WG Process Document

**CFIA - AQUATIC ANIMAL HEALTH**
**LABORATORY ASSESSMENT WORKING GROUP**
**National Emergency Response Team (NERT)**

## General objective:

To assess the functional laboratory capability for the diagnosis of the infectious salmon anaemia (ISA) virus, an OIE reportable aquatic animal health disease, at the following two laboratories:

- DFO – NAAHL, Moncton, NB
- ISA OIE Reference laboratory, Atlantic Veterinary College, Charlottetown, PEI

This assessment will be used by the NERT team to interpret the test results on samples submitted for ISA testing during the month of October 2011 from two sources in British Columbia.

## Specific Objectives

- To assess laboratory capability on: a) bio containment, b) quality assurance program, and c) validation of ISA test methods performed.

- To assess conformity of ISA testing with acceptable practices (e.g. OIE standards)

## Key steps in carrying out the laboratory assessment:

- **Step 1:** Obtain documentation from the Atlantic Veterinary College (AVC) and DFO Moncton Labs for both PCR and cell culture, and the records of the test procedure for the ISA samples. (See Annex 1).

- **Step 2:** Development of a questionnaire/ checklist tool for the assessment to ensure completeness and to facilitate comprehensive documentation.

- **Step 3:** Identify experts to carry out the assessment (Document evaluation and on site visit) (See Annex 2)

- **Step 4:** On site visit to the Laboratories.

- **Step 5:** Data analysis and report writing.

## Timelines

A progress report will be submitted to the Science and Policy Team leader by 10 November 2011. The Laboratory assessment report will be completed by the end of the week of November 21, conditional on the cooperation and timely response by the laboratories to the request for documentation and execution of the on site visit.

## Working Group Membership

- Representation from CFIA who have scientific expertise relevant to aquatic animal health disease, and/or laboratory assessment.
- Additional focused expertise and participation will be solicited as required.

Members:

- Ingrid Van der Linden (Chair) Animal Health Risk Assessment, Science Branch
- Shelagh McDonagh, National Manager Lab QA & Accreditation, Science Branch
- Victoria Pederson, Area Veterinary Specialist, Atlantic, Programs
- Tim Davis Meat Hygiene Animal Health Programs – Atlantic, Operation Branch
- Peter Wright, National Manager, National Aquatic Animal Health Laboratory System, Oceans & Science Branch, DFO

## Roles and responsibilities

| Team | Responsibilities |
|---|---|
| Ingrid Van der Linden (Chair) | • Liaise with National Emergency Response Team (NERT) Structure, Science and Policy Group<br>• Call and chair meetings of the Laboratory Assessment Working Group (WG)<br>• Ensure effective communication among members of the Laboratory Assessment WG<br>Coordinate and participate in the writing of the Laboratory Assessment Report<br>• Ensure deliverables are achieve<br>• Provide advice/leadership to Secretariat and Internal Working Group |
| Shelagh McDonagh<br>Victoria Pederson<br>Tim Davis | • Regularly participate in meetings<br>• Project oversight<br>• Provide expertise and input<br>• Share scientific information with WG members<br>• Participate in the writing and review of the Laboratory Assessment Report<br>• Guide, review and approve deliverables |
| Peter Wright | • Regularly participate in meetings<br>• Project oversight<br>• Provide expertise and input<br>• Share scientific information with Working Group members |
| **Diagnostic Laboratory Expert** | |
| Shelagh McDonagh<br>John Pasick | • Review the documentation provided by the laboratories in preparation for the on-site visit.<br>• Assess the laboratories for the specific objectives identified above<br>• Give their expert opinion on the findings<br>• Contribute to the writing of the laboratory assessment report<br>• Sign off on the final report |
| **Diagnostic Laboratory Expert Third Party Observer** | |
| Dr. Davor Ojkic, Animal Health Laboratory | • Review the documentation provided by the laboratories in advance in preparation for the on-site visit.<br>• Participate in the on site visits to observe and provide advice on the assessment process as necessary, in order to ensure that the process is comprehensive and objective.<br>• Review and sign off on the final report |

Note: People with specialized areas of expertise may be called on to assist with the review of specific areas of the documentation and to review sections of the report

# APPENDIX 1: ANNEX 1

# Information Request for the Infectious Salmon Anaemia Laboratory Assessment

## Documentation related to infectious salmon anaemia (ISA) testing

- **PCR testing methodology:**

  Documents and records:
  - Method or protocol used
  - Validation procedures
  - Procedures related to general QC for PCR work in the lab
  - Test validation data, report or summary
  - Process for ensuring competency of the people performing the testing
  - Results of any performance testing (PT) or interlab comparison (ILC)

- **Cell culture testing methodology:**

  Documents and records:
  - Method or protocol used
  - Validation procedures
  - Procedures related to general QC for cell culture work in the lab
  - Test validation data, report or summary
  - Process for ensuring competency of the people performing the testing
  - Results of any performance testing (PT) or interlab comparison (ILC)

- All peer reviewed publications on the ISA testing methodologies being performed in the laboratory

- Testing records (copies of worksheet or workbooks) for samples received for ISA testing in the month of October 2011 from two sources in BC

## General laboratory documentation:

- ISO Certification, or information on stage of certification process

- Any other pertinent information that will help the assessment

# APPENDIX 1: ANNEX 2

## Candidate Experts for the Laboratory Assessment:

- Dr. John Pasick – Head, CSF/Avian Diseases Unit, NCFAD, CFIA
  Head, CSF/Avian Diseases Unit
  National Centre for Foreign Animal Disease (Winnipeg Laboratory - Arlington)
  1015 Alington Street
  Winnipeg R3E 3M4
  Telephone: (204) 789-2013

- Shelagh McDonagh
  National Manager Lab QA & Accreditation
  Laboratory Quality Assurance and Accreditation
  1400 Merivale Rd.
  Ottawa K1A 0Y9
  Telephone: (613) 773-5314

- Participant from Animal Health Laboratory, Laboratory Service Division, University of Guelph, with PCR expertise identified by Dr. Grant Maxie:

  Dr. Davor Ojkic
  Avian Virologist and Immunologist
  Animal Health Laboratory
  Laboratory Services Division
  Building 89, 419 Gordon Street
  University of Guelph
  Guelph, Ontario N1G 2W1
  Telephone: (519) 824 4120, ext. 54524

Candidates with specialized areas of expertise may be called on to assist with the review of specific areas of the documentation.

## Appendix 2

| The ISA OIE Reference Laboratory Assessment | | |
|---|---|---|
| **Procedure or Element Reviewed** | **Details** | **Assessment Team Comments** |
| Quality management or Quality assurance procedures | The lab provided some key SOPs related to the method and procedures used for analysis of the samples, however some that would be expected in a lab doing diagnostic testing were not provided. These include; calibration and maintenance of key equipments such as pipettors, suitability and traceability of reagents and materials used in analysis, result reporting. *Clarification received from the laboratory Pippettor calibration documents were available.* | The laboratory has started to develop a quality management system, but many elements are not yet in place. The CFIA did request testing records (worksheets or lab books), the validation procedure, test validation data, procedures related to general QC for both PCR and cell culture, as well as any other pertinent information that would help in the assessment (See Appendix 1 for details). While calibration records for pipettors were available, there were no records of which pipettors were used in each step of the analysis. Team was advised that each room had a set of pipettors. |
| Cleaning and decontamination | Cleaning and decontamination before and after working on samples was reported as always being done, and is specified in SOP#ISAV-R RT-PCR-12-08 Infectious Salmon Anemia Virus Real Time RT-PCR (TagMan Assay). No requirement for environmental monitoring was included in the SOP. | There were no records of cleaning, decontamination or environmental monitoring, therefore, no verification of the procedure could be done. |
| Documents | SOPs were provided for the real time RT-PCR ISAV method and related processes (RNA extraction setting up a standard curve). SOPs for Cell culture were also provided. No details for reporting results are included in the SOPs. *The laboratory clarified that it follows the requirements established by the OIE and federal regulations.* | For diagnostic testing, instructions for result reporting should be included in an SOP either with the method or in a separate document. |

## Appendix 2

| Procedure or Element Reviewed | Details | Assessment Team Comments |
|---|---|---|
| Records | Records related to these sample were in an Excel sheet but did not include all the steps nor the equipment used. Only one analyst was involved. | As per the OIE Quality Standard and Guidelines for Veterinary Laboratories there should be records with sufficient information to allow the activity to be recreated, to identify factors affecting the quality of the test, and to enable the test to be repeated under conditions as close as possible to the original. Verification was not possible due to incomplete records. |
| Training | No procedure for method or protocol specific training was provided. | Records that were provided showed overall qualifications and expertise. There were no records (such as results from blind panels or interlaboratory comparisons) demonstrating analyst proficiency in the specific method used for the ISAV testing of samples in question. The analyst has been trained and demonstrated proficiency in specific methods for avian influenza testing, but this certification is method specific and is not evidence of the analyst's proficiency in the ISAV assay. |
| Sample receiving Room 329S | The room where the fish samples were initially received is also used for inoculating fish cell lines with ISAV. PCR associated with cloning and sequencing of ISAV has also been carried out in this room. *Clarification was received from the laboratory that the samples were not opened in the room where they were received.* | The potential for cross contamination was minimized but still exists. |

## Appendix 2

| Procedure or Element Reviewed | Details | Assessment Team Comments |
|---|---|---|
| RNA extraction Room 326S | Combination of Trizol + RNeasy Mini Kit - whole tissue and homogenates. The room and biosafety cabinet where RNA extraction took place (which includes extraction of positive control material) is the same room and biosafety cabinet where RNA template is added to the PCR master mix. Although the biosafety cabinet where both these procedures take place does not have a UV light, the cabinet is disinfected with Virkon between uses. The same set of pipettors was used for RNA extraction and addition of template to the PCR master mix. | Potential for cross contamination exists. The biosafety cabinet where the extraction of strong positive control material takes place is the same biosafety cabinet used for adding template to PCR master mix. The same pipettors are used for both procedures. Controls and samples are extracted separately. There were no records of decontamination of the biosafety cabinet. |
| PCR targets | Segment 8 - primers and probe as described in 2009 Manual of Diagnostic Tests for Aquatic Animals - 104 bp product; Segment 7 as in Manual described in protocol; Segment 6 to discriminate between NA and European viruses - confidential IP | No comments |
| One-step or two-step RT-PCR? | One-step RT-PCR; The DNA polymerase + Aptamers provides hot start capabilities and increased sensitivity down to 0.1 pg of total RNA using kit's special enhancer. *The laboratory clarified the correct reference for the method was Workenhe et al, (2008) Absolute Quantitation of Infectious Salmon Anemia Virus Using Different Real-time RT_PCR Chemistries, Journal of Virological Methods 154, (128-134)* | No comments |
| PCR chemistry utilized | LC480® Master Hydrolysis Probe (Roche) | No comments |
| Primer and probe concentrations | Calculations in Master mix table in SOP#ISAV-R RT-PCR-12-08 were confusing and needed clarification (720 nM for primers and 200 nM for probe). The laboratory provided an explanation. | Primer and probe concentrations in combination with the chemistries used can affect the analytic sensitivity of PCR assays. Using the explanation provided during the on-site visit and additional information subsequently provided the team was not able to reproduce the laboratory's calculations. |

## Appendix 2

| Procedure or Element Reviewed | Details | Assessment Team Comments |
|---|---|---|
| Internal control | Atlantic salmon elongation factor 1α (ELF1α) is described in the SOP but upon questioning is rarely used. ELF1α internal control was not used in any of these submissions. Neither was RNA quantitation by nanodrop or other spectrophotometric means. | Use of an internal control is a quality control measure that can be used to assess sample quality as well as operator, reagent and equipment performance. Lack of data makes it difficult to assess the quality of the samples for which positive results were obtained. |
| Instrument platform used | Roche LC480 | Instrument platforms and associated analysis software can have some affect on analytic sensitivity/specificity and result interpretation. |
| Cycling conditions | 63oC x 3 minutes; 95oC x 30 seconds; 45 cycles @ 95oC x 15 sec, 60oC x 1 min, 72oC x 1 sec | No comments |
| Interpretation of Ct values | Cts ≤ 30 are considered strong positive; Cts ≥ 30.1 and ≤35 are considered weak positive; Cts ≥ 35.1 and ≤40 are considered very weak positive; Cts ≥ 40.1 and ≤ 45 are considered suspicious; No Ct is interpreted as negative. | The interpretation of these ranges, particularly at the higher Ct values ≥ 35.1 may differ depending on the laboratory. In addition to the Ct values, consideration also has to be given to the shape of the curve, Ct of the internal control, etc in the final interpretation. On site the team saw curves produced by the instrument software, and noted some that were atypical were identified as a positive result. The laboratory sent the data files to the team and subsequent analysis confirmed atypical curves. |

## Appendix 2

| Procedure or Element Reviewed | Details | Assessment Team Comments |
|---|---|---|
| Validation data | Reference - Snow et al., 2006. Development, application and validation of a taqman® real-time RT-PCR assay for the detection of infectious salmon anemia virus. In New Diagnostic Technology: Applications in Animal Health and Biologics Controls. Dev. Biol., Basel Karger. 126, 133-145. *The laboratory clarified the reference Workenhe et al (2008) Absolute Quantitation of Infectious Salmon Anemia Virus Using Different Real-time RT-PCR Chemistries, Journal of Virological Methods, 154, 128-134.* | While a publication in a journal may be the outcome of a method validation procedure, it is not sufficient information to assess the completeness of the validation. |
| Positive control | ISAV RNA extracted from TO cell culture lysates - Ct values of the positive controls are low (<20) which can be a source of contamination. | ISAV RNA is a potential source of cross-contamination. Furthermore, because genomic RNA is used, it makes distinguishing between true positives and contamination with the positive control difficult and dependent on sequencing the RT PCR products. |
| Analysts certified to carry out test Proficiency testing | Dr. Molly Kibenge is the only analyst that performs diagnostic PCR assays for ISAV. A description of an ISAV real-time RT-PCR Ring Test Phase II with Fish Diagnostic Laboratories in Chile May 2009 was provided. The values obtained by Dr. Molly Kibenge appear to have been used as the expected values. *The laboratory clarified the reference for the ringtest Kibenge et al, (2011) Infectious Salmon Anemia Virus (ISAV) Ringtest: Validation of the ISAV Diagnostic process using Virus-spiked Fish Tissues and ISAV Taqman Real-time RT-PCR. Journal of Aquaculture Research and Development, 2,110.* | In lieu of a record of analyst competency based on proficiency testing (PT) participation or other methods such as interlaboratory comparisons or in-house prepared blind panels; a description of a ring test that was organized by the laboratory and distributed to other laboratories was provided. However, this indicates that Dr. Molly Kibenge created a PT panel for use by others, it is not a record of her performance of a PT panel. No results from and PT or ILC done by Dr. Molly Kibenge was provided. |

## Appendix 2

| Procedure or Element Reviewed | Details | Assessment Team Comments |
|---|---|---|
| Cell Culture | Cell cultures were inoculated in 329S. There were no worksheets or logbooks showing the details related to the inoculation. *The laboratory clarified that the sample IDs were written directly on the culture plates.* | A lack of records makes it difficult to verify the process. |
| | | |
| Note: Clarifications received from the laboratory have been added in the details column in Italics. | | |

# Content of information to provide from an OIE Reference Laboratory to inform the OIE on positive results of samples on OIE listed diseases

This form is intended to provide guidance to OIE Reference Laboratories wishing to submit their positive results on OIE listed diseases to the OIE. Please sent it by email to information.dept@oie.int
Please note that filling in this form does not avoid the requirement for the Reference Laboratories to inform the OIE Delegate of the Member Country or Territory from which the samples originated. It also doesn't replace the responsibility of the OIE delegate to inform the OIE Central Bureau, of any positive results for OIE listed diseases according to OIE' disease notification requirements.

Name of the OIE Reference Laboratory: Atlantic Veterinary College

Name of the designated OIE expert: Dr. Fred Kibenge

Name of the OIE listed disease or other identified disease in the sample(s): Infectious salmon anaemia (ISA)

Country of origin of the sample(s): Canada

Name, address and position of the person having sent the sample(s):

Dr. Rick Routledge
Professor
Dept. of Statistics & Actuarial Sci.
Simon Fraser University
8888 University Drive
Burnaby, BC
V5A 1S6, Canada

Nicole Gerbrandt
Hakai Scholar
The Hakai Network for Coastal People, Ecosystems and Management
Department of Biological Sciences
Simon Fraser University
8888 University Drive
Burnaby, BC
V5A 1S6, Canada

Date when the sample(s) were received by the laboratory: October 04, 2011.

Date(s) of laboratory results: October 12, 2011.

Date when the results were sent to the applicant: October 13, 2011.

| Sample id | Sample description | | | | Type of test(s) | Date | Result(s) | Serotype (if applicable) |
|---|---|---|---|---|---|---|---|---|
| | Type | Species | Location | Date of collection | | | | |
| 1-48 | heart tissues from smolts | Sockeye salmon (*Oncorhynchus nerka*) | British Columbia | 22/05/2011 to 18/07/2011 (attached) | Real-time RT-PCR | 05/10/2011 & 08/10/2011 | two samples positive | European genotype |

Other comments:

All samples were tested for ISAV using our real-time RT-PCR with TaqMan probe for ISAV segment 8 (Workenhe *et al.,* 2008). Samples that were positive were then further tested using our real-time RT-PCR with TaqMan probes for ISAV segment 6 for genotyping (Kibenge *et al.,* 2009). On the basis of our test results, samples #26 and #36 tested positive for ISAV of the European genotype. All the submitted material for samples #26 and #36 was used up in this testing, and no further testing (for example virus isolation and DNA sequencing) was attempted.

Cited references:

Kibenge, F., Kibenge, M., Simard, S., Riveroll, A., Pallapothu, M., and Salonius, K. 2009. Development of a DIVA system for an infectious salmon anaemia (ISA) virus vaccine using a qRT-PCR test based on segment 6 of the ISA virus. 14th *European Association of Fish Pathologists International Conference on Diseases of Fish and Shellfish, Prague, Czech Republic, September 14-19, 2009.*

Workenhe, S.T., Kibenge, M.J.T., Iwamoto, T., and Kibenge, F.S.B. 2008. Absolute Quantitation of Infectious Salmon Anaemia Virus Using Different Real-time Reverse Transcription PCR Chemistries. *Journal of Virological Methods, 154:128-134.*

Date and signature:

October 15, 2011. F.S.B. Kibenge

| Lab # | Sample ID | ISAV seg 8 Probe, Cts<br>Detects all ISAV | ISAV Seg 6 Probe 52 Cts<br>Detects European genotype | ISAV Seg 6 Probe 82 Cts<br>Detects North American genotype |
|---|---|---|---|---|
| **VT 10042011-1** | Sokeye heart _1 | 0 | not done | not done |
| **VT 10042011-2** | Sokeye heart _2 | 0 | not done | not done |
| **VT 10042011-3** | Sokeye heart _3 | 0 | not done | not done |
| **VT 10042011-4** | Sokeye heart _4 | 0 | not done | not done |
| **VT 10042011-5** | Sokeye heart _5 | 0 | not done | not done |
| **VT 10042011-6** | Sokeye heart _6 | 0 | not done | not done |
| **VT 10042011-7** | Sokeye heart _7 | 0 | not done | not done |
| **VT 10042011-8** | Sokeye heart _8 | 0 | not done | not done |
| **VT 10042011-9** | Sokeye heart _9 | 0 | not done | not done |
| **VT 10042011-10** | Sokeye heart _10 | 0 | not done | not done |
| **VT 10042011-11** | Sokeye heart _11 | 0 | not done | not done |
| **VT 10042011-12** | Sokeye heart _12 | 0 | not done | not done |
| **VT 10042011-13** | Sokeye heart _13 | 0 | not done | not done |
| **VT 10042011-14** | Sokeye heart _14 | 0 | not done | not done |
| **VT 10042011-15** | Sokeye heart _15 | 0 | not done | not done |
| **VT 10042011-16** | Sokeye heart _16 | 0 | not done | not done |
| **VT 10042011-17** | Sokeye heart _17 | 0 | not done | not done |
| **VT 10042011-18** | Sokeye heart _18 | 0 | not done | not done |
| **VT 10042011-19** | Sokeye heart_19 | 0 | not done | not done |
| **VT 10042011-20** | Sokeye heart _20 | 0 | not done | not done |
| **VT 10042011-21** | Sokeye heart _21 | 0 | not done | not done |
| **VT 10042011-22** | Sokeye heart _22 | 0 | not done | not done |
| **VT 10042011-23** | Sokeye heart _23 | 0 | not done | not done |
| **VT 10042011-24** | Sokeye heart _24 | 0 | not done | not done |
| **VT 10042011-25** | Sokeye heart _25 | 0 | not done | not done |
| **VT 10042011-26** | Sokeye heart _26 | 29.82 | 32.7 | 0 |
| **VT 10042011-27** | Sokeye heart _27 | 0 | not done | not done |
| **VT 10042011-28** | Sokeye heart _28 | 0 | not done | not done |
| **VT 10042011-29** | Sokeye heart _29 | 0 | not done | not done |
| **VT 10042011-30** | Sokeye heart _30 | 0 | not done | not done |
| **VT 10042011-31** | Sokeye heart _31 | 0 | not done | not done |
| **VT 10042011-32** | Sokeye heart _32 | 0 | not done | not done |
| **VT 10042011-33** | Sokeye heart _33 | 0 | not done | not done |
| **VT 10042011-34** | Sokeye heart _34 | 0 | not done | not done |
| **VT 10042011-35** | Sokeye heart _35 | 0 | not done | not done |
| **VT 10042011-36** | Sokeye heart _36 | 30.86 | 33.21 | 0 |
| **VT 10042011-37** | Sokeye heart _37 | 0 | not done | not done |
| **VT 10042011-38** | Sokeye heart _38 | 0 | not done | not done |
| **VT 10042011-39** | Sokeye heart _39 | 0 | not done | not done |
| **VT 10042011-40** | Sokeye heart _40 | 0 | not done | not done |
| **VT 10042011-41** | Sokeye heart _41 | 0 | not done | not done |
| **VT 10042011-42** | Sokeye heart _42 | 0 | not done | not done |
| **VT 10042011-43** | Sokeye heart _43 | 0 | not done | not done |
| **VT 10042011-44** | Sokeye heart _44 | 0 | not done | not done |
| **VT 10042011-45** | Sokeye heart _45 | 0 | not done | not done |
| **VT 10042011-46** | Sokeye heart _46 | 0 | not done | not done |
| **VT 10042011-47** | Sokeye heart _47 | 0 | not done | not done |
| **VT 10042011-48** | Sokeye heart _48 | 0 | not done | not done |
| **ADL-ISAV (European genotype)** | | 17.24 | 18.5 | 0 |
| **NBISAV01 (North American genotype)** | | 17.17 | 0 | 15.1 |
| **NTC (water)** | | 0 | 0 | 0 |

| Fish Number | Date collected |
|---|---|
| 1 | Jun-14 |
| 2 | Jun-14 |
| 3 | Jun-14 |
| 4 | Jun-14 |
| 5 | Jun-14 |
| 6 | Jun-14 |
| 7 | Jun-14 |
| 8 | Jun-20 |
| 9 | Jun-20 |
| 10 | Jun-20 |
| 11 | Jun-19 |
| 12 | Jun-21 |
| 13 | Jun-21 |
| 14 | Jun-21 |
| 15 | Jun-21 |
| 16 | Jun-20 |
| 17 | Jun-20 |
| 18 | Jun-20 |
| 19 | Jun-20 |
| 20 | Jun-22 |
| 21 | Jun-22 |
| 22 | Jun-22 |
| 23 | Jun-19 |
| 24 | Jun-19 |
| 25 | Jun-19 |
| 26 | Jun-19 |
| 27 | Jun-19 |
| 28 | Jun-19 |
| 29 | Jun-19 |
| 30 | Jun-21 |
| 31 | Jun-21 |
| 32 | Jun-16 |
| 33 | Jun-23 |
| 34 | Jun-15 |
| 35 | Jun-15 |
| 36 | Jun-05 |
| 37 | Jun-18 |
| 38 | Jun-18 |
| 39 | Jun-18 |
| 40 | Jun-18 |
| 41 | Jul-18 |
| 42 | May-22 |
| 43 | Jun-18 |
| 44 | Jun-18 |
| 45 | Jun-18 |
| 46 | Jun-18 |

| | |
|---|---|
| 47 | Jun-14 |
| 48 | Jun-14 |

**Report, 2nd November 2011**

**Testing of gill samples from juvenile *Oncorhynchus nerka* (sockeye salmon) collected in Rivers Inlet on the central coast of British Colombia, Canada.**

RNA from all gill samples was extracted as described by Devold et al 2000. The amount of RNA in each extraction sample was measured by NanoDrop ND-1000 (Spectrophotometer) (Table 1). For each tissue sample a negative control sample was included. An assay targeting the housekeeping gene, elongation factor alpha, was used as an internal control to test the quality of the RNA. We used the elf-alpha from Atlantic salmon which is not optimal for use on *O. nerka*. Two different assays targeting know ISA viruses were used: a) Assay **ISAV7** targeting segment seven from European ISA viruses (Plarre et al 2005), and b) assay **ISAV8-Uni** targeting segment 8 from all known ISA viruses (Snow et al 2006). The results of the analysis of the first tissues are presented in table 1. All samples were negative for presence of ISA virus genome. To make sure that the assays were working we included one positive control (Norwegian ISA virus) (Table 2). In the second and third run of the positive control we used a dilution of the RNA used in the first run.

According to the report from Kibenge the heart tissues from individuals 26 and 36 were positive for ISA virus using the ISAV8-Uni assay. Hence, we performed reruns on the gill samples from these two individuals and, in addition to the two ISA virus assays already used, we added one assay targeting segment 8 (European ISA viruses) and an HPR0 ISA virus assay. The most sensitive of these assays, used on European ISA viruses, are the ISAV7 and ISAV8 assays. As can be seen from table 2 the ISAV8-Uni is less sensitive compared to the ISAV7 assay. The ISAV-HPR0 assay is targeting segment 6 and is three ct values less sensitive then the ISAV7 assay. The results of the rerun are presented in table 3. Sample 36 was positive for ISA virus genome in one of the replicas. The ct value of 36.3 is close to the detection limit for the ISAV7 assay which may explain why only one of the replicas was positive, ie. the amount of ISA virus genome is to low to give a reproducible result. It should be added that none of the negative controls were positive in the first run or in the reruns of samples 26 and 36.

To test our results we performed a new extraction of RNA from the remaining gill tissues from the two individuals 26 and 36. These samples were run as five replicas. All samples

were negative for presence of ISA virus (Table 4). The positive controls were positive and the negative controls were negative.

As additional controls we also tested for two other pathogens, the parasitic flagellate *Ichthyobodo* spp. and the gill chlamydia Candidatus Clavochlamydia salmonicola. The latter is associated with epithelicystis in fresh water (Karlsen et al 2008). The results are presented in table 1. All samples tested were positive for presence of these two pathogens. However, when we tried to sequences the SSU from both we were not able to obtain a PCR product when using the RNA after cDNA synthesis. This indicates that the quality of the RNA is poor. Since the target for all ISA virus assays used in this study is RNA the poor quality may have influenced on the results.

**Conclusion**

We were able to detect ISA virus genome in gill sample 36, but this result was not reproducible. The ct value of the positive sample was close to the detection limit for the assay. The results obtained by Kibenge (using heart tissue) could not be reproduced by us using gill tissues from the same individuals. This could be explained as a result of tissue tropism for ISA virus in *O. nerka*, or a combined result of tissues tropism and poor quality of the RNA in the gill tissues sent to us. To my knowledge nothing is known about the susceptibility of *O. nerka* to ISA viruses, and the tissue distribution of the virus in this species is unknown.

**Table 1.** Results of the first testing of gill tissues.

| | | Gills | Gills | Gills | Gills | Gills | Gills |
|---|---|---|---|---|---|---|---|
| | RNA | **Elongation f.** | **Negative** | **ISAV** | **ISAV** | ***Ichthyobodo*** | **Clavochlamydia** |
| **Sample** | **ngram/µl** | **alpha** | **Control** | **ISAV7** | **ISAV8-uni** | **spp.** | **salmonicola** |
| 1 | 522,3 | 19,4 | Neg | Neg | Neg | 35,1 | 25,4 |
| 2 | 373,7 | 22,0 | Neg | Neg | Neg | 33,1 | 25,3 |
| 3 | 460 | 22,9 | Neg | Neg | Neg | 26,7 | 27,5 |
| 4 | 162,1 | 21,3 | Neg | Neg | Neg | 14,1 | 37,7 |
| 5 | 137,6 | 23,0 | Neg | Neg | Neg | 25,7 | 36,2 |
| 6 | 298,2 | 22,0 | Neg | Neg | Neg | 18,2 | 26,4 |
| 7 | 272,3 | 23,7 | Neg | Neg | Neg | 30,4 | 30,9 |
| 8 | 2,2 | 26,8 | Neg | Neg | Neg | | |
| 9 | 223,8 | 25,6 | Neg | Neg | Neg | | |
| 10 | 480,5 | 23,7 | Neg | Neg | Neg | | |
| 11 | 702,4 | 25,4 | Neg | Neg | Neg | | |
| 12 | 5,9 | 28,3 | Neg | Neg | Neg | | |
| 13 | 447,9 | 23,3 | Neg | Neg | Neg | | |
| 14 | 1752,6 | 29,3 | Neg | Neg | Neg | | |
| 15 | 1067,7 | 22,3 | Neg | Neg | Neg | | |
| 16 | 813,5 | 23,8 | Neg | Neg | Neg | | |
| 17 | 813,3 | 24,0 | Neg | Neg | Neg | | |
| 18 | 86,2 | 19,1 | Neg | Neg | Neg | | |
| 19 | 446,7 | 20,2 | Neg | Neg | Neg | | |
| 20 | 475,4 | 20,2 | Neg | Neg | Neg | | |
| 21 | 485,4 | 20,2 | Neg | Neg | Neg | | |
| 22 | 902,6 | 17,5 | Neg | Neg | Neg | | |
| 23 | 779 | 21,4 | Neg | Neg | Neg | | |
| 24 | 0 | 25,3 | Neg | Neg | Neg | | |
| 25 | 693,6 | 17,1 | Neg | Neg | Neg | | |
| 26a | 440,5 | 21,7 | Neg | Neg | Neg | 32,7 | 18,4 |
| 26b | 330,1 | 22,9 | Neg | Neg | Neg | 32 | 17,5 |
| 27 | 455 | 20,4 | Neg | Neg | Neg | | |
| 28 | 1361,8 | 21,4 | Neg | Neg | Neg | 22,2 | |
| 29 | 498,7 | 19,5 | Neg | Neg | Neg | 27,0 | |
| 30 | 304,1 | 20,3 | Neg | Neg | Neg | 31,5 | |
| 31 | 507,8 | 18,7 | Neg | Neg | Neg | 35,0 | |
| 32 | 425,8 | 19,5 | Neg | Neg | Neg | 29,9 | |
| 33 | 457,6 | 19,1 | Neg | Neg | Neg | 25,1 | |
| 34 | 52,9 | 20,8 | Neg | Neg | Neg | 32,3 | |
| 35 | 776,5 | 19,6 | Neg | Neg | Neg | 33,5 | |
| 36 | 531,8 | 21,9 | Neg | Neg | Neg | 30,9 | 26,2 |
| 37 | 108,2 | 18,0 | Neg | Neg | Neg | 29,6 | |
| 38 | 986,9 | 18,9 | Neg | Neg | Neg | 20,2 | |
| 39 | 747,5 | 17,8 | Neg | Neg | Neg | 35,8 | |
| 40 | 835,7 | 19,6 | Neg | Neg | Neg | 29,4 | |
| 41 | 293,7 | 19,8 | Neg | Neg | Neg | 26,8 | |
| 42 | 924,6 | 19,0 | Neg | Neg | Neg | 30,8 | |
| 43 | 696 | 18,7 | Neg | Neg | Neg | 21,6 | |
| 44 | 567,6 | 18,5 | Neg | Neg | Neg | 24,7 | |
| 45 | 488 | 18,7 | Neg | Neg | Neg | 28,8 | |
| 46 | 87,9 | 19,4 | Neg | Neg | Neg | 30,1 | |
| 47 | 295,1 | 18,8 | Neg | Neg | Neg | 32,1 | |
| 48 | 537,4 | 16,9 | Neg | Neg | Neg | 31,4 | |

**Table 2.** Positive controls of the three assays (Norwegian isolate used).

| Sample | ELF | ISAV7 | ISAV8-uni | Date | Dilution |
|---|---|---|---|---|---|
| Pos control | 14,8 | 21,7 | 26,3 | 26.10.2011 | |
| Pos control | 17,3 | 24,0 | 29,8 | 27.10.2011 | 1+ 9 |
| Pos control | 17,7 | 24,2 | 27,8 | 31.10.2011 | 1+ 9 |

**Table 3**. Rerun of samples 26 and 36.

| | rerun | rerun | rerun | rerun |
|---|---|---|---|---|
| **Sample** | **ISAV7** | **ISAV7** | **ISAV7** | **ISAV7** |
| 26a | Neg | Neg | Neg | Neg |
| 26b | Neg | Neg | Neg | Neg |
| 36 | 36,3 | Neg | Neg | Neg |

| | rerun | rerun | rerun | rerun |
|---|---|---|---|---|
| **Sample** | **ISAV8** | **ISAV8** | **ISAV8** | **ISAV8** |
| 26a | Neg | Neg | Neg | Neg |
| 26b | Neg | Neg | Neg | Neg |
| 36 | Neg | Neg | Neg | Neg |

| | rerun | rerun | rerun | rerun |
|---|---|---|---|---|
| **Sample** | **ISAV8-Uni** | **ISAV8-Uni** | **ISAV8-Uni** | **ISAV8-Uni** |
| 26a | Neg | Neg | Neg | Neg |
| 26b | Neg | Neg | Neg | Neg |
| 36 | Neg | Neg | Neg | Neg |

| | rerun | rerun | rerun | rerun |
|---|---|---|---|---|
| **Sample** | **ISAV-HPR0** | **ISAV-HPR0** | **ISAV-HPR0** | **ISAV-HPR0** |
| 26a | Neg | Neg | Neg | Neg |
| 26b | Neg | Neg | Neg | Neg |
| 36 | Neg | Neg | Neg | Neg |

**Table 4**. New run of the second extraction of RNA from gill samples 26 and 36 (five replicas). The positive control was run as two replicas.

| Sample | ISAV7 | ISAV7 | ISAV7 | ISAV7 | ISAV7 |
|---|---|---|---|---|---|
| 26 | Neg | Neg | Neg | Neg | Neg |
| 36 | Neg | Neg | Neg | Neg | Neg |
| Pos control | 15,7 | 15,7 | | | |

| Sample | ISAV8 | ISAV8 | ISAV8 | ISAV8 | ISAV8 |
|---|---|---|---|---|---|
| 26 | Neg | Neg | Neg | Neg | Neg |
| 36 | Neg | Neg | Neg | Neg | Neg |
| Pos control | 15,0 | 14,9 | | | |

| Sample | ISAV8-Uni | ISAV8-Uni | ISAV8-Uni | ISAV8-Uni | ISAV8-Uni |
|---|---|---|---|---|---|
| 26 | Neg | Neg | Neg | Neg | Neg |
| 36 | Neg | Neg | Neg | Neg | Neg |
| Pos control | 16,3 | 16,9 | | | |

| Sample | ELF |
|---|---|
| 26 | 18,9 |
| 36 | 18,8 |
| Pos control | 24,9 |

**Literature**

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Karlsen M, Nylund A, Watanabe K, Helvik JV, Nylund S, Plarre H.(2008). Characterization of 'Candidatus Clavochlamydia salmonicola': an intracellular bacterium infecting salmonid fish. Environ Microbiol. Jan;10(1):208-18. Epub 2007 Sep 24.

Are Nylund
Bergen 02.11.2011

| Lab # | Sample ID | ISAV seg 8 Probe, Cts Detects all ISAV | ISAV Seg 6 Probe 52 Cts Detects European genotype | ISAV Seg 6 Probe 82 Cts Detects North American genotype |
|---|---|---|---|---|
| **VT 10142011-49** | Coho heart-1 | 0 | not done | not done |
| **VT 10142011-50** | Chinook heart-2 | 0 | not done | not done |
| **VT 10142011-51** | Coho heart-3 | 0 | not done | not done |
| **VT 10142011-52** | Chinook heart-4 | 0 | not done | not done |
| **VT 10142011-53** | Coho heart-6 | 0 | not done | not done |
| **VT 10142011-54** | Sokeye heart-7 | 0 | not done | not done |
| **VT 10142011-55** | Coho heart-8 | 0 | not done | not done |
| **VT 10142011-56** | Coho heart-9 | 0 | not done | not done |
| **VT 10142011-57** | Chum heart-10 | 0 | not done | not done |
| **VT 10142011-58** | Coho heart-11 | 33.61 (1/2) | 33.06 | 0 |
| **VT 10142011-59** | Coho Gill-1 | 0 | not done | not done |
| **VT 10142011-60** | Chinook Gill-2 | 32.99 | 0 | 0 |
| **VT 10142011-61** | Coho Gill-3 | 0 | not done | not done |
| **VT 10142011-62** | Chinook Gill-4 | 0 | not done | not done |
| **VT 10142011-63** | Coho Gill-6 | 0 | not done | not done |
| **VT 10142011-64** | Sokeye Gill-7 | 0 | not done | not done |
| **VT 10142011-65** | coho Gill-8 | 0 | not done | not done |
| **VT 10142011-66** | Coho Gill-9 | 0 | not done | not done |
| **VT 10142011-67** | Chum Gill-10 | 33.77 (1/2) | 0 | 0 |
| **VT 10142011-68** | Coho Gill-11 | 0 | not done | not done |
| **ADL-ISAV (European genotype)** | | 16.85 | 18.45 | 0 |
| **NBISAV01 (North American genotype)** | | 16.43 | 0 | 14.73 |
| **NTC (water)** | | 0 | 0 | 0 |

**EXPLANATORY NOTES:**

1. All samples were tested for ISAV using real-time RT-PCR with TaqMan probe for ISAV segment 8. The result is the number of PCR cycles to reach reliable detection of product (cycle threshold or Ct).
2. Based on this PCR test, 3 of the tissue samples provided to the laboratory were RT-PCR positive. This test means only that 3 tissues contained ISAV sequences of genome segment 8.
3. The 3 tissues that tested positive were further tested using real-time RT-PCR with TaqMan probes for ISAV segment 6 for genotyping.
4. 1 of the 3 tissues tested positive for ISAV of the European genotype. This test did not detect any segment 6 sequences in the other 2 samples.
5. The ISAV sequences detected from the samples could be from viable or non-viable virus. The virus could be pathogenic or non-pathogenic.
6. The presence of ISAV sequences in the tissue samples does not imply that the subject fish had ISA or that ISA is present in the area where the subject fish were collected from.
7. Confirmation of ISAV infection requires virus isolation in cell culture and identification. This additional testing is underway, and results will not be known for another 6 weeks.
8. The laboratory did not participate in the collection of the samples or in the custody of the samples prior to receipt of the samples. The laboratory therefore cannot guarantee the integrity of the samples.
9. For convenience, the samples are identified using the labels provided by the party who requested testing by the laboratory.
10. The samples were tested as received at the laboratory.
11. In accordance with the Health of Animals Act, the test results have been reported to representatives from the CFIA by the laboratory.

**INTERPRETATION:**

1. Ct up to 40 are positive. Ct between 40.1 and 45 are considered suspicious. Sample is negative if there is no Ct value.

| AVC Lab # | Sample ID | ISAV seg 8 Probe, Cts Detects all ISAV |
|---|---|---|
| **VT 10312011-69** | Sokeye smolts-Gill and Heart 1 | 0 |
| **VT 10312011-70** | Sokeye smolts-Gill and Heart 3 | 0 |
| **VT 10312011-71** | Sokeye smolts-Gill and Heart 4 | 0 |
| **VT 10312011-72** | Sokeye smolts-Gill and Heart 5 | 0 |
| **VT 10312011-73** | Sokeye smolts-Gill and Heart 6 | 0 |
| **VT 10312011-74** | Herring - heart-17 | 0 |
| **VT 10312011-75** | Herring - heart-18 | 0 |
| **VT 10312011-76** | Herring - heart-19 | 0 |
| **VT 10312011-77** | Herring - heart-20 | 0 |
| **VT 10312011-78** | Herring - heart-21 | 0 |
| **ADL-ISAV (European genotype)** | | 18.65 |
| **NBISAV01 (North American genotype)** | | 17.95 |
| **NTC (water)** | | 0 |

**EXPLANATORY NOTES:**

1. All samples were tested for ISAV using real-time RT-PCR with TaqMan probe for ISAV segment 8. The result is the number of PCR cycles to reach reliable detection of product (cycle threshold or Ct).
2. Based on this PCR test, all 10 tissue samples provided to the laboratory were RT-PCR negative. This test means none of the tissues contained ISAV sequences of genome segment 8.
3. The laboratory did not participate in the collection of the samples or in the custody of the samples prior to receipt of the samples. The laboratory therefore cannot guarantee the integrity of the samples.
4. For convenience, the samples are identified using the labels provided by the party who requested testing by the laboratory.
5. Case history on the submitted samples was requested and is attached to this report.
6. The samples were tested as received at the laboratory.
7. In accordance with the Health of Animals Act, the test results have been reported to representatives from the CFIA by the laboratory.

**INTERPRETATION:**

1. Ct up to 40 are positive. Ct between 40.1 and 45 are considered suspicious. Sample is negative if there is no Ct value.

Raincoast Research Society, Box 399, Sointula, BC V0N 3E0

**Samples shipped October 27, 2011 to:**
**Dr. Fred Kibenge, Atlantic Veterinary College, PEI.**

Owner name and address: Alexandra Morton, Box 399, Sointula, BC V0N 3E0

**Vials # 17, 18, 19, 20, 21**

Date collected: Oct. 26, 2011
Location: South side of Malcolm Island, Pacific Management Area 12
Species: 5 Herring
Storage: Fish were placed in RNALater less than one hour after death
Shipped the next day
Clinical symptoms/lesions: bleeding from fins, blood speckled eyes, rolling over and sinking
Samples: hearts – whole, one per container

**Vials # 1, 3, 4, 5, 6,** (#2 omitted)

Date collected: June 2011
Location: Okisollo Channel, Pacific Management Area 13
Species: 5 Sockeye Smolts
Storage: Fish were placed in household type freezer and put in RNALater October 26
Shipped the next day
Clinical symptoms/lesions: bleeding from fins, popped eyeballs
Samples: hearts and gills, one fish per container

Thank you,

Alexandra Morton
250-973-2306
250-974-7086 (cell)
gorbuscha@gmail.com

| AVC Lab # | Sample ID | ISAV seg 8 Probe, Cts<br>Detects all ISAV |
|---|---|---|
| **VT 11072011-79** | Sockeye Gill and Heart Fish -1 | 0 |
| **VT 11072011-80** | Coho Gill and Heart Fish -2 | 0 |
| **VT 11072011-81** | Sockeye Gill and Heart Fish -3 | 0* |
| **VT 11072011-82** | Sockeye Gill and Heart Fish -4 | 0 |
| **VT 11072011-83** | Sockeye Gill and Heart Fish -5 | 0 |
| **VT 11072011-84** | Sockeye Gill and Heart Fish -6 | 0 |
| **VT 11072011-85** | Sockeye Gill and Heart Fish -7 | 0 |
| **VT 11072011-86** | Sockeye Gill and Heart Fish -8 | 0 |
| **VT 11072011-87** | Pink Gill and Heart Fish -9 | 0 |
| **VT 11072011-88** | Sockeye Gill and Heart Fish -10 | 0 |
| **VT 11072011-89** | Coho Gill and Heart Fish -11 | 0 |
| **VT 11072011-90** | Sockeye Gill and Heart Fish -12 | 0 |
| **VT 11072011-91** | Coho Gill and Heart Fish -13 | 0 |
| **VT 11072011-92** | Coho Gill and Heart Fish -14 | 0 |
| **ADL-ISAV (European genotype)** | | 18.5 |
| **NBISAV01 (North American genotype)** | | 18.06 |
| **NTC (water)** | | 0 |

**EXPLANATORY NOTES:**

1. All samples were tested for ISAV using real-time RT-PCR with TaqMan probe for ISAV segment 8. The result is the number of PCR cycles to reach reliable detection of product (cycle threshold or Ct).
2. Based on this PCR test, all 14 tissue samples provided to the laboratory were RT-PCR negative. This test means none of the tissues contained ISAV sequences of genome segment 8.
3. The laboratory did not participate in the collection of the samples or in the custody of the samples prior to receipt of the samples. The laboratory therefore cannot guarantee the integrity of the samples.
4. For convenience, the samples are identified using the labels provided by the party who requested testing by the laboratory.
5. Case history on the submitted samples was requested and is attached to this report.
6. The pooled tissue samples (heart and gill) were received at the laboratory and immediately stored at -80°C freezer until they were processed.
7. In accordance with the Health of Animals Act, the test results have been reported to representatives from the CFIA by the laboratory.

**INTERPRETATION:**

1. Ct up to 40 are positive. Ct between 40.1 and 45 are considered suspicious. Sample is negative if there is no Ct value.

*sample had a Ct value in one of the replicates with one of the algorithms but upon visual inspection had no amplification curve, hence the interpretation as a negative result.

November 20, 2011

**Raincoast Research Box 399, Sointula, B.C. V0N 3E0 Canada**

**Sample Owner**: Alexandra Morton
**Owner Address**: Box 399, Sointula, BC, V0N 3E0
**Collection site:** Harrison River (mill)
**Storage prior to shipping:** on ice
**Date samples taken from fish:** Nov. 4, 2011
**Clinical Symptoms:** Dead before spawning
**Tissues:** in individual whirl pack bags
**Fish Species:**
Fish # 1 - sockeye
Fish# 2 – Coho
Fish# 3 – sockeye
Fish # 4 – sockeye
Fish #5 – sockeye
Fish #6 – sockeye
Fish #7 – sockeye
Fish #8 – sockeye
Fish #9 – pink
Fish #10 – sockeye
Fish #11 – coho
Fish #12 – sockeye
Fish # 13 – coho
Fish # 14 - coho

.
Alexandra Morton

# DEVELOPMENT OF A DIVA SYSTEM FOR AN INFECTIOUS SALMON ANAEMIA (ISA) VIRUS VACCINE USING A qRT-PCR TEST BASED ON SEGMENT 6 OF THE ISA VIRUS


F.Kibenge[1], M.Kibenge[1], N. Simard[2] , A. Riveroll[2] , M. Pallapothu[2] and K. Salonius[2]

1 *University of Prince Edward Island, Atlantic Veterinary College, Charlottetown Canada*
2 *Novartis Animal Health, Aqua Health Ltd., Victoria, Prince Edward Island, Canada*


## Introduction:

Infectious salmon anaemia (ISA) virus is an important fish pathogen that has caused disease outbreaks in marine-farmed Atlantic salmon in Norway, Canada, Scotland, USA, Faeroe Islands and more recently in Chile in Godoy *et al.* (2008) and Scotland. The recent ISA outbreaks particularly in Chile have resulted in heavy morbidity and mortality, threatening to collapse the country's massive Atlantic salmon industry. Vaccination against ISAV has been permitted in Chile (and Norway) in 2009, as part of a regulatory control strategy by health authorities to effectively protect fish against ISA. A useful characteristic of a vaccine particularly for surveillance purposes, is the ability to differentiate naturally infected and vaccinated animals (DIVA). ISA virus is a member of the genus *Isavirus* within the family *Orthomyxoviridae,* possessing 8 single-stranded RNA segments of negative polarity. The virus occurs in two genotypes, I (European genotype) and II (North American genotype). Both genotypes have been associated with ISA in Canada and USA whereas only the European genotype has caused disease outbreaks in Norway, Scotland, Faeroe Islands and Chile. We have designed primers and TaqMan® probes for real-time (quantitative) RT-PCR targeting segment 6 of ISAV. This assay should differentiate between the two genotypes of ISAV, and could serve as a more discriminating diagnostic tool to differentiate between Chilean clinical isolates (Genotype I) and a commercial ISAV vaccine strain (Genotype II). The benefit would be that a diagnostic test would be available that would allow for the vaccination of fish in Norway, Scotland, Faeroe Islands and Chile without concern for detection of the Genotype II vaccine virus in diagnostic tests used for clinical surveillance programs for Genotype I field viruses. The objective of this study was to establish a diagnostic test for differentiation between vaccinated and ISAV-infected fish. We report the development of a qRT-PCR TaqMan® assay targeting segment 6 of ISAV. This assay was evaluated with fish tissues collected from vaccinated and experimentally infected Atlantic salmon.

## Materials & Methods:

Several ISA virus-specific primer-probe sets were designed using ProbeFinder online software (Roche). The ISAV segment 8 TaqMan® probe was as described by Snow *et al.* (2006) and was used to test fish tissues from Atlantic salmon vaccinated or challenged with ISA Genotype I in the laboratory (Figure 1). The TaqMan® qRT-PCR assay for the detection of ISAV was carried out in the LC 480 instrument (Roche) as described by Workenhe *et al.* (2008). The different primer-probe sets were evaluated using various ISAV isolates of the North American and European genotypes, grown in cell culture (Table 1). Figure 3 shows further evaluation of the selected primer/probes used fish tissues collected from vaccinated and experimentally infected Atlantic salmon, vaccinated with a monovalent ISA vaccine (Figure 2).

## Results:

Figure 1: Screening of fish tissue from laboratory studies for evaluation of vaccine interference using universal probe for ISA (Snow *et al*, 2006).

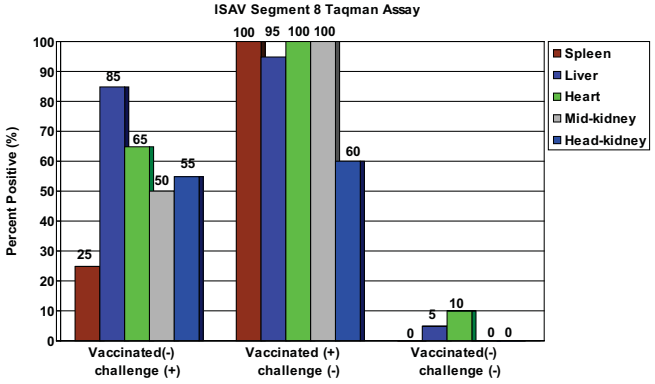



Table 1: Ct values (2 rep.) using 6 primer probe sets (Taqman segment 6): Evaluation and selection of probe primer sets for discrimination of genotype I and II virus grown in cell culture

| Virus isolate (Genotype) | Probe #E1 AnT=60°C | Probe #E1 AnT=61°C | Probe #E2 AnT=60°C | Probe #E2 AnT=61°C | Probe #NA1 AnT=60°C | Probe #NA1 AnT=61°C | Probe NA#2 AnT=60°C |
|---|---|---|---|---|---|---|---|
| ADL-Chile (Genotype I) | 18/18 | 18/17 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| U5575-1(Genotype I) | 30/35 | 27/34 | 18/18 | 16/18 | 32/36 | 0/0 | 0/0 |
| 390-98(Genotype I) | 20/19 | 20/20 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| NBISA-01(Genotype II) | 0/0 | 0/0 | 0/0 | 0/0 | 17/17 | 12/13 | 0/0 |
| RPC NB-98-280-2 (Genotype II) | 0/0 | 0/0 | 30/32 | 0/0 | 19/19 | ND | 19/19 |

**Probe #E1 and Probe #NA1 were selected for further evaluation

Atlantic salmon (20-30 g), i.p. vaccinated with 0.05ml of three batches of monovalent ISA (Genotype II) vaccine # 350011, 350021 and 350031 and saline controls Mock vaccinated with 0.05ml of 0.9% saline challenged by cohabitation with ISAV strain (Genotype I, HPR7b) infected Trojan fish at 627 dd (Figure 2).

**Figure 2: Cross protection Genotype II vaccine against Genotype I challenge (Chilean HPR7b)**

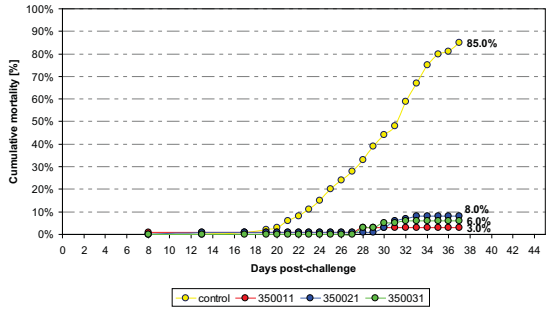
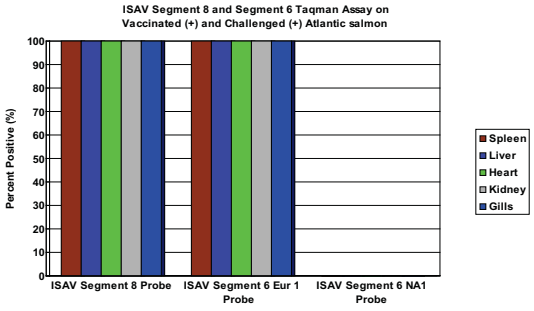



Figure 3: Screening of fish tissue from laboratory studies for evaluation of selected probe/primer sets** for potential value as a DIVA assay




## Discussion:

We are reporting that there is a significant level of interference of the vaccine status of fish with diagnostic tests using Segment 8 Taqman assays (Figure1) when analyzing tissues from laboratory vaccinated fish. Preliminary evaluation of the primer-probe set based on Segment 6 in a qRT-PCR test of virus grown in cell culture suggests a discriminating primer/probe set (Probe #E1) will detect infectious virus of Genotype I (European) but not Genotype II (NA). Validation of the robustness of the primer/probe sets, specific for Genotype I and Genotype II (#NA 1) is necessary. So far, analysis of tissue from vaccinated and challenged fish in the laboratory have demonstrated high positive reaction (Figure 3) with both Segment 8 and Segment 6 (Probe #E1) Taqman Probe assays. Work will continue to determine the relevance of the findings in clinical field settings given that reports of interference of vaccine status with other ISA vaccines and field sampled fish have not yet reported interference of vaccine in diagnostic tests.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Research Article Open Access

# Infectious Salmon Anaemia Virus (ISAV) Ringtest: Validation of the ISAV Diagnositic Process using Virus-spiked Fish Tissues and ISAV TaqMan® Real-time RT-PCR

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## Summary

Fourteen laboratories validated their procedure for detection of infectious salmon anaemia virus (ISAV) in fish tissues using TaqMan® real-time RT-PCR targeting ISAV RNA segment 8. The participants included 12 laboratories from South America, one from Asia and one from Europe. The OIE Reference Laboratory for ISA at the Atlantic Veterinary College in Canada served as the standard. All laboratories received a panel of 36 blind-coded samples representing six different ISAV preparations in homogenized fish liver or L-15 medium, each in six replicates. A clearly positive control ISAV of known titer was also included. From the results obtained, the 14 laboratories that submitted results reported no false positives. False negatives were mostly observed in the ISAV-spiked fish liver homogenate samples. The lowest virus titer to be detected in the fish liver homogenate was $10^1$ $TCID_{50}$/ml, but the virus titer that could be detected accurately by most laboratories was $10^3$ $TCID_{50}$/ml in L-15 medium. Within those laboratories that accurately detected presence of virus in a sample, there was great variation in the $C_t$ values making it impossible to recommend a single cut-off $C_t$ value. A significant factor influencing the $C_t$ values obtained and therefore the diagnostic sensitivity might be the thermocycler software used. The repeatability of the test within each laboratory was high, but the reproducibility between laboratories was low. Presumably this could be improved if all the laboratories used the same RNA extraction method since the starting quality and the quantity of the RNA template is the main determinant of the quality of results once reagents have been optimized. The low reproducibility of the test between laboratories is also suggestive of the need to standardize the threshold fluorescence line of the thermocycler software and to use properly trained personnel to perform the test.

## Introduction

Infectious salmon anaemia (ISA) virus (ISAV) is a major viral pathogen of marine-farmed Atlantic salmon (*Salmo salar*). ISAV belongs to the genus *Isavirus*, family *Orthomyxoviridae* [1]. The first registered outbreak of ISA occurred in Norway in 1984 [2]. Subsequently the disease was reported in Canada [3], Scotland [4], Faeroe Islands [5], and in Maine, USA [6]. In Chile, ISAV was first detected in 1999 in marine-farmed Coho salmon (*Oncorhynchus kisutch*) and was shown to be of the North American genotype [7], which subsequently became widespread in the Atlantic salmon industry in Chile but without signs of clinical disease [8]. ISAV of the European genotype caused a major epizootic in Chile's massive Atlantic salmon industry starting in June 2007 [8]. This virus was shown by phylogenetic analysis to be most closely related to Norwegian ISAVs isolated in 1997 and to have circulated in Chile for sometime prior to the index case in June 2007 [9]. Since the confirmation of the original ISA outbreak in Chile [8], the principal procedure of ISA laboratory diagnosis has been reverse transcription-polymerase chain reaction (RT-PCR), used directly on fish tissue samples, with primers targeting ISAV RNA segments 6 and 8, and sequencing of the PCR products (Sernapesca). RT-PCR testing in Chile is primarily performed by private diagnostic laboratories following the procedures outlined in the World Organization for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals [10].

The OIE Manual of Diagnostic Tests for Aquatic Animals recommends that when the PCR assay is used as a routine test, it is important to maintain the internal quality control [11]. The assay needs to be consistently monitored for repeatability and accuracy. Reproducibility between laboratories (ringtests) is recommended by the OIE to be estimated at least twice a year [12]. It is also advisable to regularly sequence the selected genomic regions in the viral isolates from the target country. This is especially true for the primer sites, to ensure that they remain stable so that the validation of the assay cannot be questioned.

## Materials and Methods

### Preparation and shipment of test samples

To simulate field samples, liver tissue was harvested from clinically healthy Atlantic salmon kept at the Atlantic Veterinary College (AVC) Aquatic Animal Facility. The fish were obtained as fingerlings from a local hatchery and were raised as non-challenge controls in strict isolation. The livers were weighed, homogenized, and then a 10% suspension in Leibovitz L-15 medium (L-15) was made. The liver homogenate was confirmed to be free of ISAV by conventional and

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real-time RT-PCR using ISAV RNA segment 8 primers in the OIE Reference Laboratory for ISA at AVC as detailed below (*RNA extraction and TaqMan® real-time RT-PCR*). The homogenate was then spiked with a known concentration of ISAV $10^5$ $TCID_{50}$/ml of ISAV strain ADL-PM 3205 ISAV-07 (ADL-ISAV-07) of European genotype [13], which then served as stock for making 10-fold serial dilutions from $10^4$ $TCID_{50}$/ml to $10^1$ TCID50/ml. Then ISAV-spiked liver samples with ISAV $10^5$ $TCID_{50}$/ml, $10^2$ $TCID_{50}$/ml and $10^1$ $TCID_{50}$/ml dilutions were used in the Ringtest. The liver spiked samples were to mimic field fish tissue samples which when received are macerated and suspended either in phosphate buffered formalin (PBS) or cell culture medium. In addition, two ISAV dilutions of $10^4$ $TCID_{50}$/ml and $10^3$ $TCID_{50}$/ml in L-15 medium were included in the Ringtest to represent cell culture lysate and/or the clarified supernatant of homogenized fish tissues. The liver homogenate in L-5 medium served as the negative control for the Ringtest panel. Also included were three replicates of ISAV $10^6$ $TCID_{50}$/ml in L-15 medium to be used as the positive control. The participating laboratories were instructed to provide their own negative sample, which was to check for ISAV contamination in individual laboratories. For each preparation, six replicates of 200 µl of the sample was mixed with 600 µl of AVL buffer (Qiagen), which inactivated the virus so that the sample was no longer infectious [14]. Samples were shipped from Canada to 12 laboratories in South America, one laboratory in Asia and one laboratory in Europe. Each participating laboratory received a total of 36 blind-coded samples, of which 24 were from experimentally spiked liver homogenate and 12 were experimentally spiked L-15 medium. Three vials were marked "ISAV", and contained ISAV strain ADL-ISAV-07 capable of giving a low cycle threshold ($C_t$) value (~ $C_t$ of 20) which was to be used as positive control. In total, each participating laboratory received 39 vials of samples, which were shipped frozen. Instructions were provided to each participating laboratory relative to its procedures and response time. Upon receiving the materials, each participating laboratory was instructed to immediately store the materials at -20°C until tested. The reference lab also used similarly handled samples for their subsequent testing.

## RNA extraction

Each participating laboratory was provided a table showing the order in which the samples were to be tested (Table 1). Each laboratory used their own RNA extraction kit to prepare the RNA samples and then performed an ISAV TaqMan® real-time RT-PCR procedure(s) to the 6 samples of one set, including a positive control sample and a negative control sample (lab's own, for example "no template control" or "water") for a total of 8 samples per day. Thus RNA extraction and TaqMan®

| **Day test performed** | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **Sample code** | 0A | 0B | 0C | 0L | 0J | 0K |
| | 0H | 0F | 0D | 0N | 0M | 0P |
| | 01 | 0G | 0E | 0R | 0Q | 0S |
| | 04 | 05 | 02 | 012 | 0T | 013 |
| | 08 | 06 | 03 | 015 | 014 | 016 |
| | 09 | 011 | 07 | 019 | 017 | 018 |
| **Positive control** | ISAV | | ISAV | | ISAV | |

**Table 1:** Coded samples provided to each laboratory and the respective day each sample was tested.

| Testing Lab | RNA Extraction Kit | RT-PCR Kit | Probe source | Thermocycler | | # Cycles | CT cut-off |
|---|---|---|---|---|---|---|---|
| | | | | Instrument | Software | | |
| OIE Ref Lab, A | QIAamp viral RNA extraction kit Qiagen | LightCycler 480 RNA Master Hydrolysis Probes | IDT | LightCycler 480 | LightCycler software release 1.5.0 | 45 | No CT is negative |
| Lab B | High Pure RNA Tissue kit Roche | LightCycler 480 RNA Master Hydrolysis Probes | IDT or Applied Biosystems | LightCycler 480 | LightCycler 480 software release 1.5.0 | 45 | No CT is negative |
| Lab C | High Pure RNA Tissue kit Roche | LightCycler 480 RNA Master Hydrolysis Probes | Roche | LightCycler 480 | LightCycler second derivative max | 45 | No CT is negative |
| Lab D | Total RNA kit I (E.Z.N.A) | Express One-Step qPCR Super-Script Mix | Applied Biosystems | StepOne (Applied Biosystems) | StepOne Software v2.0 FAST mode | 45 | No CT is negative |
| Lab E | Total RNA kit I (E.Z.N.A) | Invitrogen | | Stratagene MX 3000P | MxPro | | >38 is inconclusive No CT is negative |
| Lab F | Total RNA kit I (E.Z.N.A) | Invitrogen | | Stratagene MX 3000P | MxPro | | >38 is inconclusive No CT is negative |
| Lab G | Total RNA kit I (E.Z.N.A) | TaqMan® RNA-to-Ct™ 1-Step Kit | Applied Biosystems | StepOnepPlus Real-Time PCR System (Applied Biosystems) | StepOne Software v2.0 | 45 | >37.81 or no CT is negative |
| Lab H | No report | No report | No report | No report | No report | No report | No report |
| Lab I | High Pure RNA Tissue kit Roche | LightCycler 480 RNA Master Hydrolysis Probes | Roche | LightCycler 480 | LightCycler 480 second derivative max | 45 | No CT is negative |
| Lab J | High Pure Viral Nucleic Acid kit Roche | LightCycler RNA Master Hydrolysis Probes | | LightCycler 1.5 | LightCycler 4.0 | 45 | No CT is negative |
| Lab K | RNeasy Mini Kit Qiagen | One-step real-time RT-PCR Quantitech Probe Kit Qiagen | | Stratagene MX 3000P | MxPro | | No CT is negative |
| Lab L | Total RNA kit I (E.Z.N.A) | SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) | | Stratagene MX 3000P | MxPro | 40 | >38 or no CT is negative |
| Lab M | QIAamp viral RNA extraction kit Qiagen | SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) | IDT | Stratagene MX 3000P | MxPro 3005 V 4.01 build 369 | 45 | Seg 8 >35 & ELF1α <33 is negative |
| Lab N | Total RNA kit I (E.Z.N.A) | Stratagene Brilliant®II qRT-PCR Master Mix Kit | Applied Biosystems | Stratagene MX 3000P | MxPro QPCR default settings | 45 | No CT is negative |
| Lab O | MagMAX-96 Viral RNA Isolation Kit (MagMAX™ Express-96 Magnetic Particle Processors) | VetMAX Multiplex RT-PCR Reagents | confidential | Applied Biosystems 7500 Fast System | 7500 System SDS Software Version 1.4.0.25 | 40 | ≥38 is negative |
| Lab V | Total RNA kit I (E.Z.N.A) | SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) | | Stratagene MX 3000P | MxPro | 45 | >40 is negative |

**Table 2:** Real-time RT-PCR protocols used by each laboratory for amplification of ISAV segment 8.

| **Sample Identity** | Negative control | Liver in L-15 medium | ISAV $10^1$ in liver **homogenate** | ISAV $10^2$ in liver **homogenate** | ISAV $10^5$ in liver **homogenate** | ISAV $10^3$ in L-15 medium | ISAV $10^4$ in L-15 medium | Positive control **(ISAV $10^6$ in L-15 medium)** |
|---|---|---|---|---|---|---|---|---|
| **Sample Codes** | Lab's own | 01, 0B, 0D, 012, 0M, 018 | 0A, 0G, 0E, 015, 0T, 0K | 08, 011, 0C, 0L, 0Q, 016 | 0H, 05, 07, 019, 017, 013 | 04, 06, 02, 0R, 0J, 0P | 09, 0F, 03, 0N, 014, 0S | ISAV |
| **Testing Lab** | | | | | | | | |
| **OIE Ref Lab, A** | 0 | 0 | 34.98±0.39 | 31.97±0.38 | 31.27±0.64 (3/6) | 32.55± 0.58 | 29.63±0.38 | 22.7±0.55 |
| **Lab B** | 0 | 0 | 0 | 32.0± 0.4 (2/6) | 28.89±1.1 | 31.41±0.37 (2/6) | 31.04±0.99 | 25.98±1.93 |
| **Lab C** | 0 | 0 | 35.98±0.34 | 33.67±0.68 | 31.8±0.53 | 32.46±0.98 | 30.62±0.78 | 24.14±0.41 |
| **Lab D** | 0 | 0 | 30.9 (1/6) | 31.4±1.0 | 24.9±0.99 | 29.5±0.98 | 26.18±0.77 | 19.5±0.28 |
| **Lab E** | 0 | 0 | 40.87±1.1 | 36.85±0.77 | 30.0 ± 0.59 | 35.11±1.03 | 31.78±0.64 | 24.09±0.44 |
| **Lab F** | 0 | 0 | 39.15±0.69 | 35.62±0.62 | 30.32± 1.3 | 34.26±1.0 | 30.91±0.4 | 24.16±0.89 |
| **Lab G** | 0 | 0 | 0 | 31.11±0.66 | 26.59 ±1.18 | 29.39±0.27 | 26.58±52 | 20.58± 0.58 |
| **Lab H** | No report | No report | No report | No report | No report | No report | No report | No report |
| **Lab I** | 0 | 0 | 35.84±0.56 | 33.97±0.39 | 30.81±1.8 | 33.95±0.56 | 30.74±0.4 | 23.82±1.1 |
| **Lab J** | 0 | 0 | 29.64 ±2.17 (3/6) | 29.06 ±1.69 | 27.05 ± 3.64 | 28.96 ±0.83 | 27.49 ±0.45 | 21.05±1.1 |
| **Lab K** | 0 | 0 | 0 | 38.12±0.60 | 29.04±0.85 | 34.41±0.60 | 31.76±0.87 | 25.2±1.27 |
| **Lab L** | 0 | 0 | 38.85±0.62 | 36.18±0.86 | 28.13±0.62 | 34.39±0.79 | 31.28±0.62 | 23.72±0.78 |
| **Lab M** | 0 | 0 | 37.06±0.99 | 34.2±0.33 | 28.41±1.4 | 32.0±0.23 | 28.94±0.26 | 22.34±0.19 |
| **Lab N** | 0 | 0 | 37.82±1.3 (5/6) | 35.22±1.61 | 28.04±1.18 | 33.3±1.45 | 30.14±1.4 | 24.63±0.98 |
| **Lab O** | 40 | 39.9±0.2 | 36.39±1.2 | 33.57±0.45 | 24.2 ± 0.59 | 31.23±1.4 | 28.08±1.43 | 22.1±0.11 |
| **Lab V** | 0 | 42.97±0.51[1] (3/6)[2] | 41.02±0.49 | 31.14±15.37(5/6) | 29.9±0.91 | 35.41±0.33 | 32.24±0.32 | 25.13± 0.17 |

In Blue: denotes mean $C_t$ values that are ≥3 more than expected.
In green: denotes unexpected false negative(s).
In magenta: denotes sensitivity of real-time RT-PCR for ISAV is below that of most laboratories.
In red: denotes poor performing laboratory based on having more than two days (a) with mean Ct values that are ≥3 more than expected, and/or (b) with unexpected false negative(s), and/or (c) unable to detect a virus titer of $10^3$ $TCID_{50}$/ml.

**Table 3:** Overall performance (inter-lab and inter-assay) of different laboratories using real-time RT-PCR protocols for amplification of ISAV segment 8 Mean $C_t$ value ± SD[1] for assays performed on 6 different days. Where a sample had no $C_t$ value (i.e., $C_t$ = 0), the number of days in which it had a $C_t$ value ($C_t$ = >0) out of the six days it was tested is given in brackets[2].

| Sample ID | Repeatability of Reference Lab | Overall repeat-ability | Reproducibility | Performance above bench-mark* | Performance at benchmark* | Performance below bench-mark* | Performance above repro-ducibility | Performance above overall repeatability | Same as the overall repeat-ability or below |
|---|---|---|---|---|---|---|---|---|---|
| ISAV $10^1$ in liver **homogenate** | 1.11 | 2.04 | 4.61 | D, E, F, I, J, L, M, N, O | A, C, V | B, G, K | D, J | E, M, N, O | A, B, C, F, G, I, K, L,V |
| ISAV $10^2$ in liver **homogenate** | 1.07 | 2.54 | 5.54 | B, C, D, E, F, G, J, K, L, N, V | A, I, M, O | - | B | D, J, N, V | A, C, E, F, G, I, K, L, M, O |
| ISAV $10^3$ in L-15 medium | 1.63 | 2.43 | 5.46 | B, C, D, E, F, J, L, N, O | A, I, K | G, M, V | B, J | C, D, E, F,N, O | A, G, I, K, L, M, V |
| ISAV $10^4$ in L-15 medium | 1.08 | 2.23 | 4.65 | B, C, D,E, F, G, J, K, L, N, O | A, I | M, V | - | B, K, N, O | A, C, D, E, F, G, I, J, L, M, V |
| ISAV $10^6$ in L-15 medium | 1.54 | 1.53 | 4.51 | B, F, I, J, K, L, N | A, G | C, D, E, M, O, V | B | F, I, J, K, L, N | A, C, D, E, G, M, O, V |

*The repeatability and repeatability of the reference lab (Lab A) served as the standard. On this basis, most laboratories performed well.

**Table 4:** Summary of the laboratory performances (repeatability) relative to the repeatability, reproducibility and repeatability of the reference lab for individual ISAV titers.

RT-PCR assays were to be repeated 6 times except for the positive control, which was only extracted on days 1, 3 and 5. The different RNA extraction kits that were used by the different laboratories are listed in Table 2. The reference lab extracted the viral RNA using QiAamp Viral RNA mini extraction kit following the kit manual (Qiagen). RNA was extracted on six different days as shown in Table 1. On days 1, 3 and 5, one tube of the positive control was included. The viral RNA was eluted using 60 µl of AVE buffer (Qiagen) and stored at -80°C until used in ISAV TaqMan® real-time RT-PCR.

## TaqMan® real-time RT-PCR

Since the tissue samples in the Ringtest contained whole virus, each laboratory had the option to use a TaqMan® real-time RT-PCR targeting any ISAV RNA segment. All participating laboratories used ISAV TaqMan® probe targeting RNA segment 8; only two laboratories also reported results with probes to RNA segments 5 and 6. Therefore, the results in this report are for TaqMan® real-time RT-PCR targeting ISAV RNA segment 8 [15,16] except for lab O which did not disclose the probe used. The source of the ISAV RNA segment 8 primers, probes and kits were individually procured by the different laboratories. Thus, five different real-time PCR machines were used in this exercise (Table 2). Primers and probes were also ordered from several suppliers. However, all labs used the same primer and probe sequences previously described by Snow et al. (2006) [15]. The reference lab obtained the HPLC purified ISAV RNA segment 8 primers from Invitrogen Life Technologies and the ISAV RNA segment 8 dual-labeled probes from Integrated DNA Technologies Inc (IDT). The real-time RT-PCR with TaqMan® probe and primers targeting ISAV RNA segment 8 was performed as described by Workenhe et al. (2008) [16] using LightCycler 480 RNA Master Hydrolysis Probes (Roche) and LightCycler 480 machine. The data were analyzed by LightCycler software release 1.5.0. The RT-PCR was deemed to be sensitive if there was a 3 $C_t$ difference between 10-fold dilutions or a 7 $C_t$ difference between 100-fold dilutions, since it is generally accepted that a 3.3 $C_t$ difference between two samples is equal to a 10-fold difference in starting sample concentration [17,18].

## Statistical analysis

The results were analyzed and a performance report of all participating laboratories prepared. All participating laboratories received all results after analysis, with each laboratory identified only by its Code (to protect their identity) so that they could see how they compared among the other participating laboratories. The analysis

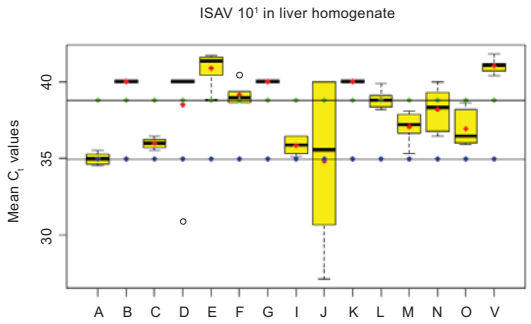


**Figure 1:** Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the $C_t$ values for each lab, error bars represent the lowest and the highest $C_t$ value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab median $C_t$ value and the line with (-♦-) represents the overall median for all laboratories.

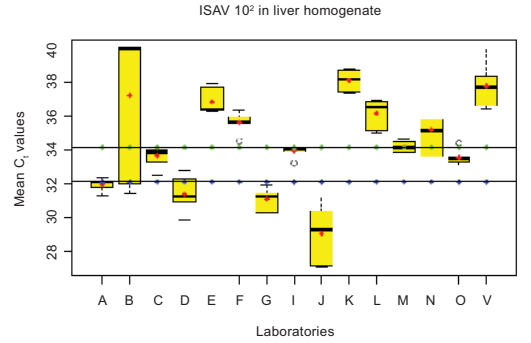


**Figure 2:** Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the $C_t$ values for each lab, the error bars represent the lowest and the highest $C_t$ value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab for ISA at AVC median $C_t$ value and the line with (-♦-) represents the overall median for all laboratories.

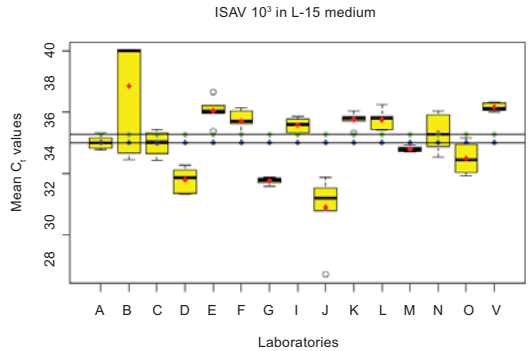


**Figure 3:** Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the $C_t$ values for each lab, error bars represent the lowest and the highest $C_t$ value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab for ISA at AVC median $C_t$ value and the line with (-♦-) represents the overall median for all laboratories.

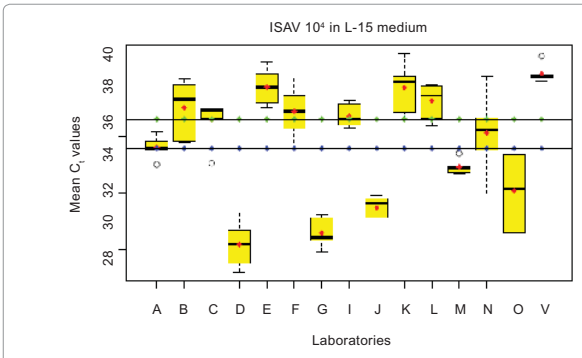


**Figure 4:** Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the Ct values for each lab, error bars represent the lowest and the highest Ct value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab for ISA at AVC median $C_t$ value and the line with (-♦-) represents the overall median for all laboratories.

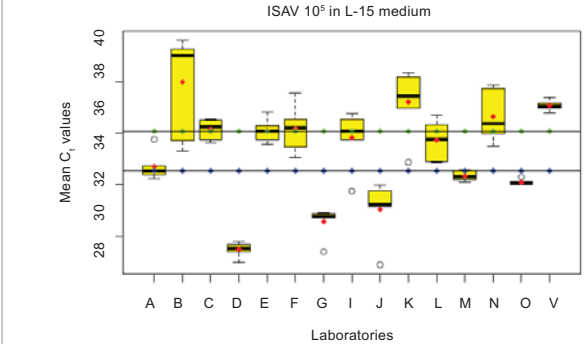


**Figure 5:** Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the $C_t$ values for each lab, error bars represent the lowest and the highest $C_t$ value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab median $C_t$ value and the line with (-♦-) represents the overall median for all laboratories.

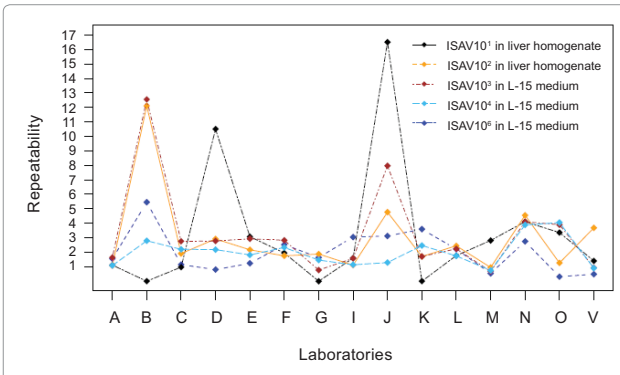


**Figure 6:** Repeatability from 15 laboratories across 6 dilutions.

included determination of analytical sensitivity and specificity and predictive values of the ISAV TaqMan® RT-PCR procedure for each participating laboratory. By using known varying amounts of ISAV in AVL buffer for RNA extraction, it allowed not only the establishment of the reproducibility of the ISAV TaqMan® RT-PCR procedure, but

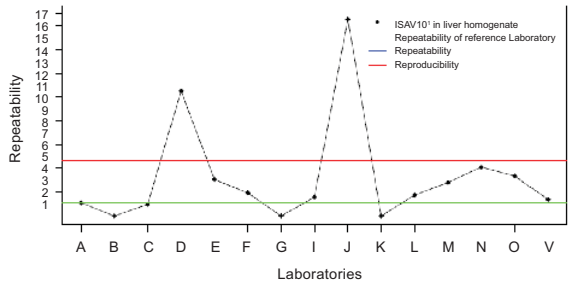


**Figure 7:** Repeatability from 15 laboratories represents the ISAV10$^1$ $TCID_{50}$/ml in liver homogenate dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

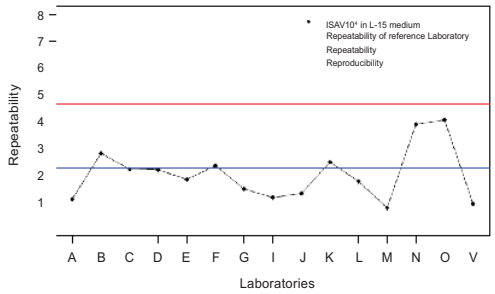


**Figure 10:** Repeatability from 15 laboratories represents the ISAV $10^4$ $TCID_{50}$/ml in L-15 medium dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

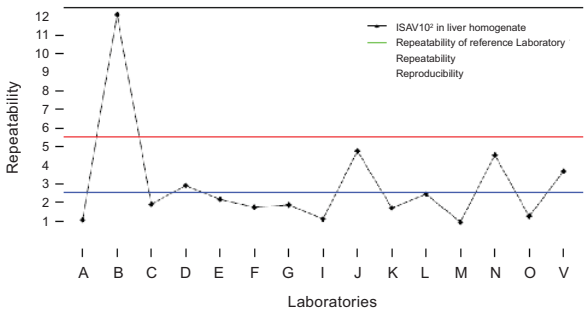


**Figure 8:** Repeatability from 15 laboratories represents the ISAV $10^2$ $TCID_{50}$/ml in liver homogenate dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

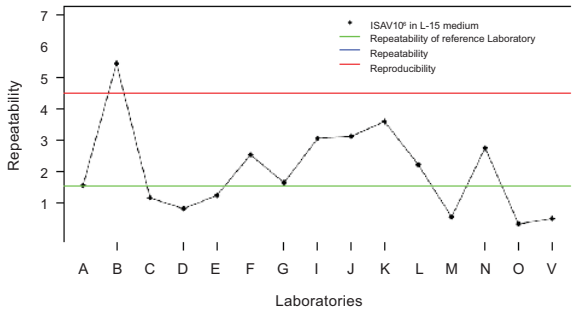


**Figure 11:** Repeatability from 15 laboratories represents the ISAV $10^6$ $TCID_{50}$/ml in L-15 medium dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

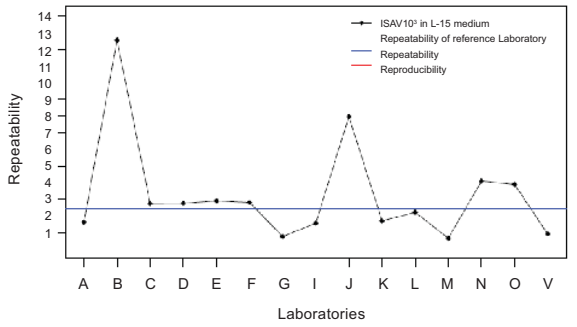


**Figure 9:** Repeatability from 15 laboratories represents the ISAV $10^3$ $TCID_{50}$/ml in L-15 medium dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

also to objectively compare the relative performance of the different laboratories' total RNA extraction step, ISAV TaqMan® assay protocol, thermocyclers, and technologists combination against the reference lab as standard.

Statistical analysis on the data was performed using R software [19] for statistical computing and graphics. Results were compiled and analyzed as per ISAV dilution and each participating laboratory. For the statistical analyses, all negative results (see Table 3) were replaced with a $C_t$ value of 40. Box plot summary statistics were generated. Repeatability and reproducibility were calculated according to the international standard [20] ISO 5725. Random effects model [21] was used to estimate the variance components between laboratories and within laboratories' $C_t$ values for each dilution, which were used to estimate the repeatability and reproducibility. Then the residual variance was used to calculate the repeatability and the total variance was used to calculate the reproducibility [22]. The repeatability is defined by ISO as "the value less than or equal to which the absolute difference between two test results obtained under repeatability conditions may be expected to be with a probability of 95%". The reproducibility is defined by ISO as "the value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions may be expected to be with a probability of 95%". In all cases, two external benchmarks were used. The first benchmark, represented in Figure 7, Figure 8, Figure 9, Figure 10, Figure 11 by the blue line, is based on the international standard ISO 5725 [20], where if the repeatability of a participating laboratory exceeds the repeatability of the corresponding ISAV dilution investigated (i.e., the blue line) then the laboratory's repeatability is not up to standard. The second benchmark, represented in Figure 7, Figure 8, Figure 9, Figure 10, Figure 11 by the green line, is based on the repeatability of the reference lab (Lab A), where if the repeatability of a participating laboratory exceeds the repeatability of the reference lab (i.e., the green line) but is below the repeatability of the corresponding ISAV dilution investigated (i.e., the blue line) then the laboratory's repeatability is considered acceptable. Therefore, the repeatability for each laboratory was calculated and plotted across several dilutions. Repeatability for each laboratory was also plotted against the repeatability of the reference lab, as well as the repeatability

and the reproducibility for each dilution.

## Results

The study was set up to compare the efficiency of RNA extraction from serial dilutions of ISAV-spiked fish liver tissue, and the sensitivity of TaqMan® real-time RT-PCR assay of several diagnostic laboratories that test fish samples for ISAV. For purposes of this Ringtest, the reference lab used five criteria for interpreting the RT-PCR results (strong positive, weak positive, very weak positive, suspicious, and negative). The sample was considered strong positive if the $C_t$ value was below ≤30, weak positive when $C_t$ was ≥ 30.1 but ≤35 $C_t$, the sample was very weak positive when the $C_t$ value was between 35.1 and 40, was considered suspicious if $C_t$ was between 40.1 and 45.0, and was negative if there was no $C_t$ value. In this study each participating laboratory set its own cut-off $C_t$ value as shown in Table 2. A sample was considered negative if there was no $C_t$ value but the cut-off $C_t$ value for negative samples ranged between ≥40 and ≥35. One laboratory included the result of ELF1α assay (Snow et al., 2006) [15] in the interpretation of the $C_t$ values (Table 2).

The results of the 15 participating laboratories are summarized in Table 3 as mean $C_t$ plus standard deviation. None of the laboratories reported a false positive result; all liver homogenates without virus and all negative controls were reported negative. In contrast, false negatives were reported in some ISAV dilutions, mostly in liver homogenate samples. The sample ISAV $10^5$ $TCID_{50}$/ml in liver homogenate was prepared as the stock from which the two ISAV-spiked liver samples, $10^2$ $TCID_{50}$/ml and $10^1$ $TCID_{50}$/ml, were prepared as serial 10-fold dilutions. During RNA extractions in the reference lab, it was noted that this stock sample tended to clog the Qiagen column in contrast to the serial dilutions, and gave very variable results. It was included in the Ringtest panel to check on the efficiency of extractions in the participating laboratories. Consequently, the results of the ISAV $10^5$ $TCID_{50}$/ml in liver homogenate sample were not included in the statistical analysis.

For most laboratories, with a few exceptions, the $C_t$s obtained reflected the serial dilution of the samples particularly for ISAV in L-15 medium. The $C_t$s increased with lower ISAV titer (for example for the reference lab, ISAV at $10^6$, $10^4$, and $10^3$ $TICD_{50}$/ml in L-15 medium had $C_t$s of 22.7±0.55, 29.63±0.38, and 32.55± 0.58, respectively), and the sensitivity of the RT-PCR was achieved within each laboratory whereby there was a 3 $C_t$ difference between 10-fold dilutions ($10^3$ $TCID_{50}$/ml and $10^4$ $TCID_{50}$/ml in L-15 medium) and a 7 $C_t$ difference between 100-fold dilutions ($10^4$ $TCID_{50}$/ml and $10^6$ TCID50/ml in L-15 medium) (Table 3). The summary statistics results for the sample dilutions are presented in Figures 1-5, showing the mean(◆), the median (-) as well as the Inter-Quartile range (▪) of the $C_t$ values for each laboratory; the error bars represent the lowest and the highest $C_t$ value. Also shown is the extreme value (○) where applicable. The median $C_t$ value (-◆-) is for the reference lab and the overall median $C_t$ value (-◆-) for all laboratories is also shown. The larger the box, the more spread out the $C_t$ values are. The repeatability for all dilutions is shown in Figure 6 while repeatability and reproducibility for individual dilutions for all laboratories are shown in Figure 7, Figure 8, Figure 9, Figure 10, Figure 11. The closer the points appear for the same laboratory in Figure 6 (for example, labs A, C, F, G, L), the better the consistency of the laboratory results across dilutions. Generally, the variations between laboratories (reproducibility) are expected to be larger than the variations within the laboratories (repeatability). Thus, excess variability within a particular laboratory (for example, labs B, D, J) reveals a strong indication of inconsistency in results. Therefore, repeatability of a particular laboratory that is closer or larger than the reproducibility (--) is not detrimental (Figure 7, Figure 8, Figure 9, Figure 10, Figure 11), and vice versa for the overall average (--).

### ISAV in the ISAV $10^1$ $TCID_{50}$/ml in liver homogenate:

Three laboratories (B, G, K) did not detect ISAV at $10^1$ $TCID_{50}$/ml in liver homogenate sample while eight laboratories (D, E, F, J, L, M, N, V) reported some false negatives in this sample. All laboratories reported mean $C_t$ values that were more than the reference lab median $C_t$ (Figure 1) and four laboratories (E, F, L, V) $C_t$ values were above the overall median $C_t$s (Figure 1). The data were analyzed for both repeatability and reproducibility, and with a 95% confidence, the difference of two values (repeatability) from the same laboratory did not exceed 2.04, whereas two values (reproducibility) from different laboratories with 95% confidence do not differ by more than 4.61. Nine laboratories (A, B, C, F, G, I, K, L, V) had the same or lower repeatability than the repeatability of all laboratories whereas for 4 laboratories (E, M, N, O) the repeatability was above the repeatability of all laboratories. Laboratories D and J had repeatability greater than the reproducibility value (Table 4, Figures 6 and 7), which was due to the larger variability that was created by replacing the negative $C_t$ values (Table 3). Also, laboratories B, G, and K showed repeatability below that of the reference lab (Lab A) because they consistently had all negative $C_t$ values (i.e., they did not detect any viral RNA) in this sample dilution (Table 3).

### ISAV in the ISAV $10^2$ $TCID_{50}$/ml in liver homogenate:

Two laboratories (B, V) reported some false negatives for the ISAV at $10^2$ $TCID_{50}$/ml in liver homogenate sample. Another five laboratories (E, F, K, L, N) had $C_t$ values that were ≥3 more (i.e., 10-fold less virus titer) than expected. The estimated repeatability and reproducibility for this sample were 2.54 and 5.54, respectively. Four laboratories showed repeatability above the repeatability (D, J, N, V), and the repeatability of laboratory B exceeded the reproducibility (Table 4, Figures 6 and Figure 8).

### ISAV in the ISAV $10^3$ $TCID_{50}$/ml in L-15 medium

False negatives were reported by laboratory B (4/6) in ISAV at $10^3$ $TCID_{50}$/ml in L-15 medium sample, and for statistical analysis, these were replaced by a $C_t$ value of 40. The repeatability and reproducibility for the sample of this dilution were 2.43 and 5.46, respectively. Six laboratories (C, D, E, F, N, O) showed repeatability above the repeatability but below the reproducibility (Figure 9). The repeatability of two laboratories (B, J) exceeded the reproducibility (Figure 9). The box plot for these laboratories was large indicating larger variability in the $C_t$ values reported (Table 4, Figures 3 and 9).

### ISAV in the ISAV $10^4$ $TCID_{50}$/ml in L-15 medium:

No laboratory had a missing value in this sample. In the box plot eight laboratories had $C_t$ values above the overall median and five were below the laboratory A median (Figure 4). The repeatability and reproducibility were 2.23 and 4.65, respectively. All laboratories had repeatability below the dilution's reproducibility (Figure 10). However, the repeatability of four laboratories (B, K, N, O) was above the repeatability. The box plot of these laboratories were large (Figure 4) indicating larger variability in the $C_t$ values reported (Table 4, Figures 4 and Figure 10).

### ISAV in the ISAV $10^6$ $TCID_{50}$/ml in L-15 medium

No laboratory had a negative $C_t$ value in this sample, which was the ISAV positive control. The box plots of nine laboratories (B, C, E,

F, I, K, L, N, V) were above or very close to the overall median and four laboratories (D, G, J, O) were below the reference lab median (Figure 5). The estimated repeatability and reproducibility were 1.53 and 4.51, respectively. The repeatability of laboratory B exceeded the reproducibility, while six laboratories (F, I, J, K, L, N) showed repeatability above the repeatability (Table 4, Figure 11).

The ISAV at $10^4$ $TCID_{50}$/ml and at $10^6$ $TCID_{50}$/ml in L-15 medium were identified as positive by all laboratories, although two laboratories (B, K) had $C_t$ values ≥3 more (i.e., 10-fold less virus titer) than expected for the positive control (Table 3). Overall, the repeatability of the reference lab was always below the repeatability except for the ISAV at $10^6$ $TCID_{50}$/ml in L-15 medium where the two were similar (Table 4, Figure 11). The repeatability of the samples ranged from as low as 1.53 for the ISAV at $10^6$ $TCID_{50}$/ml in L-15 medium (positive control) to as high as 2.54 for the ISAV at $10^2$ $TCID_{50}$/ml in liver homogenate. Similarly, the reproducibility ranged from 4.51 for the ISAV at $10^6$ $TCID_{50}$/ml in L-15 medium (positive control) to 5.54 for the ISAV at $10^2$ $TCID_{50}$/ml in liver homogenate.

## Discussion

This Ringtest was set up to compare the efficiency of RNA extraction from serial dilutions of ISAV-spiked fish liver tissue, and the sensitivity of Taqman® real-time RT-PCR assay of several laboratories involved with ISAV diagnostic testing. As most private fish diagnostic laboratories in South America have adopted real-time RT-PCR with Taqman® probe assay for detection of ISAV in field samples, a need for standardization and optimization of the test was requested. The purpose was to be sure that all the diagnostic laboratories were performing at the same level and the reported results were accurate. Considering that the private diagnostic laboratories do not normally quantify the RNA used in the RT-PCR and since most of the variation between laboratories is at the RNA extraction step, this Ringtest was designed to use fish tissue spiked with known amounts of ISAV provided in AVL buffer.

In the reference lab, RNA extracted from some fish tissues has sometimes given a negative real-time RT-PCR result when extracted at high concentration of tissue homogenate, but will give a positive result when either a higher dilution is extracted or conventional RT-PCR is used. Thus, this Ringtest included the ISAV at $10^5$ $TCID_{50}$/ml in liver homogenate sample (Table 3) for purposes of evaluating the RNA extraction protocols used in the different diagnostic laboratories. This sample was the initial preparation that was made, and was then diluted down using L-15 medium to generate the ISAV at $10^2$ $TCID_{50}$/ml and ISAV at $10^1$ $TCID_{50}$/ml in liver homogenate samples; the lower virus titer samples also contained less liver tissue. Consequently, the results of the ISAV at $10^5$ $TCID_{50}$/ml in liver homogenate sample were not included in the statistical analysis.

We objectively compared RT-PCR protocols and thermocylers between laboratories in their ability to reproducibly amplify segment 8 of ISAV in comparison to a reference laboratory. We avoided the risk of "importing" the virus by having all samples (including the spiked liver tissue) mixed with AVL buffer (Qiagen), which inactivated the virus so that the samples were no longer infectious [14]. Fourteen international laboratories (in South America, Asia and Europe) requested to participate in order to validate their ISAV assays. This report is only of results by all laboratories using ISAV RNA segment 8 primers and probes [15] except for lab O which did not disclose the probe used and had different working dilution solutions.

There was no standardized RNA extraction protocol; each laboratory used their protocol and their thermocyler as described in Table 2. Whether these are the reasons for the variation observed in the results is not clear because even those laboratories that used the same kits and/or thermocyler had variable results. In general, there were no false positives reported by any laboratory, even though variable $C_t$ cut-offs were used by different laboratories to call a sample negative. Within an individual laboratory, this should increase confidence that there was no cross contamination of samples.

On the basis of these results, it was more likely to obtain a false negative than a false positive result, regardless of the virus concentration in the sample. This was particularly true for the ISAV dilutions in liver tissue homogenate as compared to the dilutions in L-15 medium. All laboratories were able to detect ISAV in L-15 medium samples while there was difficulty in detecting virus in the liver homogenate samples. This could be a result of poor RNA quality (degraded or very limited quantity in the sample) or may be due to presence of RT-PCR inhibitory factors in the liver tissue homogenate. Overall, samples of ISAV in the liver homogenate had poor reproducibility. However, the fact that only one lab reported some false negatives for the ISAV at $10^5$ $TCID_{50}$/ml in liver homogenate sample implies that RNA extraction is probably not a major issue for most laboratories, and that all RNA extraction kits used in this study perform well in the diagnostic laboratory. In any case, as a safeguard, it is recommended that when a fish tissue tests negative on the initial screen with real-time RT-PCR, the test should be repeated either on the same RNA preparation or a repeat extraction of RNA. It is also a good practice to attempt virus isolation using permissive cell lines [23] and confirm the virus isolate with real-time RT-PCR before reaching a conclusive diagnosis.

Within those laboratories that accurately detected presence of virus in a sample, there was great variation in the $C_t$ values. The reference lab considered the $C_t$ values to be off if there was at least a 3 $C_t$ difference as this reflected a 10-fold difference in the template concentration [17, 18] and therefore virus titer. However, the difference could be due to the use of different thermocylers, and different software for data analysis, and this has to be accounted for. Regardless of the method of RNA extraction and DNA thermocyler used, the lowest amount of ISAV titer that was detected by almost all laboratories was $10^3$ $TCID_{50}$/ml in L-15 medium.

Because there was overlap in both methods of RNA extraction and the thermocylers used in the laboratories that did well in the Ringtest and those that performed poorly, the variability in the results could also be reflective of the level of training and competence of the individuals who performed the test. In addition, the accuracy of the equipments (pipettes), RNA extraction kits, and thermocylers in the different laboratories should be considered. Even with variable equipments, most laboratories were able to accurately detect ISAV in the correct samples. Those that performed poorly on this Ringtest may require more training of their technical staff or to upgrade their thermocylers.

An interesting observation that could easily be overlooked is the effect of the software in the different thermocyclers on the threshold fluorescence; the value that the fluorescence intensity has to exceed in order to register a $C_t$ value. From Tables 2 and 3, it is apparent that the seven laboratories that used the Stratagene software MxPro (Lab E, F, K, L, M, N, V) all reported relatively high $C_t$ values compared to the other participating laboratories for the same samples, and in fact had the highest $C_t$ values for the samples with the lowest amounts of virus (ISAV at $10^1$ $TCID_{50}$/ml and $10^2$ $TCID_{50}$/ml in liver homogenates) except for Lab V "ISAV $10^2$ $TCID_{50}$/ml in liver homogenate", but the lower mean $C_t$ value in this case had a very high standard deviation. The consequence is that these seven laboratories were flagged in Table 3 for

having mean $C_t$ values that were ≥3 more than expected and/or having unexpected false negative(s) for the ISAV-spiked liver samples that had the lowest amounts of virus. This indicated to us that a significant factor influencing the $C_t$ values obtained and therefore the diagnostic sensitivity might be the software used. In this particular case, adjusting the threshold fluorescence line in the software appropriately may address the problem of variation in the $C_t$ values, which might allow the generation of a single cut-off $C_t$ value for all laboratories irrespective of the thermocycler and software used.

## Conclusions

In conclusion, this Ringtest showed that there are various RNA extraction kits as well as thermocylers that are used in the laboratory diagnosis of ISAV, resulting in poor reproducibility of the test result as a $C_t$ value. The variation in performance of different laboratories could also result in false reporting of the fish tissue sample status. The sensitivity of TaqMan® real-time RT-PCR for ISAV for most laboratories is $10^3$ $TCID_{50}$/ml in L-15 medium, although a significant factor influencing the $C_t$ value and therefore the diagnostic sensitivity might be the thermocycler software used. It is considered that exercises such as this one if carried out regularly would encourage individual laboratories to assess their performance in comparison with the other laboratories.

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# Development, Application and Validation of a Taqman® Real-Time RT-PCR Assay for the Detection of Infectious Salmon Anaemia Virus (ISAV) in Atlantic Salmon (*Salmo salar*)

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**Key words:** Infectious salmon anaemia virus, ISAV, real-time PCR, qRT-PCR, Taqman.

**Abstract:** Infectious salmon anaemia (ISA) is a disease of cultured Atlantic salmon (*Salmo salar*) which was successfully eradicated from Scotland following its emergence in 1998. The rapid deployment of sensitive diagnostic methods for the detection of ISA virus (ISAV) was fundamental to the swift eradication of ISA disease in Scotland and continues to be of crucial importance to surveillance of the aquaculture industry. This study reports the development, validation, application and interpretation of two independent, highly sensitive and specific semi-quantitative Taqman® real-time RT-PCR (qRT-PCR) methods for the detection of ISAV. Such technology offers considerable advantages over conventional RT-PCR methods in current routine use for ISAV surveillance. These include an increased sensitivity, enhanced specificity, semi-quantification using endogenous controls, a lack of subjectivity in results interpretation, speed of processing and improved contamination control.

## Introduction

Infectious salmon anaemia virus (ISAV) is a pathogen of cultured Atlantic salmon (*Salmo salar*) which has caused significant production losses in Norway, Scotland, Canada, the U.S.A., and the Faroes. Emergence of ISA disease in Scotland in the late 1990s prompted a swift and apparently successful eradication programme [1]. The disease has, however, become established in Norway and Canada where in 1999 alone it was estimated to be responsible for losses totalling $11 and $14 million, respectively [2].

The rapid deployment of sensitive diagnostic methods for the detection of ISAV

was fundamental to the swift eradication of ISA disease in Scotland and remains equally important in managing the impact and spread of this virus in counties where it is endemic. Three diagnostic assays are in widespread use for ISAV diagnosis, which include virus isolation on permissive cell lines such as SHK-1 [3], indirect fluorescent antibody testing (IFAT) [4] and reverse transcriptase polymerase chain reaction (RT-PCR) [5]. Of these assays, the RT-PCR is generally regarded as the most sensitive [6-8] although a recent study highlighted a lack of reproducibility between laboratories and emphasized a need to standardize inter-laboratory protocols [9]. Much of the variability was attributed to the use of different external controls and methods applied to interpret and confirm results [9].

Recent developments in real-time PCR technology offer the potential to overcome many of the limitations of conventional PCR including an improved sensitivity of ISAV diagnostics. Indeed, a sensitivity increase of 100-fold over conventional RT-PCR was recently reported using a SYBR green-based assay for the detection of ISAV [10]. Further advantages of Taqman® real-time PCR chemistry include improved specificity of amplification, since signal generation is reliant on the annealing of a highly specific secondary probe to the primary amplification product. In addition the development of an endogenous control allows the confirmation of target integrity as well as facilitation of relative quantification of viral load and subsequent comparison between samples. Real-time PCR thus offers considerable advantages over conventional RT-PCR-based protocols and has the potential to reduce problems in the interpretation and reproducibility of results between laboratories.

In this report we describe the development, validation and application of Taqman®-based assays targeting two independent genes of ISAV coupled to an assay targeting the constitutively expressed endogenous control translation elongation factor 1 alpha (ELF1$\alpha$) [11]. The comparative efficiencies of these assays were determined and a method for the relative quantification of viral target-level developed. Assays were compared with the conventional RT-PCR in widespread use for ISAV diagnostics [5] throughout an experimentally induced clinical outbreak of ISAV and in samples analysed for routine ISAV diagnostics in our laboratory in 2004.

## Materials and Methods

### Development of Taqman Real-Time RT-PCR method

#### *Primers and probe design*

RT-PCR primer pairs and Taqman® MGB probes targeting ISAV segments 7 and 8 were selected from alignments of all available sequences using published sequence data and the Primer Express software (Applied Biosystems), and are detailed in Table 1. Both primers and probes were targeted to conserved gene regions to ensure detection of all documented variants of ISAV. Primers designed for amplification of the salmon ELF1$\alpha$ gene were those recently published by Moore et al. (2005). The ELF probe was designed to target mRNA specifically by spanning two exons such that it would not amplify contaminating salmon genomic DNA.

*Table 1:* Real-time PCR primers and Taqman® MGB probes.

| Organism | Gene target | Upstream primer | Downstream primer | Taqman® probe | Amplicon size (bp) |
|---|---|---|---|---|---|
| ISAV | Segment 7 | CAGGGTTGTA TCCATGGTTG AAATG | GTCCAGCCCT AAGCTCAACT C | FAM-CTCTCTCATTG TGATCCC-MGB | 155 |
| ISAV | Segment 8 | CTACACAGCA GGATGCAGAT GT | CAGGATGCCG GAAGTCGAT | FAM-CATCGTCGCT GCAGTTC-MGB | 104 |
| *Salmo salar* | ELF1α mRNA | CCCCTCCAGG ACGTTTACAA A | CACACGGCCC ACAGGTACA | FAM-ATCGGT#GGTA TTGGAAC | 57 |

### *RNA extraction*

Tissues were stored in 1 ml RNALater (Ambion) and stored at -20°C before RNA extraction. For field samples, 50-100 mg tissue was homogenised in 1ml TRIZOL using a disposable pestle and RNA subsequently extracted according to the manufacturer's protocol (Invitrogen). RNA was finally resuspended in DEPC-treated $H_2O$, quantified by measuring the OD at 260 and 280 nm on a Genequant Pro spectrophotometer (Amersham Biosciences) and stored at -80°C. In the case of experimental samples, 10mg tissue was homogenised in 600 µl RLT buffer (Qiagen) for 1min using the TissueLyser system (Qiagen). RNA was subsequently extracted using the RNeasy kit according to the manufacturer's protocol for the extraction of RNA from animal tissues (Qiagen). Total RNA was finally resuspended in 200 µl DEPC-treated $H_2O$ and stored at -80°C. For routine diagnostics, negative extraction controls (designated A control) were conducted by performing a blank extraction. Controls were taken through subsequent RT and PCR steps.

### *Reverse transcription*

For field diagnostic samples, reverse transcription reactions were conducted by first preparing dilutions containing 2 µg RNA in 9.5 µl DEPC-treated dH2O. RNA samples were incubated at 55-60°C for 10min and then placed on ice. Reverse transcription was conducted in a total volume of 20 µl which contained 9.5 µl RNA prepared as above, 5 ngµl$^{-1}$ random hexamers (Promega), 1x 1$^{st}$ strand buffer, 1mM each dNTP, 1Uµl$^{-1}$ RNase OUT and 10Uµl$^{-1}$ MMLV reverse transcriptase (all from Invitrogen). Reactions were incubated at 37°C for 1h. In the case of experimental samples, reverse transcription was performed using the Taqman® RT Kit (Applied Biosystems) in a final reaction volume of 25 µl. Firstly, 9.125 µl RNA & 1.25 µl of 50 µM oligo $d(T)_{16}$ were mixed and denatured at 70°C for 10 min. Reactions were placed on ice and mixed with 1x reverse transcriptase buffer, 5.5 mM $MgCl_2$, 2 mM dNTPs, 0.4U RNase Inhibitor and 1.25U Multiscribe reverse transcriptase. Reactions were incubated at 48°C for 90 min followed by 95°C for five min. Negative reverse transcription controls were conducted by performing reactions containing no target (designated B control). Controls were taken through subsequent PCR steps.

### *Real-time RT-PCR (qRT-PCR) reagents and cycling*

Assays were performed on an Applied Biosystems 7000 Sequence Detection System programmed to conduct the following cycling profile: 50°C for two min (AmpErase uracil N-glycosylase incubation), 95°C for 10 min (AmpliTaq Gold polymerase activation) followed by 45 cycles of 95°C for 15s (denaturation) and 60°C for one min (annealing/extension). Reactions were conducted in a final volume of 25 µl containing 1 µl cDNA template, 1x Taqman® Universal PCR mastermix, 900nM each primer and 250 nM Taqman® probe. Controls were conducted for each primer and probe set containing no target (designated C control).

*Validation of reaction efficiencies and relative quantification*

To determine whether the salmon host cell endogenous ELF1a assay control might permit the relative quantification of ISAV and ensure efficient performance of each assay, a validation experiment was conducted to determine whether the efficiency of all assays was comparable. For each assay, triplicate reactions were conducted on each dilution of a 10-fold serial dilution of cDNA prepared from concentrated standards. Standard curves were generated by plotting the relative dilution of RNA versus the cycle number required to elevate the fluorescence signal above the threshold for the ISAV Segment 7, 8 and ELF assays. Reaction conditions with respect to probe and primer concentrations were independently evaluated as described in the Applied Biosystems literature. The effect of reducing the total reaction volume from 50 µl to 25 µl was also determined.

*Sensitivity*

The same 10-fold dilution series of ISAV cDNA was used to evaluate the comparative sensitivity of both Taqman® qRT-PCR methods with that of conventional RT-PCR. Triplicate individual reactions were conducted for each test using the methods described above.

*Specificity*

ISAV is the only orthomyxovirus from fish to be characterised at the molecular level, and as such has been designated within the genus Isavirus. Nucleotide sequence of probes was used to search GenBank using BLAST X [12] to identify any known organisms with which they might cross-react. Segment 7 and 8 probes and primer sets were tested against cDNA derived from a range of other RNA viruses capable of infecting Atlantic salmon including Atlantic salmon paramyxovirus, viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and pancreas disease virus (PD). To ensure the detection of all forms of ISAV, segment 7 and 8 primers and probes were designed from sequence alignments containing representatives of all known subtypes of ISAV, which are classified according to the sequence of a highly polymorphic region (HPR) of the haemagglutinin esterase (HE) gene. Such alignments included the HPR0 subtype from which all other HPR subtypes of the virus which are associated with clinical disease in aquaculture may have been derived [13,14].

## Application of relative quantification to the detection of ISAV in experimentally infected fish

*Pathogen-free fish*

Atlantic salmon post smolts (mean weight +/- SE, 89.4 +/- 14.69 g) were reared at the FRS Marine Research Unit, Aultbea, Ross-shire, Scotland. Before the experimental challenge, four fish were screened for the presence of ISAV using RT-PCR and qRT-PCR as described above. Fish were allowed to acclimatize for seven days and starved for 24 h before experimental infection. Water temperatures were maintained at 11°C for the duration of the experimental infection. Seawater was extracted from the North Sea and passed through a sand filter and UV disinfection treatment before entering fish tanks.

*Experimental infection with ISAV*

Atlantic salmon post smolts (n=75) were anaesthetised by immersion in methane tricaine sulphonate (3-aminobenzoic acid ethyl ester of MS-222; Sigma) at a concentration of 100 mg l-1, infected intraperitoneally with a dose of $2 \times 10^6$ $TCID_{50}$ $fish^{-1}$ in an inoculum volume of 100 µl, and distributed between two 360 l aquaria (Tanks 1 & 2). Controls consisted of 75 fish similarly anaesthetised and injected with an equivalent volume of culture medium containing no virus, and distributed between a further two tanks (Tanks 3 and 4). Two additional tanks were similarly set up, each of which contained 25 virus infected and non-infected fish respectively. These tanks were used to monitor the efficacy of the viral challenge. All fish were monitored for the duration of the experimental infection period (20d) and all dead fish were recorded and removed twice daily.

*Sampling and comparative PCR analysis*

From each set of virus-infected (Tanks 1 and 2) and control tanks (Tanks 3 and 4), a total of four fish were sacrificed and sampled on each of days 1,2,3,4,5,6,7,9,11,15 and 19 post infection. Kidney tissue was excised, sampled into RNALater (Ambion) and stored at -80°C for analysis by PCR. RNA extraction and Taqman® qRT-PCR was performed on experimental kidney samples using the methods described above for amplification of ISAV segment 7, 8 and ELF1a. Samples were also processed for conventional RT-PCR detection of ISAV segment 8 which was previously described [5] and is in routine diagnostic use within our laboratory.

### Application of qRT-PCR to the detection of ISAV in field samples during routine ISAV surveillance in Scotland

Field samples intended for ISAV diagnosis during the routine ISA surveillance programme conducted by FRS in Scotland were processed in parallel throughout a nine month period in 2004, using both the RT-PCR method and qRT-PCR methods reported in this study. During this period the presence of ISAV was suspected in Scotland, although it was not attributed as the cause of clinical disease [15]. During this investigation a total of 279 kidney and 87 gill tissue samples were processed for ISAV diagnosis by both qRT-PCR and conventional RT-PCR.

## RESULTS

### Development of Taqman real-time RT-PCR method

#### *Validation of reaction efficiencies and relative quantification*

The amplification efficiency graphs obtained from serial dilutions of ISAV-positive fish tissue are indicated in Figure 1. Each of the ISAV assays exhibited comparable efficiencies with that of ELF1α as indicated by them exhibiting a difference in slope (ΔS) of <0.1 [16]. This indicates that the ELF1α endogenous control is a suitable reference for the relative quantification of ISAV over the range of starting concentrations used in this study. For each reaction series, there was also a high correlation between cycle number and dilution factor ($R^2$ =1.0 in all cases) and slope values approached the optimum theoretical value of -3.32, indicating efficient amplification of all assays. Primer and probe concentrations were optimised at 900 nM and 250 nM respectively and no loss in sensitivity was recorded by reducing the total reaction volume to 25 μl.

#### *Sensitivity*

The segment 8 Taqman® qRT-PCR assay consistently detected ISAV at a log higher dilution that the segment 7 Taqman® qRT-PCR assay which itself consistently detected ISAV at a log higher than the conventional RT-PCR (data not shown). In addition, very faint products were observed nearing the endpoint of detection for conventional RT-PCR which required experience in their interpretation.

#### *Specificity*

Both segment 7 and 8 assays yielded no detectable product when using cDNA derived from viruses other than ISAV. Virus identified in field samples during 2004 proved to be of the HPR0 type, which demonstrated efficient detection of variants of ISAV [15].

### Application of relative quantification to the detection of ISAV in experimentally infected fish

#### *Experimental infection of salmon with ISAV*

The mortality obtained in the unsampled tanks used to monitor the efficacy of the experimental infection is shown in Figure 2. Mortality commenced on day 11 post-infection and all fish in the ISAV infected group were dead by day 20. All dead fish showed clinical signs consistent with ISA disease which included pale gills, darkened livers and the presence of ascites. No mortalities were recorded in the control tanks throughout this period.

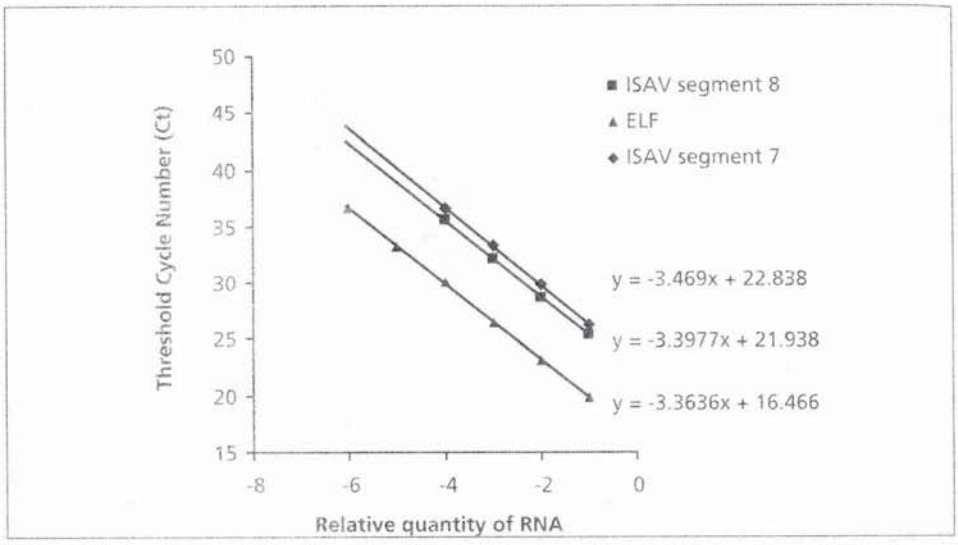


*Fig. 1:* Standard curves obtained by plotting the relative dilution of RNA versus the cycle number required to elevate the fluorescence signal above the threshold for the ISAV Segment 7, 8 and ELF assays. Equations were derived from linear regression.

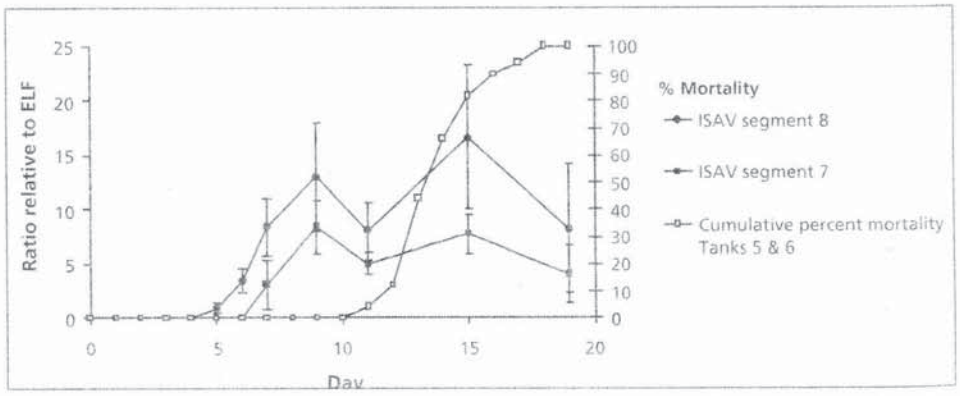


*Fig. 2:* **Graph indicating the mean level of ISAV segment 7 and 8 detected at different time-points following the experimental infection of Atlantic salmon with ISAV.** Mean levels were derived from four fish sampled at each time-point and represent the level (in arbitrary units) of each ISAV gene expressed as a proportion of the endogenous ELF-1α control signal detected in the same sample. Error bars indicate the standard error of the mean. The mean cumulative percent mortality obtained in un-sampled ISAV challenge reference tanks is indicated.

*Sampling and comparative PCR analysis*

Results obtained from the qRT-PCR analysis are detailed in Figure 2. The relative signal corresponding to target ISAV RNA was normalized to that obtained with the ELF1α endogenous control in each case. Values were expressed in arbitrary units. This calculation was performed according to the comparative Ct method described in the literature supplied with the ABI 7000 Sequence Detection System. All samples tested positive for ELF1α indicating efficient RNA extraction and reverse transcription in all cases. All fish testing positive for ISAV segment 8 also tested positive for ISAV segment 7. At day 1 post infection, two of the four fish sampled tested positive for ISAV, but at all subsequent time-points all fish tested positive. The mean values obtained for segment 8, however, were consistently higher that those obtained for the segment 7 assay, when expressed as a ratio to all ELF1α (Fig. 2). No contamination was recorded in any of the control fish sampled or in any of the RNA extraction (A), reverse transcription (B), real-time or conventional PCR controls (C) performed as described above.

Results obtained on the same sample set using a conventional RT-PCR targeting ISAV segment 8 corresponded with those obtained with the qRT-PCR methods, with the same two fish testing negative. In many of the positive cases, however, very weak products were observed on conventional agarose gels, which required experienced interpretation. In some cases non-specific products were also observed alongside the expected 155bp product.

## Application of relative quantification to the detection of ISAV in field samples during routine ISA surveillance in Scotland

Two hundred and seventy nine kidney samples (each a pool of up to five fish) were screened for the presence of ISAV using Taqman® qRT-PCR assays targeting segments 7 and 8 in addition to conventional RT-PCR which also targets segment 8. Six samples were identified as positive using the conventional RT-PCR. All six samples were also positive using both ISAV segment 7 and 8 Taqman® qRT-PCR assays. qRT-PCR assays for segment 7 and 8 identified a further 11 and 12 positive samples, respectively (corresponding to 17 individual samples), which tested negative by conventional RT-PCR. Of these, six samples were positive using both assays, but five and six samples were positive using only the segment 7 or segment 8 assay respectively. Higher relative quantities of ISAV segment 7 and 8 were identified in samples testing positive for conventional RT-PCR as opposed to those testing negative for RT-PCR and positive by either one or both of the Taqman® assays (Figure 3). Interestingly, some of the values obtained from field samples were of a similar magnitude to those obtained during the course of clinical infection under experimental conditions.

During surveillance for ISAV, gill samples were also taken from some fish. Of a total of 87 fish samples processed, 41 yielded positive results using conventional RT-PCR and both segment 7 and segment 8 Taqman® qRT-PCR assays. No samples were positive using only conventional PCR. Seventeen samples were positive using both segment 7 and segment 8 assay but were negative using the conventional RT-PCR assay. Three samples were positive using the segment 7 Taqman® qRT-PCR assay only and a further 12 samples were positive using only the ISAV segment 8 Taqman® qRT-PCR. Relative quantities of virus detected are indicated in Figure 4. Samples which were positive using all methods yielded high relative quantities of virus. For those which tested negative using conventional RT-PCR, but positive using

both Taqman® qRT-PCR assays, low relative values were obtained for both segment 7 and segment 8 assays. Similarly samples testing positive using only one of the qRT-PCR assays also yielded low relative values.

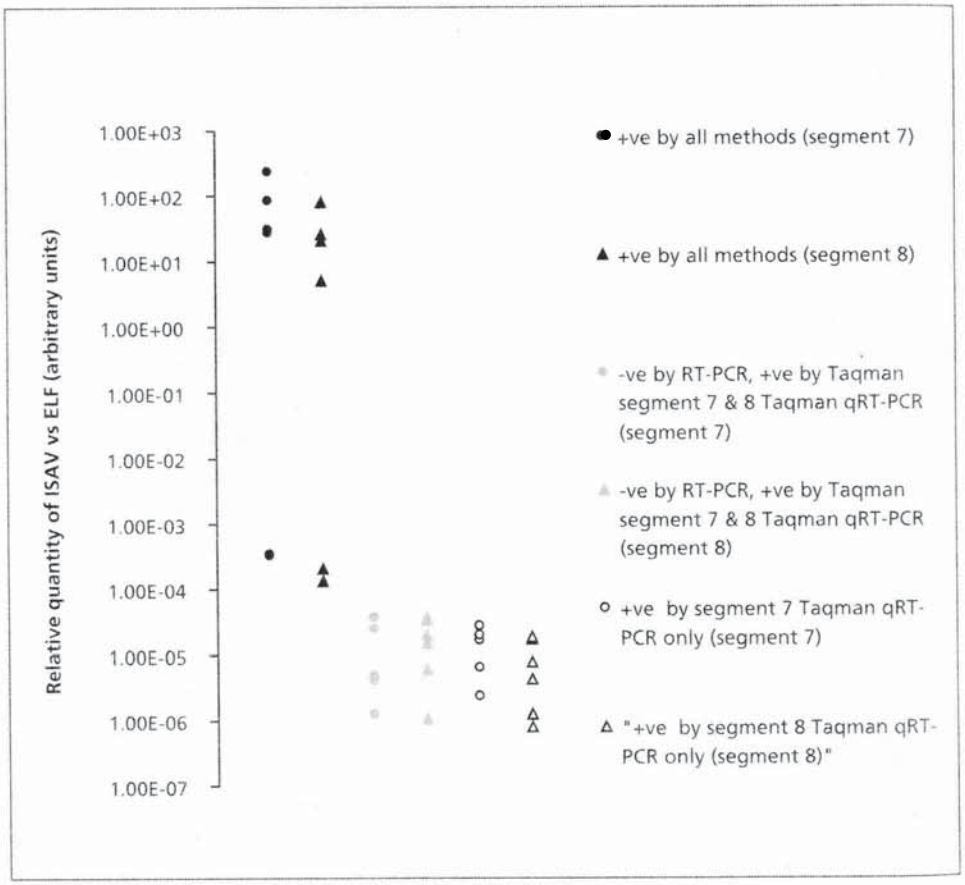


*Fig. 3:* **Relative levels of ISAV segment 7 and 8 detected in field kidney samples analysed during routine surveillance for ISAV in Scotland during 2004.** ISAV Levels obtained using Taqman® segment 7 (indicated by circles) and segment 8 (indicated by triangles) semi-quantitative qRT-PCR assays and expressed as a proportion of the endogenous ELF-1α control signal detected in each sample are detailed. Results are indicated according to the number of the three diagnostic tests (RT-PCR, Taqman® qRT-PCR segment 7, Taqman® qRT-PCR segment 8) generating positive results in each case.

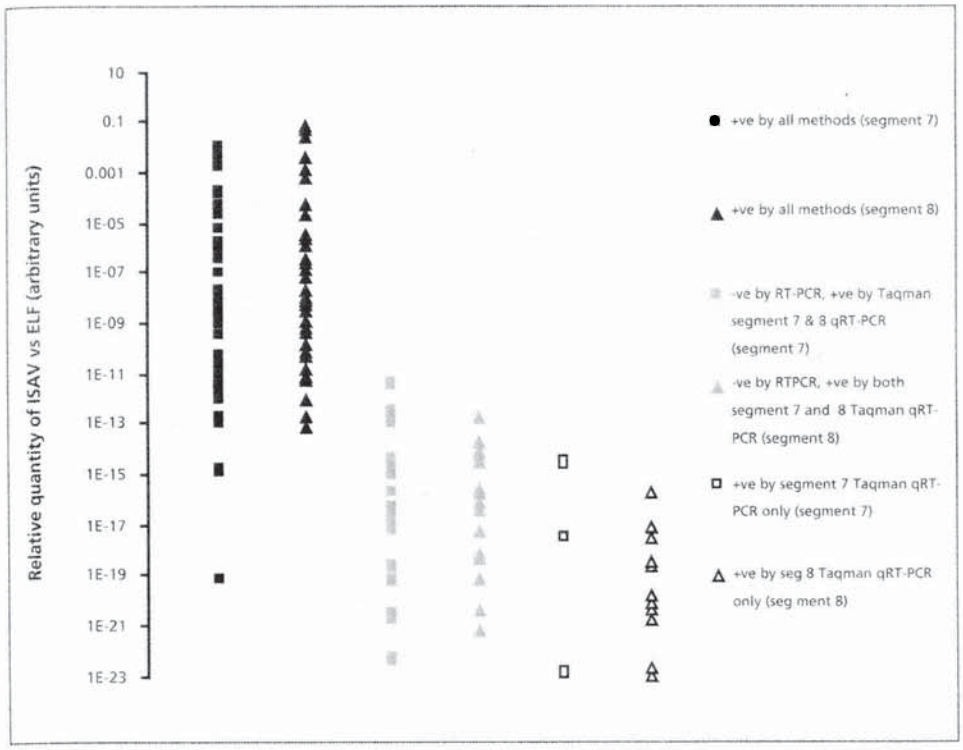


*Fig. 4:* **Relative levels of ISAV segment 7 and 8 detected in field gill samples analysed during routine surveillance for ISAV in Scotland during 2004.** ISAV Levels obtained using Taqman® segment 7 (indicated by squares) and segment 8 (indicated by triangles) semi-quantitative qRT-PCR assays and expressed as a proportion of the endogenous ELF-1α control signal detected in each sample are detailed. Results are indicated according to the number of the three diagnostic tests (RT-PCR, Taqman® qRT-PCR segment 7, Taqman® qRT-PCR segment 8) generating positive results in each case.

## Discussion

The results presented describe two independent highly sensitive and specific two-step Taqman® qRT-PCR methods for the detection of ISAV in Atlantic salmon tissues. Both methods proved to be more sensitive than the conventional RT-PCR method in routine use for ISAV diagnosis [5]. In addition, the application of semi-quantitative Taqman® qRT-PCR to ISAV diagnostics offers a number of advantages associated with this technology including an increased sensitivity, enhanced specificity, quantitative expression against endogenous controls, a lack of subjectivity in the interpretation of results, speed of processing and contamination control.

The segment 8 qRT-PCR assay was shown to be more sensitive than the segment 7 assay during initial experimental dilution series experiments. This was supported by the consistent detection of higher levels of segment 8 in experimentally infected fish during a clinical outbreak of ISA disease and the fact that more field kidney and gill

samples were positive by the segment 8 assay only than by the segment 7 assay only. Since the assays displayed similar theoretical efficiencies, such a finding is most likely related to the differential expression of segment 7 and 8 segments throughout the viral infection cycle. Both segments 7 and 8 of ISAV have been shown to encode two proteins, based on removal of an intron from the primary transcript [17] or from overlapping open reading frames, respectively [18]. Segment 8 has been shown to encode a major structural protein [19], while segment 7 has recently been demonstrated to encode at least one protein which mediates the host interferon defence system [20]. An increased abundance of segment 8 mRNA generated during the viral life cycle may thus explain the apparent improved sensitivity of the Taqman® segment 8 assay.

Both Taqman® assays performed at least as well as the RT-PCR segment 8 assay in the analysis of experimentally infected fish. This was perhaps not surprising since the fish had been injected with a relatively high dose of ISAV which, based on the severity of the challenge, was actively replicating. Analysis of serial dilutions, however, suggested an increased sensitivity of the Taqman® assays. This increased sensitivity may be related to the specificity and sensitivity of Taqman® chemistry which permits additional cycles of PCR to be conducted (n=45) when compared to conventional PCR (n=35). Furthermore, the generation of the very small amplicons in both ISAV Taqman® assays may contribute to the improved sensitivity observed. Improved sensitivity of both Taqman® assays over conventional RT-PCR was also demonstrated during evaluation of both kidney and gill samples collected as part routine diagnostic surveillance of Scottish salmon aquaculture during 2004. Indeed, many samples testing negative by conventional PCR tested positive by both segment 7 and segment 8 Taqman® assays. However, the relative quantity of ISAV detected was lower than in those samples which tested positive by conventional RT-PCR. In these cases, the agreement of the independent Taqman® assays, coupled to the detection of signals beyond the apparent detection threshold for conventional RT-PCR strongly supports the fact that these were indeed true positive samples. In some cases, samples were negative by conventional RT-PCR and positive by only one of the segment 7 or 8 Taqman® assays. In all these cases, relative levels of ISAV detectable were also very low. The demonstrated difference in sensitivity of the segment 7 and 8 assays, which is probably related to differences in viral gene expression could account for such differences and supports the view that the results observed are not due to a lack of assay specificity.

The specificity of detection using Taqman® chemistry is significantly improved as compared to either conventional RT-PCR or real-time PCR based on chemistry such as SYBR green. Indeed, detection of Taqman® PCR products is dependent on the annealing of highly specific probes. Experience in our laboratory and in other studies has shown the method to be capable of the absolute discrimination of PCR products based on the presence of a single nucleotide mismatch in the probe binding region. Such specificity raises the prospect of false negatives using a single probe, should mutation occur within this region. To reduce the likelihood of false negatives, probes in this study were designed from absolutely conserved regions based on alignments of all known ISAV sequence types. The parallel use of two independent assays in this study provides further confidence in the characterisation of true negatives and adds significant power to the detection of rapidly mutating organisms such as RNA viruses.

The development of a quantitative method for ISAV diagnosis using the ELF1α endogenous control provides a significant advantage over the conventional RT-PCR method in ensuring quality control of RNA and subsequently generated cDNA quality and quantity. The elongation factor 1 alpha gene has been previously demonstrated to

be stably expressed between different individuals, and as such represents a suitable calibrator for semi-quantitative real-time PCR [21]. ISAV load expressed as a function of ELF1α is thus a measure which can be compared across individuals sampled at different times. In a diagnostic context such information can be used to express confidence levels in positive results obtained and as a basis to predict the likelihood of other tests (e.g. conventional RT-PCR or virus culture) also yielding positive results. Results might also be used to predict the clinical status of fish once a more comprehensive understanding of the relationship between viral RNA levels and pathology emerges. Such a method thus offers considerable scope both for improving the understanding and interpretation of PCR results, generating more information than a simple positive/negative result obtained using conventional RT-PCR. Indeed, within the current study of ISAV experimentally infected fish, we have demonstrated the range of signals expected in kidney tissues throughout a clinical disease outbreak. Results from kidney samples obtained in field samples during 2004 [15] were in some cases of a similar magnitude to these values, although in others relative values were significantly lower. Such knowledge might in future assist us in identifying carrier fish populations and those in which clinical disease is expected. In this case, however, further characterization of detected virus identified it as an HPR0 ISAV subtype, the relevance of which to development of ISA disease is not fully understood.

Taqman® qRT-PCR does not require any post-PCR processing and in particular does not require the subjective interpretation of weak products visualised on agarose gels. As such, interpretation of its results is more objective and less variable and suited to implementation in routine viral diagnostic testing. The lack of post-PCR processing also reduces the time taken to complete the method (approximately four to five h) and minimises the potential for obtaining false positives due to cross contamination. The Taqman® method also replaces dTTP with dUTP during PCR amplification. Subsequent PCR mixes are treated with the enzyme uracil N-glycosylase (UNG) which specifically degrades PCR products from previous amplifications in order to reduce the risk of amplicon carry-over resulting in sample contamination [22].

In conclusion, two highly sensitive and specific Taqman® qRT-PCR assays targeting ISAV segment 7 and segment 8 have been developed and evaluated. Use of an internal endogenous control ELF1α allows semi-quantitative expression of viral transcript levels. The application of Taqman® technology to ISAV diagnosis offers a number of significant advantages over conventional RT-PCR and lends itself well to implementation within a routine viral diagnostic environment.

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# Absolute quantitation of infectious salmon anaemia virus using different real-time reverse transcription PCR chemistries

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Routine laboratory diagnosis of infectious salmon anaemia virus (ISAV) infection is primarily by reverse transcription polymerase chain reaction (RT-PCR) because of the high sensitivity and rapid turnaround time of the test. This paper describes methods for highly reproducible absolute viral load measurements using external standard curves generated with either ISAV recombinant plasmid DNA (pDNA) standards or transcribed RNA standards prepared by *in vitro* transcription with T7 RNA polymerase, and using a two tube real-time or quantitative (q)RT-PCR with SYBR® Green I chemistry and a single tube qRT-PCR with TaqMan® probe chemistry. When applied to virus samples of known virus titer for the highly pathogenic ISAV strain NBISA01 and the low pathogenic ISAV strain RPC/NB-04-085-1, both methods showed a 100-fold lower detectable titer for RPC/NB-04-085-1 but with a higher number of viral RNA molecules compared to NBISA01. Overall, the SYBR® Green I method overestimated copy numbers in samples having equivalent Ct values with the TaqMan® probe method. Taken together, the findings suggest that the TaqMan® probe method with the *in vitro* transcribed RNA standard curve is the preferred method for reliable and rapid quantitation of ISAV in samples.



## 1. Introduction

Infectious salmon anaemia (ISA) is a highly fatal viral disease of marine-farmed Atlantic salmon caused by ISA virus (ISAV), an orthomyxovirus belonging to the genus *Isavirus* within the family *Orthomyxoviridae* (Kawaoka et al., 2005). The genome is composed of eight segments of linear, single-stranded (ss)RNA of negative sense ranging in length from 1.0 to 2.4 kb with a total molecular size of approximately 14.3 kb (Clouthier et al., 2002). The fish disease is reportable with the World Animal Health Organization (OIE). Virus can be detected in fish tissues by RT-PCR (Mjaaland et al., 1997), electron microscopy (Hovland et al., 1994), indirect fluorescent antibody test (IFAT), and by virus isolation using permissive fish cell lines (Dannevig et al., 1995; Bouchard et al., 1999; Kibenge et al., 2001). Comparison of the different methods showed the RT-PCR method to be the most sensitive for virus detection (Snow et al., 2003).

Data generated by real-time or quantitative (q) RT-PCR can be analyzed using either absolute or relative quantitation (reviewed by Bustin, 2005). Absolute quantitation requires construction of a standard curve using relevant standards such as a known copy number of plasmid DNA (pDNA) or *in vitro* transcribed RNA standards (Bustin, 2000; Wong and Medrano, 2005). Relative quantitation describes the change in expression of the target gene relative to some untreated reference sample and normalized to a reference gene, usually a housekeeping gene (reviewed in Giulietti et al., 2001; Livak and Schmittgen, 2001). It is becoming increasingly apparent that more than one reference gene is required for proper use of relative quantitation (a minimum of three reference genes is recommended), making it cumbersome to use let alone to compare test performance between different laboratories. Moreover, housekeeping genes are not necessarily appropriate references for qRT-PCR data normalization (Sellars et al., 2007). In contrast, absolute quantitation analysis is useful in determining absolute viral RNA copies based on a constant, allowing straight forward comparison of data from different PCR runs on the same day or on different days, and more importantly between different laboratories.

There are two chemistries used most commonly for detection of PCR products during qRT-PCR. These are the DNA binding fluorophore SYBR® Green I (Simpson et al., 2000), and the sequence-specific fluorescently labeled probes (Holland et al., 1991; Lay and Wittwer, 1997). Quantitation of ISAV by qRT-PCR first utilized the SYBR® Green I format, targeting RNA segment 8 (Munir and Kibenge, 2004), and then subsequently used TaqMan® probes initially targeting RNA segment 8 (Mjaaland et al., 2005) and then comparing RNA segments 7 and 8 (Snow et al., 2006). The authors found the segment 8 TaqMan® qRT-PCR assay to be more sensitive

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than the segment 7 TaqMan® qRT-PCR assay (Snow et al., 2006). All the previous ISAV quantitation reports have used relative quantitation of ISAV transcripts calibrated to housekeeping genes (Mjaaland et al., 2005; Kileng et al., 2006; Jørgensen et al., 2007; Snow et al., 2006), however, there has never been correlation with biological significance of the amount of viral RNA detected in a sample. Using expression of reference/housekeeping genes is relevant when studying gene expression, but has less relevance in viral quantitation except for estimating the quality of the RNA in a sample and detecting presence of inhibitory effects. This report describes the use of ISAV segment 8 pDNA and *in vitro* transcribed RNA standards for absolute quantification of ISAV RNA copy number equivalents in both a two tube qRT-PCR using SYBR® Green I and a single tube one-step qRT-PCR with a TaqMan® probe. Moreover, this study established the relationship of qRT-PCR cycle threshold (Ct) value to median tissue culture infectious dose ($TCID_{50}$) when used to assess viral load in a sample for ISAV isolates of differing pathogenicities. Considering the replication strategy of influenza viruses, ISAV replication is expected to generate viral mRNA and cRNA from the vRNA genome. When primed with gene specific primer or random hexamers, the total RNA from ISAV-infected cultures will have a population of cDNA generated from viral mRNA, cRNA, and vRNA. It is possible to specifically amplify ISAV vRNA by priming the non-coding UTR region in the RT step with sequence specific primer but this requires a two-step RT-PCR to allow RNase treatment before addition of a second gene specific primer in the PCR step. Thus, the idea of relating transcript copies to ISAV genome equivalents is limited when using cDNA primers that are not specific for vRNA, although this method was used for absolute quantitation of coronavirus (Vijgen et al., 2005), a non-segmented ssRNA virus of positive sense. When quantitating segmented RNA viruses the question would be how many individual genome segments are contained in an infectious virus particle? ISAV is not well studied in this respect; but influenza virions containing more than eight individual RNA segments have been isolated (Flint et al., 2004). Thus for the present study, in order to extrapolate the segment 8 ISAV copies as ISAV RNA copy number equivalents, an assumption was made that the genome in a single infectious ISAV particle has at least one molecule of each RNA segment.

## 2. Materials and methods

### 2.1. Viruses and virus culture

Two ISAV isolates of differing genotypes and pathogenicities were compared. NBISA01 is a highly pathogenic strain belonging to the North American genotype, whereas RPC/NB 04-085-1 is a low pathogenic strain of the European genotype found in Eastern Canada (Kibenge et al., 2006). The two isolates have variations in the amino acid sequence of the haemagglutinin-esterase (HE) protein, with deletions of 13 and 17 amino acids in the highly polymorphic region (HPR) for RPC/NB 04-085-1 and NBISA01, respectively (Kibenge et al., 2007). In an experimental trial using equal viral doses, NBISA01 induced very high mortality in Atlantic salmon (95%) and moderate mortality in rainbow trout (50%), whereas RPC/NB 04-085-1 induced very low mortality in Atlantic salmon (18.2%) and no mortality in rainbow trout (Kibenge et al., 2006). These ISAV isolates were propagated in the TO cell line (Wergeland and Jakobsen, 2001) and the cell lysates were titrated on TO cell monolayers as described previously (Kibenge et al., 2001). For serial sampling during virus replication, virus was propagated in 24 h-old TO cell monolayers (~80% confluent) in six-well tissue culture plates. Infected cells were incubated at 16 °C in maintenance medium. Sampling was done at days 0, 3 and 6 by freezing the whole plate at −80 °C prior to the total RNA extraction step.

### 2.2. Sample extraction

Total RNA was extracted from virus samples and fish tissue samples using 1.25 ml of TRIZOL Reagent (Invitrogen) and 375 μl of sample volume. For the fish tissue samples, each tissue was weighed and macerated to a 10% suspension (w/v) in PBS with 10× antibiotics. The extracted RNA was eluted in 20–50 μl of nuclease-free water and was treated with DNAse I using the Roche DNAse treatment kit following the manufacturer's procedure. RNA was quantitated by UV spectrophotometry.

### 2.3. First strand cDNA synthesis

For use in the two tube SYBR® Green I method, first strand cDNA synthesis was performed using the Transcriptor reverse transcriptase first strand cDNA synthesis kit (Roche). Different amounts of RNA were used in cDNA synthesis depending on the source of the RNA. cDNA synthesis of ISAV segment 8 transcribed RNA used 1 μl of RNA per reaction. cDNA synthesis of total RNA extracted from the different virus samples of known virus titer (in $TCID_{50}$) and ISAV-positive fish tissues used 1 μl of RNA per reaction. cDNA synthesis of total RNA extracted from serial sampling during virus replication in TO cells used 300 ng of RNA per reaction. Three different primers were used for cDNA synthesis; random hexamer primers and oligo-dT primers that come with the cDNA synthesis kit (Roche), and the gene specific F5/R5 primers. The F5/R5 primers were first described by Devold et al. (2000) to amplify 220 bp of the ISAV segment 8, and previously described for single tube one-step qRT-PCR (Munir and Kibenge, 2004). The cDNA synthesis master mix consisted of 4 μl of 5× RT reaction buffer, 2 μl of dNTP mix (200 μM), primer (2 μl of random hexamer (600 μM) or 2 μl of oligo-dT primer (0.8 μg/μl) or 1 μl of gene specific F5/R5 primer (20 μM)), 0.5 μl RNase inhibitor (40 U/μl), 0.5 μl of Transcriptor reverse transcriptase (20 U/μl), and nuclease-free water to adjust the 20 μl volume. The reactions were incubated at 25 °C for 10 min followed by 55 °C for 30 min with a final enzyme denaturation at 85 °C for 5 min (Workenhe et al., 2008).

### 2.4. Preparation of plasmid DNA standards

The pDNA standard was obtained by cloning the 878 bp genomic RNA of ISAV segment 8 RT-PCR product (Cunningham and Snow, 2000) into the pCRII-TOPO vector (Invitrogen); the clone was designated pCRIITOPODNA-NBISA01-S8. The recombinant plasmid was purified using the High Pure Plasmid Purification kit (Roche). The plasmid DNA concentration was determined in triplicate by UV spectrophotometry. The mass of a single pDNA molecule was calculated using the formula 1 bp ~ 660 g/mol and the 4880 bp size of the recombinant plasmid, following the method in the ABI Manual of absolute real-time RT-PCR quantification (Anon., 2003).

### 2.5. In vitro transcription of ISAV RNA segment 8

The pCRIITOPODNA-NBISA01-S8 clone was also used for *in vitro* transcription with T7 RNA polymerase in the sense direction in order to generate *in vitro* transcribed RNA. For this, 200 ng of recombinant plasmid was linearized by digestion with BamHI enzyme (New England Biolabs) in a 20 μl reaction volume following the manufacturer's protocol. The linearized DNA was then purified using the QIA quick PCR purification kit (Qiagen), and was recovered in 30 μl of elution buffer. *In vitro* transcription was carried out in a 40 μl volume using 20 μl of linearized plasmid DNA, 1× T7 RNA polymerase buffer, 2 μl of 100 mM DTT, 16 μl of 10 mM NTPs (Invitrogen), 1 μl RNase OUT (40 U/μl) (Invitrogen), and 1 μl of T7 RNA

polymerase (50 U/μl) (Invitrogen). The reaction was incubated for 2 h at 37 °C. RNA purification was carried out using RNeasy kit (Qiagen), and was eluted in 30 μl of nuclease-free water. Nucleic acid concentration was determined by UV spectrophotometry. DNase treatment was done using 1 unit of RQ1 RNase-free DNase I (1 U/μl) (Promega) per μg of RNA following the manufacturer's procedure. This treatment was performed twice to ensure complete elimination of any residual plasmid DNA (which could potentially yield a positive result in two-step RT-PCR even in the absence of RT). RNA was cleaned up using RNeasy kit and eluted in 30 μl of nuclease-free water and the concentration was again determined by UV spectrophotometry. The ISAV segment 8 *in vitro* transcribed RNA was analyzed using a native 1% agarose gel to check the integrity of the RNA before use.

### 2.6. Construction of ISAV segment 8 in vitro transcribed RNA standards

The concentration of the ISAV *in vitro* transcribed RNA was determined by UV spectrophotometry in triplicate. The copy number of the *in vitro* transcribed RNA per microliter was calculated as described by Fronhoffs et al. (2002). Serial 10-fold dilutions of the RNA transcripts were prepared starting with the highest concentration of $2.79 \times 10^{11}$ copies/μl. For use in the two tube SYBR® Green I method, cDNA synthesis was carried out using 1 μl of each *in vitro* transcribed RNA serial dilution. The single tube one-step qRT-PCR TaqMan® method used 8 μl of each *in vitro* transcribed RNA serial dilution per reaction.

### 2.7. Two tube qRT-PCR with SYBR Green I chemistry, and standard curves and quantitation

QPCR was performed on the first strand cDNA using the LightCycler (LC) 1.2 instrument (Roche) with Fast Start DNA Master SYBR® Green I (Roche) and the ISAV segment 8 primer pair F5/R5 amplifying 220-bp product (Devold et al., 2000; Munir and Kibenge, 2004). Briefly, the 20 μl reaction consisted of 2 μl of cDNA and 18 μl of the master mix prepared using 0.3 μl of the 20 μM of the forward and reverse primers (final concentration of 0.3 μM), 2 μl SYBR® Green I, 3.2 μl of the 25 mM stock $MgCl_2$ (a final concentration of 0.005 μM), and 12.2 μl of nuclease-free water. The cycling conditions consisted of 10 min denaturation at 95 °C to activate the hot start polymerase followed by 50 cycles of 95 °C for 5 s, 59 °C for 10 s, 72 °C for 10 s, and detection at 80 °C for 2 s. Melting curve analysis was performed from 70 to 95 °C in 0.1 °C/s increments to assess the specificity of the RT-PCR products. For generation of the standard curves, the pDNA and *in vitro* transcribed RNA standards were run in triplicates. In order to use standard curves to calculate the ISAV segment 8 genome copies, pDNA and *in vitro* transcribed RNA standards were run alongside the unknown samples. For calculating viral genome copy numbers/ml of unknown sample, the viral genome equivalents/20 μl PCR reaction was multiplied by a factor of $20/11 \times 1000/375$. The factor is based on cDNA synthesis using 11 μl of the total 20 μl RNA eluted from 375 μl of virus lysate, and the use of 2 μl of cDNA from the 20 μl cDNA synthesis reaction. The Ct values were used to generate a standard curve plot of cycle number (*Y*-axis) versus log concentration (*X*-axis). The quality of standard curves was judged by the slope of the standard curve and the correlation coefficient (*r*). The slope of the line was used to estimate the efficiency of the target amplification using the equation $E = (10^{-1/\text{slope}}) - 1$. In case of the SYBR® Green I qRT-PCR, melting curve analysis was used to check the specificity of the RT-PCR product. In some cases, the RT-PCR products were resolved in 1% agarose gel electrophoresis.

### 2.8. Single tube one-step qRT-PCR with TaqMan® chemistry, and standard curves and quantitation

The single tube one-step qRT-PCR with TaqMan® primers and probe targeting ISAV segment 8 is a modification of the TaqMan® qRT-PCR assay for the detection of ISAV described by Snow et al. (2006), which uses relative quantitation methods. The modifications made in this study included use of a single tube with a one-step RT-PCR kit (Roche) and TaqMan probe in a 96-well plate in the LC 480 instrument (Roche) with absolute quantitation methods. Briefly, 8 μl of RNA are added to 12 μl of master mix consisting of 9.28 μl LC 480 RNA Master hydrolysis probe, 1.88 μl of activator $Mn(OAc)_2$ (50 mM), 1 μl of enhancer (20×), 1.13 μl of ISAV Segment 8 Forward primer and Reverse primer (Snow et al., 2006) (20 mM each) and 1.04 μl of ISAV segment 8 probe (Snow et al., 2006) (6 μM). The primers and probe binding sequences are identical for both of the virus isolates used in the present study. The cycling conditions consisted of 1 cycle of RT for 3 min at 63 °C followed by denaturation at 95 °C for 3 s, and 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min and amplification and detection at 72 °C for 1 s. For generation of the standard curve, the *in vitro* transcribed RNA standards were run in 5 replicates. The standard curve was constructed automatically with LC software version 4.0 (Roche) using the Ct values obtained when the serial 10-fold dilutions of the *in vitro* transcribed RNA samples with known numbers of RNA transcripts were used as templates. The standard curve obtained was then used as an external standard curve in all subsequent TaqMan® qRT-PCR assays on LC480. For calculating ISAV RNA copy number equivalents per ml of unknown sample, the ISAV RNA copy number equivalents/20 μl RT-PCR reaction was multiplied by a factor of $20/8 \times 1000/375$ based on the use of 8 μl of the total 20 μl RNA eluted from 375 μl of virus lysate used for RT-PCR reaction.

### 2.9. Construction of a standard curve for estimating $TCID_{50}$ from Ct values

To construct a standard curve for relating the virus titer of a sample expressed as $TCID_{50}$ with the Ct values obtained in qRT-PCR, total RNA extracted from 10-fold dilutions of virus lysates of ISAV strains NBISA01 and RPC/NB-04-0851 was tested with both the two tube qRT-PCR with SYBR® Green I chemistry and the single tube one-step qRT-PCR with TaqMan® chemistry. The $TCID_{50}$ (*X*-axis) of each sample was then plotted against the respective Ct value (*Y*-axis), and a linear fit was constructed as described in Falsey et al. (2003).

## 3. Results and discussion

### 3.1. Generation of ISAV RNA segment 8 recombinant plasmid DNA standards and absolute quantitation of ISAV with SYBR® Green I chemistry

In order to develop a qRT-PCR method for absolute quantitation of ISAV segment 8 RNA transcripts, the full segment 8 genomic RNA sequence was cloned into a pCRII-TOPO vector for use as the standard. The copy numbers of the pDNA standards prepared ranged from $3 \times 10^1$ to $3 \times 10^9$. Preliminary qRT-PCR analysis showed that copy numbers below $3 \times 10^2$ gave inconsistent Ct values within the triplicates. Thus pDNA standards of $3 \times 10^2$ to $3 \times 10^7$ copies were used to generate the standard curve. The curve had a PCR amplification efficiency of 2.0 with high linearity (correlation coefficient $r = 0.9979$). The pDNA standard curve was used to quantify the ISAV segment 8 cDNA copy number/ng

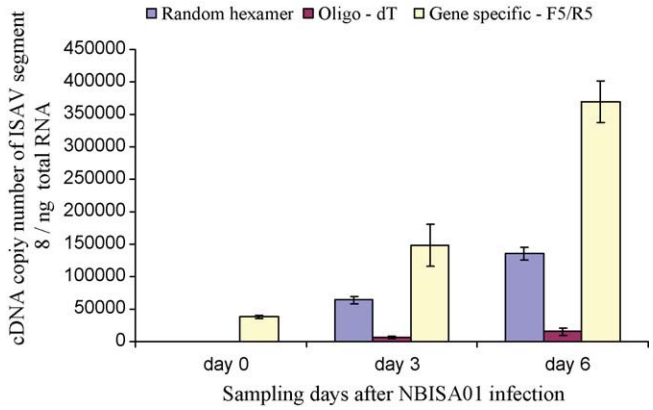


**Fig. 1.** cDNA copy number of ISAV segment 8 from unknown samples of cDNA generated using RNA extracted from TO cells infected with ISAV strain NBISA01 and three priming strategies (random hexamer, oligo-dT and gene specific primer) (data are average ± S.D. of three separate triplicates). The qRT-PCR used SYBR® Green I chemistry with absolute quantitation based on the external standard curve of the ISAV RNA segment 8 pDNA standards.

of total RNA extracted from ISAV-infected TO cells and primed for cDNA synthesis using three different priming strategies (oligo-dT, random hexamers, and gene specific F5/R5 primers). In all the cDNA priming strategies the 0 h samples showed the lowest copy number compared to the 3-day and 6-day samples, indicating specific increase in ISAV transcripts due to virus replication. cDNA generated using the gene specific primer showed an overall highest absolute copy number of the ISAV segment 8 $ng^{-1}$ of total RNA, followed by that of the random hexamer and then the oligo-dT primer (Fig. 1). The agarose gel electrophoresis of the RT-PCR products showed the expected 220 bp PCR product for the three different priming strategies (data not shown), confirming specificity of the PCR reactions. Each primer has a different way of priming cDNA synthesis from RNA of ISAV-infected TO cell lysates. The oligo-dT primer binds to the poly(A) tails of mRNA to generate first strand cDNA, whereas the gene specific F5/R5 primer anneals specifically to ISAV segment 8 genomic RNA, cRNA or mRNA to generate first strand cDNA. Random hexamers are capable of priming cDNA at many points along the viral RNA, cRNA, or mRNA template as well as cellular RNA templates, generating fragmentary copies of entire populations of RNA molecules (Sambrook and Russel, 2001).

As expected from the limiting RNA populations that can be primed using oligo-dT primers, this priming strategy had the lowest copy number of ISAV transcripts at all the sampling points. The process of mRNA synthesis from the ssRNA genome of ISAV is not well studied. However, influenza A virus which belongs to the *Orthomyxoviridae* family similarly to ISAV has been well studied in this respect. Influenza A virus negative-strand RNA (vRNA) serves as a template for the synthesis both of capped, polyadenylated viral mRNA and of full-length positive-strand RNA or complementary RNA (cRNA) (Cros and Palese, 2003). The poly(A) tail of influenza virus mRNA is synthesized by reiterative copying of a 5–7 nt long U sequences of 16 nt from the 5′ end of the viral RNA template. The cRNA is associated with the same viral proteins as the vRNA and serves as a template for the synthesis of new vRNA molecules, which in turn serve as templates for mRNA and cRNA, particularly early in the infection (Robertson et al., 1981; Fodor and Smith, 2004; Amorim and Digard, 2006). Even though there is no detailed characterization of molecular replication strategy of ISAV, sequencing of 3′ and 5′ ends of segments 7 and 8 has revealed that ISAV mRNA is polyadenlyated (Sandvik et al., 2000).

### *3.2. Generation of ISAV RNA segment 8 in vitro transcribed RNA standards and absolute quantitation of ISAV with SYBR® Green I chemistry and TaqMan® probe chemistry*

Since ISAV has a ssRNA genome, it was necessary to use *in vitro* transcribed RNA of the full segment 8 coding sequence to construct a standard curve. It was considered that *in vitro* transcribed RNA templates would estimate more accurately template amounts in the RNA inputs and therefore give a more accurate quantitation as they would be subjected to the same RT reaction (unlike the pDNA standards for qPCR). For initial calibration, first strand cDNA synthesis used the gene specific F5/R5 primer from 10-fold diluted *in vitro* transcribed RNA of $10^1$–$10^{10}$ copies. The F5/R5 primer showed non-specific amplification signals in transcribed RNA preparations with $\leq 10^5$ copies. Thus, for comparison of the F5/R5 with the random hexamer priming, *in vitro* transcribed RNA standards were prepared in serial 5-fold dilutions with copies ranging from $3.2 \times 10^6$ to $1 \times 10^9$. The qPCR was carried out under the same conditions as for the pDNA standard curve. The F5/R5 primed cDNA had a higher amplification efficiency ($E$) of 2.14 compared to that for random hexamers primed cDNA ($E = 1.94$), which was mainly a result of the lower dilutions of the *in vitro* transcribed RNA standards, which generated closer Ct values between dilutions. These low template reactions in the F5/R5 primed cDNA were associated with primer-dimers. Oligo-dT priming was not attempted on the *in vitro* transcribed RNA templates since they were not polyadenylated.

From the standard curves obtained using *in vitro* transcribed RNA standards with the SYBR® Green I chemistry, we selected one default method for estimating the viral load in the unknown samples. For this, the utility of the two primers (random hexamers and gene specific F5/R5; Oligo-dT primers were excluded since they would not be specific for T7 *in vitro* transcribed non-polyadenylated RNA standards) to prime cDNA synthesis from all ISAV templates (vRNA, cRNA, and mRNA) and optimality of the PCR amplification efficiency were compared. The F5/R5 showed non-specific primer-dimers in reactions using $\leq 10^5$ *in vitro* transcribed RNA copies whereas the quantitation limit with random hexamer primers was $10^3$ *in vitro* transcribed RNA copies; the standard curves generated with random hexamer primers also had a better PCR efficiency compared to those with the gene specific F5/R5 primers. Thus, the random hexamer cDNA priming-based two step method was selected as the default for absolute quantitation with the SYBR® Green I chemistry.

In order to provide a method for absolute quantitation of ISAV using TaqMan® probe chemistry, the segment 8 TaqMan® probe-based qRT-PCR assay developed and validated by Snow et al. (2006) was modified for use in a single tube with a one-step RT-PCR kit (Roche) in a 96-well plate in the LC 480 instrument (Roche) with absolute quantitation methods. Preliminary qRT-PCR analysis showed that the *in vitro* transcribed RNA preparations with $\geq 2.2 \times 10^{11}$ copies (or $\geq 15.16$ ng/μl cRNA) inhibited the RT-PCR, giving Ct values >34.0 whereas for preparations with $2.2 \times 10^{10}$ copies, the Ct value was <5.0 and increased in proportion to the dilution of the *in vitro* transcribed RNA preparation with a detection limit of $2.2 \times 10^1$ *in vitro* transcribed RNA copies. Based on these observations, serial 10-fold dilutions were prepared, and those in the range from $2.2 \times 10^9$ to $2.2 \times 10^2$ were used to establish a standard curve for ISAV segment 8 RNA transcripts with 2–5 replicates per dilution point. The standard curve had an amplification efficiency of 1.965 and error of 0.00866.

Table 1 summarizes a comparison between the two methods (SYBR® Green I-based two tube qRT-PCR versus TaqMan® probe-based single tube one-step qRT-PCR) when applied to RNA extracted from eight serial 10-fold dilutions of virus lysates of NBISA01 and RPC/NB 04-085-1 in terms of their dynamic range

**Table 1**
Comparison of the dynamic range of ISAV segment 8 two tube qRT-PCR with SYBR Green I chemistry and single tube one-step qRT-PCR with TaqMan probe chemistry

| | Two tube qRT-PCR with SYBR Green I | | Single tube one-step qRT-PCR with TaqMan probe | |
|---|---|---|---|---|
| | NBISA01 ($10^{8.75}$ $TCID_{50}$/ml) | RPC/NB 04-085-1 ($10^{5.75}$ $TCID_{50}$/ml) | NBISA01 ($10^{8.75}$ $TCID_{50}$/ml) | RPC/NB 04-085-1 ($10^{5.75}$ $TCID_{50}$/ml) |
| Dynamic range ($TCID_{50}$/ml) | $10^{0.75}$ | $10^{0.75}$ | $10^{1.75}$ | $10^{1.75}$ |
| Reliable detection[a] limit ($TCID_{50}$/ml) | $10^{4.75}$ | $10^{2.75}$ | $10^{4.75}$ | $10^{2.75}$ |
| Reliable detection[a] limit (ISAV RNA copy number equivalents/ml) | 5956.4 ± 24.3 | 18568.1 ± 539.1 | 27.7 ± 1.8 | 227.2 ± 55.8 |

[a] Reliable detection is defined as the dilutions run in triplicate giving similar Ct values.

relative to the virus titres expressed as $TCID_{50}$/ml. Both methods had the same $TCID_{50}$ detection limits for NBISA01 and RPC/NB-04-085-1, $10^{4.75}$ $TCID_{50}$/ml and $10^{2.75}$ $TCID_{50}$/ml, respectively. For the same titers of the two isolates the SYBR® Green I method showed 215- and 81-fold higher copy numbers for NBISA01 and RPC/NB-04-085-1, respectively. The data suggest that there is 100-fold lower detectable virus titer of the low pathogenic ISAV strain RPC/NB-04-085-1, which was accompanied by 3- (in the SYBR® Green I method) and 8-fold (in the TaqMan® method) higher copy number of RPC/NB-04-085-1 compared to NBISA01. The difference in the $TCID_{50}$ detection limit for the two virus strains is probably related to the fact that qRT-PCR also detects viral RNA in non infectious or defective virus particles which are probably more in the lower pathogenic ISAV. This would imply that the molecular basis for the virulence difference between the two viruses occurs at the post-transcription steps of virus replication, probably resulting in a higher production of non-infectious virus particles by the low pathogenic ISAV strain RPC/NB-04-085-1.

While using the same *in vitro* transcribed RNA standards for quantitation of ISAV RNA copy equivalents in both chemistries, the SYBR® Green I-based system reported higher RNA copies per ml of virus lysate for the same Ct value in the TaqMan® probe chemistry (data not shown). The difference can be partly explained by the sequence-specific detection chemistry of the TaqMan probe chemistry (Holland et al., 1991; Lay and Wittwer, 1997), compared to the non-specific dsDNA binding of SYBR® Green I fluorophore (Simpson et al., 2000). Moreover, the SYBR® Green I method loses reliability in reactions with low templates amounts (Ct values ≥35.0). The inconsistency of SYBR® Green I readings for low template reactions was previously reported by Walters and Alexander (2004).

### 3.3. Correlation of $TCID_{50}$ with Ct values

Using the reliable detection limit (reliable detection is defined as the dilutions run in triplicate giving similar Ct values) for the virus titrations a standard curve was constructed to estimate $TCID_{50}$/ml from Ct values for the two ISAV strains, NBISA01 and RPC/NB-04-085-1. The standard curve plots of Ct versus $\log_{10}$ $TCID_{50}$/ml for the SYBR® Green I and TaqMan chemistries are shown in Fig. 2A and B. Both plots have a linear model fit and have small slope difference between the isolates manifested by the parallel nature of the two lines. Both the SYBR® Green I and TaqMan reaction linear fits suggest that for a certain Ct value NBISA01 will have higher $\log_{10}$ $TCID_{50}$ compared to the RPC/NB-04-085-1 for the range of dilutions considered. This is consistent with NBISA01 being highly pathogenic (Kibenge et al., 2006) where with lower viral genome copies, it can give a higher titer $TCID_{50}$ compared to the less pathogenic RPC/NB-04-085-1. Similarly, using the NBISA01 dilutions that have 5 points on the standard curve, the linear fits generated using the TaqMan® one tube one-step method were compared with the linear fit generated using the SYBR® Green I two

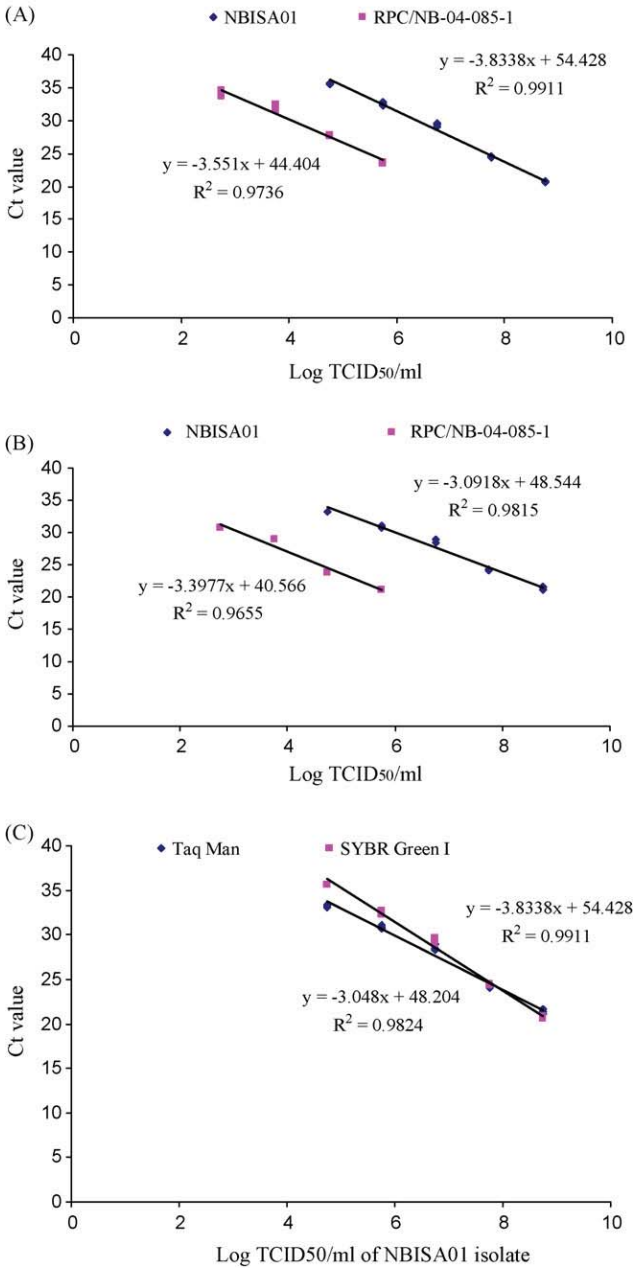


**Fig. 2.** Standard curve relating $TCID_{50}$ to Ct value from RT-PCR using 10-fold dilutions of virus lysates of known titre (A) SYBR Green I two tube method, Ct values vs. $\log_{10}$ $TCID_{50}$ for NBISA01 and RPC/NB-04-085-1, (B) TaqMan one tube method, Ct values vs. $\log_{10}$ $TCID_{50}$ for NBISA01 and RPC/NB-04-085-1, (C) SYBR Green I two-tube and TaqMan one-tube method, Ct values vs. $\log_{10}$ $TCID_{50}$ for NBISA01.

tube method (Fig. 2C). The linear fits show slight differences in the two methods in that the SYBR® Green I two tube reactions show a slightly higher Ct value for a specified $TCID_{50}$ below $10^{7.92}$ $ml^{-1}$ (the $TCID_{50}$ value where the two lines cross). The difference can be explained by the fact that the TaqMan® probe method involves a single step that uses all the cDNA from the RT-step whereas the SYBR® Green I method uses 2 μl of cDNA generated in 20 μl reaction, introducing a 10-fold dilution of the cDNA template. Thus, the slightly lower Ct values of the SYBR® Green I two tube method are related directly to the template cDNA amounts available for the PCR stage.

The standard curve for NBISA01 constructed using the TaqMan® single tube one-step method was used to estimate the $TCID_{50}$ in tissue samples of experimentally infected fish. The standard curve estimated the fish tissue samples to have virus titers ranging from $10^{4.87}$ to $10^{6.23}$ $TCID_{50}$/ml. Thus, the method can be used to estimate ISAV loads in fish tissues based on RNA copy numbers, and to estimate the viral titers ($TCID_{50}$) without use of the time-consuming virus titration in fish cell lines.

In conclusion, this report describes methods for absolute quantitation of ISAV genome copies using external standard curves generated with either ISAV pDNA standards or *in vitro* transcribed RNA standards, and for the first time report a correlation of Ct values to viral titers expressed as $TCID_{50}$/ml using two ISAV isolates of differing pathogenicities and two detection chemistries. Both SYBR® Green I and TaqMan® probe chemistries showed a 100-fold lower detectable titer for RPC/NB-04-085-1 but with a higher number of viral RNA starting molecules compared to NBISA01, indicating that the low pathogenic ISAV produces more non-infectious or defective particles than the highly pathogenic ISAV. Overall, the SYBR® Green I method overestimated ISAV RNA copy number equivalents in samples having equivalent Ct values with the TaqMan® probe method. Thus, the TaqMan® probe method with the *in vitro* transcribed RNA standard curve is the better method for reliable and rapid quantification of ISAV in samples.

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