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SCIENCE ADVICE TO GUIDE A RESEARCH STUDY USING THE FLUIDIGM® BIOMARK PLATFORM FOR MICROBE DETECTION IN WILD AND FARMED SALMON

Figure 1: Department of Fisheries and Oceans' (DFO) six administrative regions.

Context

A Strategic BC Salmon Health initiative (SSHI) funded by Genome BC, and in partnership with the Department of Fisheries and Oceans (DFO) and the Pacific Salmon Foundation (PSF) has been initiated. The goal of this multi-phase project is "to discover the microbes and potential diseases that may undermine the productivity and performance of British Columbia (wild) salmon and to determine what exchanges may happen between wild and cultured salmon". Key to the success of this project is the development and application of a new technology, the Fluidigm® BioMark quantitative polymerase chain reaction (PCR) platform, to detect prevalence and load of dozens of microbes across 1,000's of tissue samples from BC salmon.

In this advisory process, participants assessed the methodology for this research application, including an assessment of analytical sensitivity, specificity, repeatability and comparability, and the benefits, limitations, uncertainties and proposed uses of this methodology (including the design and the statistical analyses) for the identified research purposes.

This Science Advisory Report is from the December 2-4, 2014 national advisory process on the Review of the Fluidigm BioMark platform: Evaluation to assess fitness for purpose in microbial monitoring. Additional publications from this meeting will be posted on the Fisheries and Oceans Canada (DFO) Science Advisory Schedule as they become available.

SUMMARY

- Over 90% of juvenile salmon migrating from freshwater into the ocean will die before returning to freshwater to spawn. While mortality is believed to be highest during the first few months in the marine environment, there is ongoing mortality due to many different reasons. Because the bulk of the current knowledge on salmon diseases comes from observations of cultured fish, the relative importance of infectious and non-infectious causes in wild migrating pacific salmon remains largely unknown.
- The Genome BC Strategic BC Salmon Health initiative (SSHI) is underway and its strategic role will be to detect the microbes and potential resultant diseases that may affect the productivity and performance of BC salmon, their evolutionary history, and the potential role of exchanges between wild and cultured salmon. This research project is highly multidisciplinary involving several research areas namely: Genomics, Epidemiology, Histopathology, Immunology, Virology, Parasitology, Salmon Ecology and Bioinformatics. The project will proceed through four sequential phases (stages).
- The SSHI project focuses principally on microbes that are recognized globally, and either known or suspected to cause disease in salmon (or related to opportunistic infections of immune compromised fish). It utilizes genomic methods to identify and verify which microbes are present in wild and cultured (federal hatcheries and salmon farms) finfish in BC, Canada. Later phases of the project will develop challenge studies for those microbes that are agreed upon to have the greatest potential to negatively impact wild salmon. These studies will allow us to better understand under what conditions, if any, these microbes cause disease.
- The scope of this CSAS process is on **Phase 2a** of this project with the stated major objective to evaluate the sensitivity, specificity and repeatability of assays designed to quantitatively assess the presence and load of microbes, in multiple samples simultaneously using a high throughput micro-fluidics platform (the Fluidigm® BioMark; hereafter BioMark). This technology utilizes a specific targeted amplification (STA) step, the effects of which were assessed within this report.
- There are no agreed upon minimum standards for analytic performance characteristics as outlined by the World Organization for Animal Health (OIE). Stage 1 of the OIE evaluation (diagnostic tests) is perhaps the closest analogue; however, the OIE has not explicitly provided guidelines for validation of multiplex assays (e.g. the STA) or new technologies (e.g. the BioMark platform).
- In the present context, the intended purpose of the assays tested on the BioMark platform is for conducting research (which is not an OIE-listed purpose, but nor is it specifically excluded). Therefore, the conceptual testable hypothesis that can be statistically evaluated (null vs. alternate) within the context of this project is the estimation and comparison of microbe and pathogen prevalence, and thus probably the closest to the purposes listed by OIE.
- For CFIA-regulated or OIE-listed diseases, a procedure for reporting to the Canadian Food Inspection Agenda (CFIA) was discussed and agreed upon:
    - The project team (lab) use OIE recommended assays, where available.
    - If samples are suspected to contain a CFIA reportable disease agent, the project team (lab) will follow the CFIA mandatory notification directive for research

laboratories for such events (*Directive: Mandatory Notification of Reportable Aquatic Animal Diseases by Researchers*).

- If samples are suspected to contain an OIE-listed disease agent that is not CFIA reportable (or immediately notifiable to CFIA), that CFIA be notified per requirements for a diagnostic lab.
- Positive results for reportable disease agents are required to be reported to the CFIA within 24 hours of detection, and such preliminary results are not to be reported outside of the project team. CFIA has the regulatory responsibility to confirm the presence of all reportable diseases in Canada.
- The project team (lab) will liaise with the CFIA to seek clarification if required.
- The SSHI project management team will only be notified following a CFIA investigation (if applicable).

- Per the Terms of Reference, this CSAS review assessed: the analytical sensitivity, specificity and repeatability of microbial assays on the BioMark platform; the comparability of assay results between the BioMark and ABI 7900 platforms; the effect(s) of the STA; and the benefits, limitations, uncertainties and proposed uses of this methodology.
- *Assay sensitivity* – For virtually all BioMark assays, a Limit of Detection (LOD) of samples undergoing an STA enrichment step was 1-10 copies per chamber, similar to the LOD of the starting material. The threshold cycle (Ct) cutpoint associated with the LOD was between 27 and 29. Positive control samples were collected from as many reliable sources as possible and showed satisfactory results for most of the 47 assays; for two viruses, ISAV7 and IPNV, the assays did not detect all strain variants, consistent with results from other platforms and expected based on the degree of homology. The authors of the review suggested that there may also be some strain variants of bacterial microbes not included among those tested that based on *in silico* analyses may not be detected.
- *Assay specificity* – 13 viral and 12 bacterial assays showed high specificity against all closely-related species tested. Assays for 22 parasites were mostly specific but some distantly-related species appeared positive in certain tissue samples presumably because of co-infection by multiple parasite species. While the authors showed that for these pairs of microbes, co-detection was not the norm (consistent with a co-infection rather than cross-amplification scenario), the reviewers felt that nucleotide sequencing would provide better evidence for mixed infection.
- In all, there was no variance in assay sensitivity or specificity from expectations on other platforms, any variance from 100% analytical specificity (ASp) did not impede resolution of known salmon microbes (with one exception), and measures of analytical performance were within an accepted range for most assays.
- *Assay repeatability* – Twenty-six (26) endemic microbes were assessed for repeatability across 240 samples from BC salmon. Repeatability (within a dynamic array) and reproducibility (across dynamic arrays) were examined. Binary agreement (positive/negative) was assessed between replicates and found to be 98% overall.
- *Platform comparison* – The study compared the performance of BioMark and ABI 7900 HT using the primer/probe concentrations recommended for the BioMark platform, and obtained comparable results for 22 available assays. These conditions were not individually optimized for conventional quantitative PCR platforms.

- *Effect of STA* – The use of STA allows for enhanced sensitivity of detection of the target and is required because of the small assay volumes inherent in any microfluidics platform (7 nl for the 96.96 dynamic array). This volume is approximately 1:1000th the volume of a traditional quantitative PCR (qPCR) platform. The cycle threshold cutpoint and LOD per chamber for STA and non-STA are the same, Ct 27-29 and ~1-10 copies, respectively, but a minimum of ~1,000 copies per µl are required for the starting material if the STA is not applied. In essence the STA enriches targeted products by approximately 1000x. Other than the LOD effect on the starting material, no consistent biases were identified regarding the relative abundance of targets. Moreover, no significant differences between STA and non-STA frequencies of false positives were observed across the assays, suggesting that the enrichment step did not impact sensitivity or specificity of the assays. Target enrichment STA did not significantly affect repeatability and reproducibility, nor individual results (positive/negative). Overall, repeatability was good to excellent for most assays, and within ranges observed on other platforms.
- *Strengths and weaknesses* - A number of *strengths* were identified, including individual benefits with respect to: depth of coverage; identification of co-infections; cost per test; time-savings; efficiency; flexibility; expandability; refinement of purpose; analytical sensitivity; interchangeability; limited tissue requirements; upgrading to facilitate PCR-sequencing; simultaneous RNA quality assessments; detection of a second probe; and use of non-lethal samples. Conversely, a number of *weaknesses* were also identified, including: poor curve quality for some assays; enhanced training requirements; high initial cost of instrument; volume of data and level of analysis required ("big data"); need to improve software algorithms; instrument better suited for large studies and datasets; and cautions for interpretation of results. See *Assessment* section for a more comprehensive discussion on these points.
- *Uncertainty* – a number of sources of uncertainty were identified in the analytical approach, including: the use of gBlocks for closely-related species; the use of tissue samples as positive controls which may enhance possibility of detection of co-infections when multiple microbes are assessed at once; the grouping of control samples (by viruses, bacteria and parasites) for specificity studies; and the use of a single serial dilution (did not assess technical variation in pipetting accuracy). These limitations are more fully discussed in the *Assessment* section, along with associated recommendations.
- The results of this peer review indicated that this research project can proceed to Phase 2b. However, the Science advice includes a number of recommended improvements to the experimental design, lab procedures and the associated working paper (Research Document) which must be addressed. Further details are provided in the *Assessment* section of this report, but these *recommendations* include:
    - Dozens of replicates of the same assays were performed to obtain the limit of detection (LOD) and assay linearity for each primer/probe design, however, this was done using a single serial dilution of artificial positive controls (APC). The authors successfully demonstrated the accuracy of measurement within a single sample for each point, including the impact of six independent STA reactions, however the evaluation of possible variations in liquid handling (during the preparation of serial dilution of APC) was not included. To more fully address the inherent variability, the evaluation of inter-control variability using multiple independently prepared serial dilutions is recommended.
    - All positive detections obtained against positive control samples other than the one(s) targeted should be subjected to DNA sequencing for confirmation. For

sequencing, it would be best to target another gene (classical or nested PCR), which will distinguish potential cross-reactions or co-infections. Similar sequencing should be conducted on a subset of tested field samples to confirm/validate specificity associated with microbe monitoring studies.

- To ensure the same detection efficiency (i.e. same Ct values), a comparison should be conducted between both platforms using STA samples, under the prescribed conditions for both platforms (e.g., a temperature of hybridization of 60°C for all primers and probes). This comparison would ideally be conducted using real positive samples (i.e. without gBlocks), where possible.
- A framework to guide the interpretation of research findings is strongly recommended (e.g. scoring methodology). Consideration should also be given to the broader implications of potential findings and how this will be communicated both internally (within the Department) and more broadly externally (the public). Both regulators and policy makers would benefit greatly from a consistent framework for the interpretation of project results.
- To account for the uncertainty around some assay processes, it is recommended that a quality management system be implemented, which would provide methods and process checks, verification and calibration of equipment and instruments, critical reagents, and the introduction of new assays. In addition, each aspect of the quality assurance/quality control (QA/QC) process should consider and mitigate the risks identified (e.g. via standard operating procedures (SOPs) or a quality control manual).
- A verification of the effectiveness of the 'clean-up step' after the STA is recommended.
- The verification of specificity across microbe groupings (viruses, bacteria, and parasites) using real samples and excluding tissues and gBlocks across all assays is recommended.
- It is recommended that the efficiencies of the calculated Cts be tested for robustness. This can be examined by changing the efficiencies (manually in formulas) and verifying the effect on the calculated Cts.

BACKGROUND

Over 90% of juvenile salmon migrating from freshwater into the ocean will die before returning to freshwater to spawn. While mortality is believed to be highest during the first few months in the marine environment, individuals continue to drop out of the population over time due to many different reasons, including both diseases and other various causes (e.g. nutrition, environmental, predation, exploitation, etc.). Current knowledge of infectious diseases in salmon is derived almost exclusively from observations of cultured fish (both in hatcheries and in aquaculture). Consequently, there is a fair understanding of pathogens and clinical disease impacting salmon in freshwater hatcheries and sea-water net pens, but a much poorer understanding of pathogens affecting wild Pacific salmon in the ocean. Motivated by the need to fill this gap and the recognition that novel genomic approaches may provide a depth of resolution not previously available, the authors developed a 4-phase project with the Pacific Salmon Foundation and Genome British Columbia to describe the microbes (defined as viral, bacterial, fungal and protozoan microorganisms) in British Columbia (BC) salmon, and identify those that may influence productivity and performance of BC's wild, hatchery and farmed salmon populations.

The Project focuses principally on microbes that are recognized globally to cause clinical disease (or related to opportunistic infections of immune compromised fish) in salmonids, and utilizes genomic methods to identify and verify which microbes are presently detectable in wild and cultured (federal hatcheries and salmon farms) fishes in BC, Canada. Later phases of the project will attempt to develop challenge studies on top ranking microbes that have the greatest potential to negatively impact wild salmon to better understand under what conditions, if any, these microbes may cause disease. The stated strategic goal of the project is "to discover the microbes and potential diseases that may undermine the productivity and performance of BC (wild) salmon and to determine what exchanges may happen between wild and cultured salmon in the evolution of these microbes".

For the broader project, four sequential Phases were established as follows:

PHASE 1 (2012 - 2013) established a large-scale sampling program for wild, hatchery and aquaculture salmon. The initial sampling was conducted in 2012 and early 2013 but extends into other phases to include multi-year data. Collection archives are also available for hatchery and wild salmon from 2008-2011, which was supported by a previous Genome BC project.

PHASE 2 (2013 - 2016) is developing, testing, evaluating and ultimately applying a novel genomic technology to determine which microbes associated with disease in salmon worldwide are carried by wild and cultured salmon in BC. This phase also involves epidemiological studies supported by microbe distributional data, and next generation sequencing, histopathology, and functional genomics studies to identify the microbes that carry the highest probability of impact on wild fish.

PHASE 3 (2015 - 2017) will focus on the microbes identified in Phase 2, with an emphasis on microbes that have not been extensively researched in BC and that carry the highest potential for disease in wild salmon. Laboratory challenge studies will be carried out to assess under what conditions specific microbes are associated with disease in Pacific salmon. Additional studies assessing transmission dynamics of specific microbes will also take place. Genomic data on host response and adaptability to disease will be integrated into these studies. This phase may move to funding from Genome Canada and will involve a greater contribution of academic laboratories.

PHASE 4 (2017 - 2018) will include reporting of research and presentations to management agencies on the potential utility of methods developed and the application of outcomes to future microbe detection research.

It is important to note that the first two phases of the research program focus on microbes rather than specifically on disease, which is the focus in the third phase of the SSHI program. This approach was taken because there is rarely, if ever, the opportunity to observe wild fish die, especially in the ocean; hence, it would be rare to sample wild fish in their final stages of disease. However, because many microbes can be carried by fish before (and after) clinical disease occurs, identification of some infectious disease agents is possible in a representative sample of wild fish. In taking this approach, it is recognized that while many microbes can cause disease in wild salmonids, not all diseases are caused by microbes (note: this program is specifically focused on infectious disease) and not all microbes necessarily cause disease. It is also recognized that the diagnostic performance for simultaneous detection of large numbers of microbes, particularly in healthy fish populations, will be very difficult to quantify and may not reflect active infection (i.e. the assay is detecting components of the microbe and not necessarily reflecting live organisms), thus limiting the interpretation of results to trend comparisons. Hence, in Phase 2 of this program, the authors were careful to avoid assuming that all microbes detected in BC salmon are causing disease and mortality. However, the

authors argued that by identifying microbes detectable in wild Pacific salmon populations, and by restricting our surveys largely to microbes known to be associated with disease or mortality in various salmon species worldwide, one can begin to define the types of diseases that may affect them and to define the temporal and spatial distribution patterns of selected microbes. Further, statistical analyses contrasting microbe prevalence and load among salmon populations, life-history stages, and/or years may also inform the ecological interaction of specific microbes, or suites of microbes, and salmon population dynamics.

Genomic science provides a foundation through which this project will examine salmon microbes and disease processes. Over the course of the project, three genomic approaches will be used:

1) a micro-fluidic quantitative PCR platform (BioMark) for microbe detection,
2) high throughput sequencing (HTS) for microbe sequence confirmations, epidemiological studies and novel microbe (mostly viral) discovery, and
3) gene expression profiling on microarrays and the BioMark platform to assess transcriptional signatures associated with microbe carrier states (Phase 2) and disease (Phase 3).

For microbe detection, the project is using a combination of published and newly developed assays for up to 45 unique microbes that are suspected or known to associate with disease or decreased marketability (e.g. *Kudoa*) of salmon worldwide.

Description of the Fluidigm Biomark Platform

The BioMark platform uses micro-fluidics to increase the number of samples and assays run simultaneously, decrease the sample volumes by 1000-fold and increase the speed and sensitivity of the assays when compared with the ABI 7900 platform, resulting in a significant reduction in reagent use and cost. The platform's dynamic array simultaneously performs 96 assays on 96 samples, providing 9,216 data points per run. Earlier evaluations of the BioMark against the ABI 7900 have shown that the linear detection range and precision of replicate measurements to be similar between instruments. Moreover, the BioMark was recently shown to be able to detect a 1.25 fold difference in DNA copy number (when 18 to 40 replicates are applied), better than most other platforms. The assays run on this platform, and use of appropriate controls, can be the same as one would run on other quantitative PCR platforms.

The major distinguishing feature of the BioMark from other qPCR platforms is the very small sample volumes utilized (0.007 μl compared to 6-10 μl for traditional platforms). These small volumes necessitate the use of a specific targeted amplification (STA) step to enrich for targeted sequences of interest. While this protocol has been extensively evaluated for gene transcriptional studies, the analysis herein provides a thorough evaluation of any effects it may have on the relative quantification and specificity of microbe assays.

It should be noted that the BioMark platform is being developed for human pathogen and disease diagnostics in a few large biotechnology companies in the USA (one such study out of Japan is already published). There is also a potential that the BioMark system could, once appropriate levels of validation are met, be applied more broadly for use in fish health diagnostics and regulatory diagnostics, resulting in considerable cost and time savings for the application of molecular assays - the cost to run four assays in duplicate on 90 fish samples on the ABI 7900 is similar to the cost of running 48 assays in duplicate on 90 fish samples on the BioMark, with a time commitment similar to running only a single assay. The BioMark is capable of 3-4 runs per day, based on normal working hours; hence up to 360 individuals for 47 duplicated microbe assays.

ASSESSMENT

Per the established Terms of Reference, this Science advisory process provides a review and analysis of the proposed methodology (including design and statistical analyses) to assess the large-scale genomic methods used to identify and verify which microbes are presently detectable in wild and cultured (federal hatcheries and salmon farms) finfish in BC, Canada. The *strengths* and *weaknesses* detailed below are provided. One caveat of the analyses presented is that this assessment was conducted based on one BioMark instrument, and did not examine variability across a representative sample of similar instruments.

Strengths

1) **Depth of coverage**: Ascertainment of results of multiple simultaneous assays can increase the depth of microbe assessments, potentially enabling a more thorough understanding of the role of co-infections on disease development.
2) **Cost**: Cheaper cost per assay if greater than four (4) assays are required. It was found that it costs roughly the same amount to run 96 assays on the BioMark as to run four (4) assays on a traditional qPCR platform. Any claims of cost efficiency should also consider the initial (and considerable) purchase price of a BioMark instrument (approximately $250,000).
3) **Time savings**: The principal investigator reported that one technician can run over 27,000 PCR reactions in two days when starting from nucleic acids.
4) **Efficiency**: All assays are run in a 96-well format, enabling a smooth, efficient work-flow from robotic sample preparations.
5) **Flexibility**: The composition of microbe assays can be easily modified to meet the needs for different applications; refinements could include parsing out assays by environment, tissue, species, or life-history stage, among others. As well, there are different dynamic array formats that can accommodate fewer assays over more samples (24.192 array) or fewer assays over fewer samples (48.48 array).
6) **Expandability**: It is easy to incorporate additional assays to microbes or even to host biomarkers, although one might want to consider doing so in a separate STA.
7) **Refinement of purpose**: the research lab developed and tested a broad range of salmon microbe assays to assess which microbes were detectable in BC salmon; one could quite easily focus development on strain- or genogroup typing within microbes as has been done for human *Streptococcus pneumoniae* bacteria. One could also design a gill panel that included infectious agents and harmful algal bloom species.
8) **Analytical Sensitivity**: Results of the analyses conducted within this project are in accordance with recently published scientific literature and indicate that the BioMark is, more sensitive than other platforms, reliably detecting 3-10 copies per µl of starting material for most assays as opposed to 30-100 copies for most single-assay qPCR platforms. While this enhanced sensitivity may not be desirable for disease diagnostics (the platform was not assessed for this purpose, nor was it considered within this review), it could provide valuable information on the early transmission of microbes, microbe carrier states and ascertaining dilute microbes present in the water column or sediment.
9) **Interchangeability between platforms**: The BioMark uses the same assays developed for any qPCR platform, whether they are TaqMan, as applied herein, SYBR, EVA, and others.
10) **Limited tissue requirements**: Given the very small assay volumes (0.007 µl), hundreds of assays can be performed on the BioMark with as little as 1 µg of nucleic acids. This makes

possible the use of non-destructive samples and sampling from very small specimens. The lab utilized the platform with a single smolt gill filament smaller than a pin head as the starting material to run three dynamic arrays with microbes and host immune and stress biomarkers, the caveat being the smaller the tissue, the greater potential for false negatives for the microbes. Profiling microbes present in the stomachs of microparasites (e.g. sea lice) is also realizable.

11) **Upgradable to facilitate PCR-sequencing**: The BioMark can be upgraded to run the access array system that enables sequencing of all products produced on a single dynamic array, with barcoding of individual samples. This could facilitate rapid strain typing of multiple microbes simultaneously if assays were developed for such purposes or provide an extra level of validation of quantitative assay results.

12) **Simultaneous RNA quality assessments**: Housekeeping genes are often used as controls for RNA quality, and can easily be incorporated into the workflow on the BioMark.

13) **Detection of a second probe**: The lab utilized a positive control strategy developed by *Snow et al* that incorporates a second probe labeled in a different wavelength to facilitate detection of contamination of high copy number positive control material from artificial constructs. This is done seamlessly on the BioMark, which is capable of detecting multiple wavelengths at once.

Weaknesses

1) **Poor amplification curves for some assays**: The lab identified a small percentage of assays that were difficult to score due to poor quality amplification curves. It was assumed that this is an issue with the software rather than the platform, but further testing and working with the software company will be required to verify this assumption. In some cases, assays had to be removed due to curve quality issues.

2) **Enhanced training requirements**: Because so many assays are being run at once, there is likely a greater need for training of technical staff to accurately score a broad range of microbe assays simultaneously. The lab ran the first analyses with no assay-specific scoring rules, and for a small percentage of assays, they required refinement to increase the repeatability of assay results between technical staff.

3) **Purchase price**: The purchase price of the BioMark platform is approximately four times that of the single assay ABI 7900 system (~$250,000 CAD versus ~$60,000 CAD).

4) **Assesses presence and load, not disease**: As with any PCR-based assay, a molecular assay alone cannot discern whether an animal is diseased or if a microbe is causing disease.

5) **Volume of data and level of analysis ('big data')**: The BioMark platform is capable of delivering very large volumes of data, requiring a concomitantly intensive level of data management and analysis.

6) **Software algorithms**: The review suggested that some anomalies in the data might be explained by deficiencies in the algorithms within the software provided by the manufacturer. The research team is committed to working with the manufacturer to investigate potential software improvements.

7) **Better suited to large studies, less useful for small datasets**: The review found that the BioMark platform may prove more valuable for large scale research studies which assess multiple assays across many species rather than small numbers of samples obtained from a single species.

8) **Interpretation of results**: The review noted that the interpretation of results should exercise caution, and should be guided by the biological and pathological context of the ecosystems and species assessed.

Platform comparison (BioMark and ABI 7900)

In comparing the two PCR platforms, non-STA samples were tested on the ABI 7900 platform, whereas STA samples were assessed on the BioMark. To ensure the same detection efficiency (same Ct values), the comparison between the two platforms should have used STA samples, and the manufacturer's prescribed laboratory conditions for each. Analyses conducted on both respective platforms were per manufacturer's specifications. To address the STA issue, and to provide a valid comparator, LOD was calculated as the copy number within each chamber rather than the Ct of the sample. Ideally the same comparison should be done using real positive samples (without the gBlocks), recognizing that this may not be possible for all microbes.

The BioMark accepts most assays developed on other conventional quantitative PCR platforms, including the ABI 7900 HT through the addition of a target enrichment step i.e.STA. The authors compared the performance of BioMark and ABI 7900 HT using the same primer/probe concentrations and obtained comparable results for 21 of 22 available assays. It should be noted that the cycling conditions were not optimized for the ABI 7900.

The BioMark platform:

As the BioMark platform is still a novel and emerging technology, there are limited opportunities to perform comprehensive testing to ensure that the results are reproducible and transferrable between labs. In addition, there would be substantial value in approaching the manufacturer for validation of assays on multiple instruments.

Performance verification, validation and reproducibility:

The peer review of the report strongly recommended an on-going program of performance verification of equipment and validation of results. While this is a research project, it is likely labs will use these performance metrics to measure the implementation of the same or similar assays. As part of a QA/QC program, the platform and related equipment (e.g. pipettes, PCR machines, BioMark HD, balances, BioMark chip loading device, etc.) should be regularly calibrated.

It should be noted that while the associated Research Document indicates this to be an evaluation study for the BioMark platform *per se*, in reality it is only evaluating a single instrument – not the entire "platform". To claim platform validation, the performance characteristics of the assays should be evaluated on a number of identical instruments. This is important because the performance of the assays will only be as good as the manufacturers' ability to produce standardized, quality instruments and consumables.

The experimental design would be strengthened by presenting reproducibility data from the use of more than six (6) dynamic arrays. Additionally, the report would benefit from demonstrating whether the different dynamic arrays employed in the study were from the same or different manufacturing lots. Ideally, to fully assess the system, it would be beneficial to obtain a measure of the reproducibility (and hence performance) of different manufacturing lots of such a key critical reagent.

Serial dilution, LOD and Ct cutoff:

For each primer/probe design, a number of assays were performed to assess both the Limit of Detection (LOD) and assay linearity. Most assays demonstrated a high level of performance without modifications to the fixed assay condition (e.g. concentrations of amplification primers and/or probes and thermal cycle profile according to the manufacturer's instructions). However, this was done using a single serial dilution of artificial positive controls (APC), with considerable replication (40x) across STA reactions and dynamic arrays. However, one replicate is not necessarily sufficient to estimate the performance characteristics of the assay(s), especially since the quality of a dilution series may have a significant impact on the efficiency of subsequent experimental work (vis-à-vis "downstream" work flow).

The results of the study design (i.e., a single dilution series) with respect to the LOD may have a number of effects downstream including establishment of the Ct cutoff (as the LOD was used to inform the cutoff), and ASp (as the Ct cutoff was used to inform the ASp). With respect to the Ct cutoff, some assumptions were made that this cutoff would be used in the downstream work flow, and to guide the scoring of a positive/negative sample. It was noted that there is an option in the Fluidigm software to select for a Ct less than 40. However, a question remains as to how the number of cycles will be selected, and if the Ct cutoff will be used to inform scoring.

Quality Assurance / Quality Control measures (QA/QC):

To account for the uncertainty around some processes in the workflow, it is suggested that a quality control program be considered to provide methods and process checks, verification of critical reagents, calibration of pipettes, etc. Each aspect of the process should be considered to mitigate risks and these processes should be captured in SOP's and an overarching quality manual. Consideration should also be given to the fact that the analytical performance of the assays can be affected by variation between manufacturer's lots, including reagents, Taqman, etc.

The workflow is also multilayered and complex. As such, there are many different steps each of which takes additional time and manipulation potentially creating the possibility of error (e.g.; cDNA synthesis, STA, plate injection, PCR). Accordingly, a more complex process also requires more QA/QC measures to ensure quality around each step, which can further add to the cost and time incurred. Finally, it was noted that the close physical configuration/layout of the multi-chamber chips may present a risk of cross-contamination between samples/chambers. It may be possible to mitigate this risk via the process design.

The ExoSapit procedure (post-STA) was recognized as a very important step, and a critical control point. It would be beneficial to develop a series of QA/QC indicators to confirm the findings, and to ensure there is no carry-over of primers to the final reaction. Additional testing should further ensure that all primers in the STA are fully digested.

Co-infections, multiple infections:

In the specificity analysis (particularly with the parasite panel), it is important to explore all possible areas of risk for 'false positives'. Hence, sequencing of potential 'false positive' reactions (so-called multiple infections) is critical to distinguish between potential cross-reactions or co-infections. Also, it would be a good idea for samples with apparent multiple parasites, to examine the 'hits' to determine if the parasites present make sense given the life history of the sample used.

Microbe detection, infection and disease:

The peer review noted that there should be clearer distinction made between microbe (agent) detection, infection, and disease. *Detection* of an agent does not necessarily imply infection and infection is not the same as disease. These distinctions are very important to clarify; particularly as they relate to use of the technology and interpretation of results. Also, a *diagnosis* is the process of determining the causality of *disease* and generally involves more than determining possible causes of disease.

There should be no ambiguity in the Research Document of a high infection load as being proof of causality. While infectious agent load is usually high for a specific disease, detection of high loads of a potential disease-causing agent does not necessarily imply that the same agent is causing disease i.e. this is not a two-way relationship.

Selection of primers and controls:

When testing for World Organization of Animal Health (OIE) listed notifiable infections, the primer sets recommended in the Manual of Diagnostic Tests for Aquatic Animals should be used. The analytically validated primer sets for segment 7 and 8 recommended in the OIE Manual were not used for detection of ISAV in this study. Similarly, the assays used for detection of betanodaviruses were not those that appear in the OIE Manual.

There is an apparent incongruity in the selection of ISAV controls; genogroups of ISAV are based on phylogenetic analyses and includes two major groups, European and North-American and one minor European-North-American group. In addition there are 3 subgroups with the European clade. Here the choice of ISAV controls is based on HPR, which don't correlate well with genogroups, thus when a large collection of controls were selected, the major genogroups and subgroups should be represented.

Phase 2a assays were conducted at a hybridization temperature of 60°C for all of the primers and probes. Some of the assays were adopted from the literature and the recommended annealing temperature (Ta) was not always 60°C. Adapting such primers and probes to fit the Ta of 60°C could potentially be beneficial (for the published assays).

Interpretation and reporting of results and findings:

While a benefit of this project will be the insight gained into the presence of multiple microbes in wild and reared fish, there will be some uncertainty around the biological and ecological significance and meaning of such findings given the large array of microbes examined. A framework to guide the interpretation of these findings is strongly recommended, and should be collaboratively developed among both the research project team, and the regulators (CFIA and DFO). As part of this framework, consideration should be given to the mechanisms of how such findings will be communicated to the public, including the broader implications of potential findings.

For many of the included test agents, "natural" agent controls were not available, thus synthetic positive controls (gBlocks and plasmids) were used to represent some species/strains. This seems adequate for use as positive test controls, however, when used as targets during examinations of specificity, results should be reported and evaluated with great care. A 1000bp gBlock usually represents only a small portion of the total genome (for example for a typical bacterium about 0.02%). Thus, there are questions regarding the relevance of reporting 100% specificity when such synthetic antigens are used. Studies of such cross reactions are of limited value when gBlocks are used, and results should be interpreted with great care.

As reported, the cross reaction studies were only performed *within* the main microbe groups (i.e. viruses, bacteria and parasites), but the analyses should have been conducted *across* the main microbe groups. These agents have co-evolved with their hosts for millions of years, and there are several examples of gene exchange between groups during evolution. Note, however, that typical assay evaluations would not go to this level of complexity (e.g. bacteria to parasite).

The biological/pathological interpretation of results obtained from healthy fish (either farmed or wild) will be both more challenging and potentially more significant - i.e. which of the detected agents are of importance, and do they really harm the fish? There are also several questions to be addressed when referring to diseased and/or healthy fish, and these are expressed as caveats and cautions to the interpretation of positive results:

- Not all infections are generalized - they may be confined to particular organs, i.e. which organs should be tested.
- Is it relevant to test for generalized infections in healthy fish using the same type of samples as for diseased fish (typically *internal* organs)?
- In one sense it may be more relevant to test *external* organs of healthy fish (i.e. skin, gill, gut), however such samples may more reflect the environment than the fish. Meta-genomic analyses of seawater have revealed enormous amounts and diversity of microbes present, and the probability of picking up cross-reactive siblings of the test agents is significant. Examples of non-virulent siblings of virulent viruses identified during recent years include non-virulent ISAV HPR0, non-virulent marine VHSV and several reovirus species.

It is strongly recommended that a framework for the consistent biological/pathological interpretation of results be created. This framework (or protocols) should be developed collaboratively between the research project team, and the respective management and regulatory decision makers.

Translation of microbe detection (from farmed disease to wild scenarios):

Most infectious fish diseases have been detected and are well-described in farmed or cultured fish. On the other hand, the agents chosen to be included in Phase 2b of this study are not agents that have been demonstrated to cause damage to wild fish. The relevance and validity of extrapolation of disease scenarios between farmed fish and wild fish populations is uncertain. The interpretation of such results obtained from farmed and wild fish scenarios should be conducted very cautiously for several reasons:

- Farmed fish are reared under extreme conditions, thus these operations are usually highly industrialized and production is intense with large numbers (and biomass) of host within a limited space.
- Disease is a function of cause/agent, host and environment, and the farming situation cannot be compared with wild fish. Disease problems in farmed fish must largely be regarded as production-related diseases (though many have an infection component).
- One key virulence factor is the ability to transmit and sustain an infection in susceptible hosts – thus the number and density of available hosts is a key factor.
- It is rare for viruses to cause immediate death in their hosts – viruses are well adapted parasites that have co-evolved with their hosts for millions of years and thus generally don't kill their host. For instance, it has been estimated that humans are at any time infected with 5-7 different viruses that don't cause any harm.
- Infectious agent have co-evolved with their natural hosts for millions of years and are generally very host-specific (although there are exceptions like IHNV that seem to be able to infect new host species readily without any prior adaptation). On the other hand VHSV,

believed to have its natural reservoir in marine fish, must go through an adaptation process to be able to cause disease problems in Rainbow Trout.

- Infectious diseases described in cultured fish/aquaculture are generalized infections (many are septicemias). The question is whether it is relevant to test for such infections using same approaches as in diseased fish (i.e. testing internal organs). Thus - is it reasonable to assume that a healthy fish has a generalized infection/septicemia?
- There are only few examples of major kill-offs caused by infectious disease in wild fish. Recent examples of this are the VHSV epidemics in the Great Lakes and in Pacific Herring. On the other hand, infectious epidemics in animals are well known in natural regulation of population sizes.

CONCLUSIONS AND ADVICE

This peer review and advisory process revealed a number of recommended improvements to the experimental design, lab procedures and the associated working paper (Research Document) which must be addressed.

The BioMark platform is *state of the art* and represents a powerful new technology, providing excellent potential for the detection and surveillance of various microbes. This Science review found the BioMark platform to function as well as any other qPCR platform, and there were no shortcomings identified for the instrument *per se*.

Taking into consideration the *Assessment* section in this report, it was concluded:

1) Given the approach used, and based on the information provided, the measures of analytical performance appear to be within an acceptable range for most assays for the proposed research project.

2) *Sensitivity:* No significant differences between STA and non-STA frequencies of false positives were observed across the assays. The measures of analytical performance used were within an accepted range for most assays; any variance from 100% ASp did not impede resolution of known salmon microbes (with one exception).

3) *Replication (LOD):* A single serial dilution was used for the assays (and 40 replicates were performed from this single serial dilution). For more robust results, it is recommended that a minimum of 3 different serial dilutions with 5 replicates for each should be tested. Without such replicates, conclusions regarding the analytical sensitivity on an assay-by-assay basis may be problematic.

4) *Specificity*: Samples were collected from as many reliable sources as possible and showed satisfactory results for 13 viral and 12 bacterial assays. Assays for 22 parasites were mostly successful but some assay subgroups appeared positive in certain tissue samples (presumably because of co-infection by multiple parasite species). Nucleotide sequencing by species-specific segments of the genome could confirm mixed infection.

5) *Repeatability*: Twenty-six (26) endemic microbes were assessed for repeatability across 240 samples from BC salmon. Repeatability (within a dynamic array) and reproducibility (across dynamic arrays) were intensely examined. Binary agreement (positive/negative) was assessed between replicates and found to be 98% overall. Based on the data presented, there did not appear to be any issues with respect to repeatability.

6) *Platform comparison*: The study compared the performance of BioMark and ABI 7900 HT using the primer/probe concentrations recommended for the BioMark platform, and obtained comparable results for 22 available assays. Ideally it would be best to have a full

comparison of the two platforms using the optimal conditions for each, noting that conditions were not necessarily optimal for the ABI 7900 instrument.

7) Based on the number of copies in the starting material, other than the limit of detection (LOD), there were no consistent biases introduced by the STA.

8) Of the 47 assays conducted, two assays were targeted for replacement (Piscine myocarditis virus, Viral hemorrhagic septicemia virus) and one for removal (*Yersinia ruckeri*).

9) The BioMark instrument assessed has demonstrated fitness of purpose for this research project. A number of *strengths, weaknesses* and potential *uncertainties* in results obtained using the BioMark instrument have been identified and should be addressed.

Caveats to the Advice

The present research design is notably different from a traditional hypothesis-driven approach to the investigation of fish health. Accordingly, it represents a new approach to assessing the potential impact of pathogens on fish populations (i.e. interpretation of the significance of potentially low levels of pathogen on the health status of fish). There are a number of cautions and caveats regarding the limits of extrapolation of such research results.

This review did not evaluate individual microbial assays in detail beyond what is provided in this report; instead this is a more general assessment of the microfluidics qPCR platform, and limited to assessment of one individual instrument. Accordingly, this advisory report does not provide comment on the design of the individual assays used.

Per the use of any qPCR platform, of critical importance will be the biological and pathological interpretation of results and the ability to assign risk to these results (e.g. differentiation between presence of pathogen, infection, and disease).

Although mentioned in the working paper that the BioMark platform could be used for diagnostic purposes, an evaluation of this potential application was neither included in the study, nor was this an objective of this Science advisory process.

Sources of Uncertainty:

The various sources of uncertainty and relevant research recommendations have been described in the appropriate sections in this Science Advisory Report (see *Assessment* section), the Proceedings of the peer review meeting, and in the associated Research Document.

APPENDIX 1: DEFINITIONS

Disclaimer: while many of these definitions may be universally applied, others are specific to the context of this research study, so caution is advised. For example, the definition of *repeatability* would be different in other studies since it usually applies to the entire process (e.g. starting with the sample that would be split and then processed twice).

Term	Definition
ABI 7900HT™	A standard qPCR platform for microbe quantification used around the world and manufactured by Applied Biosystems.
Analytical sensitivity (ASe)	The minimum number of copies of the target gene sequence reliably detected by the qPCR assay. Typically, sensitivity is expressed as the limit of detection (LOD), which is the concentration that can be detected with reasonable certainty (95% probability is used here). Experimental results less than the theoretically possible LOD should be reported as <LOD. The term sensitivity is also used in the specificity analysis, but in this case, it defines the percentage of times that real positive samples were detected, or the "inclusivity" of the assay. In this case, assays showing <100% "sensitivity" may not amplify all known strains or some positive control samples may be concentrations below the limit of detection.
Amplification efficiency	In an ideal assay the amount of target sequence (amplicon) doubles after each cycle (exponential increase). Amplification efficiency is a measure of the closeness of the amplification rate to the exponential ideal. It is determined by creating a standard curve (plot of Ct vs. log concentration) for a series of 10 fold dilutions of the standard. The standard curve can be used to determine the dynamic range (useable working range) of the assay and to calculate the slope and efficiency of the assay in terms of r and R^2 coefficients. The efficiency of the PCR reaction is calculated using the following equation: $E = 10^{(-1/slope)} - 1$; ideally the efficiency should be between 90 and 110% and the slope of a standard curve should be −3.32, with $R^2 > 0.985$.
Analytical specificity (ASp)	The degree to which the assay does not detect (amplify) other phylogenetically related non-target microbes. This is determined empirically by comparing available nucleotide sequence information for the target species and closely related species (in silico analysis) and by running the qPCR assay designed for the target with authentic positive samples. The positive samples may be infected fish tissue, pure pathogen culture, or made-to-order synthetic gene sequences (gBlocks™).
APC clone (aka Minigene™)	Artificial Positive Control (APC) standards are constructs that contain the entire sequence targeted by each assay cloned into a plasmid vector to enable continuous production of control material. Serial dilutions of APC controls of known concentration were used to assess the analytical sensitivity of each assay—i.e. the minimum copy number that could be detected or "limit of detection".

Term	Definition
APC probe	An extra probe sequence was added to each APC clone, enabling continuous monitoring of any contamination of the APC in all qPCR reactions. The universal APC probe (which is the same for all assays) was labeled in NED™. The BioMark was set to detect both FAM (the probe specific to each assay) and NED simultaneously.
AUC	Area under curve. - see ROC
BLUP	Best linear unbiased prediction
BLASTN	BLASTN for Basic Local Alignment Search Tool is an algorithm for comparing primary nucleotide sequence information. This program, given a DNA query, returns the most similar DNA sequences from the DNA database that the user specifies.
BioMark™	The microfluidic platform being validated in this report made by the Fluidigm corporation.
cDNA	Complementary DNA is DNA produced from mRNA template and a reverse transcriptase enzyme.
CFIA	Canadian Food Inspection Agency
CHSE	Chinook salmon embryo cells are a commercially available cell line derived from Pacific Chinook salmon. While these are widely used to culture viruses, the research lab used the RNA expressed in virus-free CHSE cells as a spike-in to APC clones and gBlocks to provide a background of host nucleic acids similar to the levels expected in a tissue sample.
Cohen's kappa for dichotomous variables	A measure of agreement of pass/fail, presence/absence results that adjusts for the amount of agreement that would occur by chance. Kappa ranges from 0 – 1 with κ =0.20 showing very poor agreement and κ = 1 showing perfect agreement.
Comparability	Used here to describe the agreement between the two qPCR platforms (BioMark and ABI 7900).
Concordance Correlation Coefficient (CCC) for continuous variables	The concordance correlation coefficient (Lin 1989) is a measure of agreement between pairs of results (across platforms, technicians, methods, etc.) A concordance plot shows how closely the results lie on a line with slope=1 (45 degree angle) that passes through the origin (line of perfect concordance). The CCC (ρc in the following equation) contains a parameter for precision (ρ) and accuracy ($C_{b)}$. ρ is the Pearson correlation coefficient, a measure of goodness of fit to the line (precision) and C_b is a bias correction factor that indicates how much the best-fit line deviates from the slope and origin, and is a measure of accuracy. $\rho_c = \rho\, C_b$

Term	Definition
CI	Confidence interval
CRS	Closely-related species. These were defined herein as species with >90% sequence homology to target species. On occasion, all species within a genus were considered CRS even if there was <90% homology.
CSAS	Canadian Science Advisory Secretariat.
Ct	The threshold cycle where the fluorescence exceeds background levels seen in the no template control and where exponential growth of the PCR product ensues. Lower Ct values indicate higher sample loads.
Ct cutpoint (cutoff)	The threshold Ct above which samples are considered to be “negative” for the microbe analyte. Here the optimal BioMark Ct value for maximum agreement between the qPCR platforms was determined as the highest area under the ROC curve with the highest specificity value. The lab also used the Ct LOD, defined as the maximum Ct whereby at least 95% of known positives are repeatedly detected.
DFO	Fisheries and Oceans Canada.
Dynamic Array™	The 96.96 “chip” that runs on the BioMark platform. Dynamic arrays are available for 96 assays x 96 sample (96.96), 48 assays x 48 samples (48.48), 24 assays x 192 samples (24.192), and 6 grids x 12 assays x 12 samples (6.12.12).
EVAgreen™	A reporter molecule that fluoresces when bound to any double-stranded DNA. The binding is not sequence-specific.
False-negative reaction	A negative result (no detectable fluorescent product) in a TaqMan qPCR assay of a test sample obtained from a sample known to contain the target gene. It may be due to lack of analytical sensitivity, restricted analytical specificity or sample degradation.
FAM™	A fluorophore with an emission maximum at 518 nm.
False-positive reaction	A positive result (detectable fluorescent product) in a TaqMan qPCR assay where the target gene is not known to be present. It may arise from cross-reactivity of closely-related pathogen species, cross-contamination of the test sample or non-specific reactions, e.g. probe degradation resulting in fluorescence.
gBlock™	1,000 base pair synthetic DNA constructs produced for ASp analysis for strains or species for which “real” controls in the form of tissue samples, cell lines, or bacterial cultures were unavailable.
ISO	International Organization for Standardization.

Term	Definition
LOA	The Limit of Agreement can be determined graphically using a Bland and Altman plot. They suggest that when the difference of the paired two measurements is plotted against the mean of the two measurements that 95% of the measurements should be within ±2 standard deviations of the mean difference.
LOD	Limit of detection is a measure of the analytical sensitivity. The LOD is the estimated amount of analyte in a specified matrix that would produce a positive result at least a specified percent of the time. Typically, estimated LOD will be based on spiking of the analyte into the target matrix. Calculated values were derived from the measured concentrations of the last dilution with at least 95% detection.
MGL	Molecular Genetics Laboratory at Pacific Biological Station headed by Dr. Kristi Miller and where the laboratory analysis was performed.
Minigenes™	Same definition as APC clones.
Mx3005P platform	The qPCR platform used by NAAHP.
NAAHP	National Aquatic Animal Health Program.
NED™	A fluorophore with an emission maximum at 575 nm. In this study the artificial positive control probe was labeled with NED™.
NGS	Next generation sequencing.
Non-STA	The typical method of qPCR without the specific target amplification step. See STA.
NRP	A negative control assay without added primers and/or probes.
OIE	World Organisation for Animal Health (formerly the Office international des epizooties, or OIE).
Pr(Disagreement)	Predicted probability of disagreement.
Repeatability	Agreement between sample replicates, both within an assay run and between independent assays. For example - two results taken from the same chip run by one technician from one RNA sample plate.
Reproducibility	Agreement of results between runs. For example, agreement between two results from one sample run by two technicians from two RNA extractions on two dynamic arrays.
RFTS	Rainbow trout fry syndrome caused by *Flavobacterium psychrophilum.*

Term	Definition
ROC	Receiver operator characteristic. ROC curves are used to plot the sensitivity of a test versus the false positive rate (1-Sp) computed at a number of different cutpoints; the lab used ROC to select the appropriate Ct cutpoint for distinguishing between diseased and non-diseased animals. Since the researchers did not have known disease status for the animals from which the samples were collected, they assessed the agreement between the two platforms by artificially assigning a 'diseased' status set at four Ct cutpoints (25, 30, 35, and 40) to classify ABI7900 samples as Pass/Fail. The nonparametric ROC analysis was used to determine the optimal BioMark Ct value for maximum agreement, as determined by the highest area under the ROC curve with the highest specificity value.
SOP	Standard operating procedure.
SSU	Small subunit ribosomal RNA sequences.
STA	Specific Target Amplification. The 96.96 dynamic array uses a sample loading volume of 5 µl, and distributes this sample mixture across 96 reaction chambers in 7 nl aliquots. With these micro-volumes, detecting the specific targets requires a minimum of 500-1,000 copies per µl in the sample loaded onto the array to ensure that at least 1 copy will be present in each well. The Specific Target Amplification is a multiplex PCR using $1/10^{th}$ the normal concentration of all primers (no probes) cycled 14 times (in a normal PCR machine) to increase target concentration 1000x. Primers are removed from the STA before samples are loaded onto the dynamic array; hence amplifications on the BioMark are singleplex. This method has been proven not to negatively impact target quantitation.
SYBR™	A fluorescent intercalating dye used for DNA, with an emission maximum at 523 nm.
TaqMan™ probe	TaqMan probes are used to increase the specificity of a qPCR assay (see below). The research lab used Applied Biosystems® Custom TaqMan® MGB Probes, which incorporate a 5' reporter dye and a 3' non-fluorescent quencher (NFQ), with the MGB moiety attached to the quencher molecule. The NFQ offers the advantage of lower background signal, which results in better precision in quantitation. The MGB moiety stabilizes the hybridized probe and effectively raises the melting temperature (Tm). Source: Life technologies

Term	Definition
TaqMan™ qPCR assay	"The 5' hydrolysis chemistry utilizes two primers, a probe, and the exonuclease activity of Taq DNA polymerase. The DNA probe is non-extendable and labeled with both a fluorescent reporter and a quencher which are maintained in close proximity to each other as long as the probe is intact. Once the primers and probe hybridize to the target and the primers begin extension, the exonuclease activity of the polymerase during extension will cause hydrolysis of the probe and the connection between the reporter and quencher will be broken; this allows the reporter to fluoresce. Because all three components, the two primers and one probe, must all hybridize to the target, this approach leads to greater accuracy and specificity in the PCR product amplified than primers alone (as in SYBR PCR). In addition, different probes can have different fluorophores which will allow multiple transcripts to be simultaneously detected in a single reaction." Source: Integrated DNA Technologies
Plasmid control	A "plasmid" control probe labeled in NED was multiplexed into all assays to track contamination of the APC (same probe for all assays).
VIC™	A fluorophore with an emission maximum at 554 nm.

SOURCES OF INFORMATION

This Science Advisory Report is from the December 2-4, 2014 national advisory process on the Review of the Fluidigm BioMark platform: Evaluation to assess fitness for purpose in microbial monitoring. The accompanying Research document and Proceedings from this meeting will be posted on the Fisheries and Oceans Canada (DFO) Science Advisory Schedule as they become available.

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