General QC procedures for PCR assays, with reference to SOPs

1) General QC in the molecular laboratory
2) General precautions when handling samples
3) General precautions when setting up assays
4) Additional steps and controls in place to insure results accuracy

1) General QC in the molecular laboratory

SOPs:

GFC-GEN-9	General molecular laboratory practices	v1
GFC-GEN-6	Laboratory Cleaning	v2

a) Work space:

The molecular biology unit (MBU) comprises 3 labs.

- The largest lab is the general area, where samples are received and processed up to PCR cycling.
- A small lab adjacent to it is called "clean or DNA free zone", and is restricted to the preparation of master mixes and reagents mainly. No samples enter this area.
- A third lab is called "dirty" and is dedicated to amplification and electrophoresis. No material exits this lab without being thoroughly cleaned and disinfected if it has to return to the main area.

b) Movement from one lab to another:

Lab coats are restricted to each lab, i.e. someone moving to another lab must first take off their lab coat and take one in the new zone.
Similarly, gloves touching samples are not taken out of the sample area.

c) Cleaning and maintenance of the space:

We did a study of various disinfectants and found that only bleach (diluted) was effective at destroying DNA. The lab space is cleaned weekly.

d) Aliquots and reagents integrity:

Whenever appropriate, reagents are aliquoted in size suitable for the work. When preparing extractions, some reagents are in large sizes (e.g. ethanol). For these, we pour the amount needed in clean and sterile beakers, and dispose of any leftovers. Hence no stock of reagents is ever sampled directly, thus reducing chances of cross contamination from stock between batches of samples.
Reagents and aliquots are tracked, solutions made in the lab are captured in worksheets and any reagents or solutions used is recorded with lot or batch no.

e) Samples batches:

Samples are necropsied by case and lot. In the PCR lab, we do not mix cases of samples, i.e. we do not process together samples from different cases, even when the number of sample is low. Although this can increase the work, this reduces the chances of cross contamination between cases submitted.

f) New programs, new worksheets, etc:
Each time a program is entered in equipment software, or a template worksheet is created for an assay etc, a second person will double check the work. If this replaces an older program of worksheet, the older version is kept as an archive.

2) General precautions when handling samples
SOP:
GFC-DIA-MBU-13 RNA extraction with TRI or TRI-LS Reagent v2

a) Samples are processed outside the MBU, according to policies. To maintain tissue integrity and prevent degradation of the pathogen (if present), samples are processed according to strict guidelines regarding the time between culling and sampling, etc.

b) Samples coming in the main lab are usually non-infectious, except for cell lysates coming in for confirmation by PCR. Otherwise, they are mostly tissues preserved in RNAlater (for RT-qPCR), or tissues preserved in Et-OH (e.g. mollusc for PCR detection of protozoans). These are stored in refrigerators and freezers reserved for samples (and identified as such). They may not be processed by PCR immediately, but the RNA or DNA is stabilised to prevent degradation during storage.

c) Samples are provided in sufficient amount so that a back up piece can be kept. They are either provided as two sets, one kept for backup, or as one set. In this case, the technician will take one smaller piece and return the rest in the original tube. This subsampling is done with sterile material (e.g. sterile toothpicks) and on clean paper towels for example. The backup tissue is important: any suspect positive sample may need to be retested (decision to retest depends on the whole case history and prevalence).

d) Sample tubes are sprayed externally before processing.

e) Cases are processed separately.

f) Samples are kept in secured area (locked doors).

3) General precautions when setting up assays
Protocol:
NAT-PROT-qRT-PCR-ISAV-1 qRT-PCR Diagnostic with TaqMan Universal PCR Master Mix for the Detection of Nucleic Acids from Infectious Salmon Anemia -v1

a) Samples are processed in batches convenient for the type of equipment we have, i.e. 30 extractions at a time.

b) Critical pipettes used for each extraction batch are cleaned prior to handling samples. Cleaning means dismantling pipettes, cleaning the interior, drying and reassembly.

c) For each batch of extraction, blanks are introduced, one per 15 samples and always one after the last sample. The blanks are processed like samples throughout the PCR assay.

d) During extractions, all precautions to avoid aerosols are taken, i.e. physical space between tubes, keeping microtubes closed between steps, changing gloves frequently and immediately if contamination is suspected, etc.

e) Master mixes for Reverse transcription and for PCR are prepared in the clean area and brought to the inoculation area when ready.

f) Inoculation of samples in mixes for RT and for PCR proceeds as follows: samples are inoculated first, then positive controls, and finally negative controls.

g) For RT, two positive RNAs (*in vitro* transcripts or RNA extracts from standard material) are introduced. They will be carried on through the PCR process. One negative well (water) is included.

h) For the qPCR portion of the assay, two positive cDNA are introduced (usually taken from a previous assay). A negative well (water) is also included.

i) Samples and controls are processed in duplicates for qPCR.

j) A positive result requires reextraction using the backup piece of tissue and repetition of the results obtained in the first assay before it is considered positive. If many samples are presumptive positive, a maximum of 5 samples are selected for rextraction per case, and the weakest positives are chosen for repeated analysis. If a sample cannot be confirmed by reanalysis, it is declared inconclusive. Next steps depends on the context, i.e. when several samples in a case are positive, no additional testing is required. For index cases, sequencing would be required.

k) For most qPCR assays, our positive controls are plasmids with an insert that can be easily distinguished on gel, or by sequencing, from the true analyte. Since the true pathogen is not present, any presumptive positive can be tested to rule a possible cross contamination from positive control material.

4) <u>Additional steps and controls in place to insure results accuracy</u>

SOP:

GFC-DIA-MBU-23 Preparation of Standard Material for Molecular Assays - v3

a) Although several controls, positive and negative, are introduced during the PCR process, we are not able to insure that all samples were processed equally well. In one

batch, a sample could be accidentally processed incorrectly, etc. These events are usually detected, e.g. a dropped tube, a wrong volume of reagents, etc. Samples are submitted within guidelines, but degradation can be suspected on occasions, e.g. during a mortality event investigation.

i. An additional qPCR assay is performed in our laboratory. This assay aims at detecting a gene from the specimen submitted for testing, i.e. a reference gene (or housekeeping gene). The assay includes a positive control produced from the same specie and if possible, from the same tissue.
ii. The results of this assay should be positive for all samples, thus insuring that all samples were processed correctly. They should also be within a range that is close to the positive control. Usually, when degradation occurs, all samples will suffer a similar level of degradation. In special cases, i.e. submission of material improperly processed and preserved, we have seen degradation levels as extreme as to prevent the detection of the reference gene.

b) It is also possible to confirm presumptive results by other techniques:
i. Viral culture (samples must be placed in culture at the time of necropsy, or must be frozen and cultured upon request.
ii. Sequencing the PCR product directly
iii. Amplification of an alternate portion of the pathogen genome (and sequencing of this region if desired).
iv. etc