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Abstract

Protecting the seafood supply chain from species substitution is critical for economic, health and conservation reasons. DNA-based methods represent an effective means to detect species substitution, but current methods can be time consuming or costly, and require specialized instruments and operators. Real-time PCR provides an alternative that can be performed quickly, and in some cases even on-site. The use of commercial kits reduces the expertise required by the operator, and therefore increases accessibility to testing. This potentially increases the likelihood of adoption into the supply chain, but only if the kits are robust across multiple operators, instruments and samples. In this study the InstantID™ Atlantic Salmon kits were tested on a variety of instruments with market samples of fresh, frozen, smoked and canned Atlantic Salmon. Results were repeatable across all samples and instruments tested. This kit, and others like it that have undergone appropriate evaluation, represents a means for expanded access to testing for industry or regulators to screen seafood for species authenticity. Portable equipment can bring testing on-site, further reducing analysis time.

Key Words: real-time PCR, Atlantic salmon, food authenticity, species substitution

Introduction

Seafood represents a critical component of food security, making up almost a fifth of the global animal protein intake and representing a US$130 billion industry (FAO 2014). For these reasons, careful management of the seafood industry is critical. One aspect of this is product authenticity, which is linked to both economic impacts and sustainability. Seafood substitution and mislabelling in the commercial market has been reported in numerous studies conducted around the world. For example, recent studies have reported seafood mislabeling in countries including Malaysia (Chin et al. 2016), the Czech Republic (Kyrova et al. 2017), Russia (Nedunoori et al. 2017), South Africa (Cawthorn & Hoffman 2017), USA (Stern et al. 2017), Taiwan (Chang et al. 2016), Canada/Spain (Muñoz-Colmenero et al. 2017), and Brazil (Carvalhoa et al. 2017). Recent reports also show that consumers are concerned about the authenticity of the products they are consuming, including seafood (Charlebois et al. 2017). Regulations in North America have increased the focus on proper labelling and authenticity, for example as part of the Food Safety Modernization Act (available at: https://www.fda.gov/food/food-safety-modernization-act-fsma/full-text-food-safety-modernization-act-fsma) and the Safe Food for Canadians Act (available at: https://laws-lois.justice.gc.ca/eng/acts/S-1.1/index.html).

DNA-based methods can be used to authenticate seafood species and have been suggested as options to help the industry combat the problem of seafood fraud (e.g. Galimberti et al. 2013; Naaum and Hanner 2016). Incorporation of these testing methods into the supply chain may help the industry enhance traceability, protect brands from the impact of fraud, and meet regulatory requirements. Many DNA-based protocols for seafood species identification have been published in scientific literature (e.g. reviewed in Naaum and Hanner 2016). However, few of these are readily available for use by industry and those that are typically require shipment of samples to off-site facilities with specialized equipment and expertise. These challenges create a barrier to efficient uptake of new technologies by industry. One example of this is the existence of published, real-time PCR tests for commercial salmon identification (Rasmussen Hellberg et al. 2011), that have not seen wide use. This may be due to difficulties in translation of assays published in academic journals to a wider audience of potential users. In order to facilitate use by industry, it is important to make testing more accessible with simplified protocols, automated results interpretation, and on-site testing capabilities. Advances in instrumentation have resulted in the emergence of highly portable and automated real-time PCR instruments that have the potential to address some of these issues.

Additionally, commercial kits for use with real-time PCR technology have been developed to further facilitate the process of seafood species authentication. However, there is currently a lack of information regarding the robustness and reliability of the various instruments available when combined with commercial test kits.

In this study we evaluate a commercially available real-time PCR kit for identification of Atlantic salmon (*Salmo salar*) on a range of real-time PCR instruments to assess test performance and compare the instruments used. Instruments were selected to cover the range from benchtop research instrument to handheld device. Atlantic salmon was chosen as a target from the available test kits offered by InstantLabs due to the wide availability of commercial samples in a variety of processing types. This allowed a focus on performance across instruments. Our case study results illustrate the potential for rapid real-time PCR detection kits to streamline the process of seafood identification, making lab-based protocols more easily accessible.

Materials and Methods

Sample Collection and Verification

Thirty samples labelled as containing Atlantic salmon were collected from grocery stores in Guelph, ON. Samples represented a range of processing types, including: fresh (n=7), frozen (n=5), cooked (n=2), smoked (n=14) and canned (n=2), and were selected to cover the types of samples that a targeted species-specific kit would be designed to test as a means of confirming the presence of the target species. Samples were labelled with a random 3-digit number and subsampled into two sets of collection tubes containing 90% ethanol: one set was held at University of Guelph and one set was shipped to Chapman University.

Instruments

All samples underwent real-time PCR with six different instruments. At the University of Guelph, the following instruments were tested: Smart Cycler II (Cepheid, Sunnyvale, CA, USA), Hunter (InstantLabs, Baltimore, MD, USA), Open qPCR (Chai Biotechnology, Santa Clara, CA, USA) and two3 Device (Biomeme, Philadelphia, PA, USA). At Chapman University, two instruments were tested: Roter-Gene Q (Qiagen, Germantown, MD, USA) and CFX Connect (BioRad, Hercules, CA, USA).

Test Kits

InstantID™ Atlantic Salmon Kits were provided by InstantLabs for evaluation in this study. Two forms of the kits were used: one with individually packaged lyophilized single reaction master mixes and cartridges for use in the Hunter instrument, and one with identical DNA extraction supplies and reagents, but with bulk lyophilized master mix for use with the other real-time PCR instruments.

DNA extraction was performed in duplicate on each sample according to manufacturer guidelines, with the exception that the swab step was replaced due to the use of ethanol-preserved tissue. Instead, a subsample of tissue (~2 mm^3) was taken with sterile tweezers, rinsed in molecular grade water, and then added to the lysis buffer in a pre-made sample extraction tube. To complete the DNA extraction, 20 µL of PK buffer from the kit is then added to the sample extraction tube. Lysis was completed at room temperature for 20 minutes, followed by heating at 100 ° C for 10 minutes. Following lysis, 2.5 µL of the extracted DNA and 10 µL of molecular grade water were added to the master mix for use in real-time PCR.

Real-Time PCR

Real-time PCR was carried out according to the manufacturer instructions and Cq values were recorded for all samples. Before commercial samples were distributed to all labs, specificity of the kit was tested against other commercial salmon species on the Hunter instrument. One sample each of Sockeye salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tshawytscha*), Coho salmon (*Oncorhynchus kisutch*), Pink salmon (*Oncorhynchus gorbuscha),* Chum salmon *(Oncorhynchus keta*) were tested. In addition, a test of one product each of smoked, canned and fresh Atlantic salmon were conducted to confirm that product processing did not affect the ability to detect the target species. Reference samples were identified using DNA barcoding, carried out as described in Handy et al. (2011) to confirm their identity, except in the case of the canned product on which DNA barcoding could not be done.

For all commercial samples tested on multiple instruments, duplicate DNA extracts were tested on each instrument, except for the two3 device and Open qPCR, where only one replicate was tested. One sample that failed to amplify on the first replicate on the Open qPCR system was repeated in duplicate from the same DNA extract. Cq values under 35 cycles were considered positive for Atlantic salmon except on the Hunter instrument, where the default result output is positive or negative for the sample. This is in line with typical Cq values for real-time PCR. A positive control with synthetic Atlantic salmon DNA and a no-template negative control were run with each newly

105 re-constituted bulk batch of master mix. For the Hunter instrument, the controls were run once per each replicate of
106 sample testing.

107

108 **Results and Discussion**

109 Preliminary testing on the Hunter instrument showed negative results for all samples of non-target species tested,
110 and positive results for Atlantic salmon samples of all three processing types analyzed. For market samples tested in
111 this study, real-time PCR testing with the Atlantic salmon kit identified Atlantic salmon in every sample tested
112 across all instruments and replicates, including fresh, frozen, smoked and canned samples, with the exception of one
113 canned product that failed to amplify on the Open qPCR system. Positive and negative controls were as expected in
114 each case for all instruments. The one sample that initially failed to amplify on the Open qPCR system underwent
115 repeat testing in duplicate with the same DNA extract and tested positive for Atlantic salmon. Raw data from the
116 first replicate showed some amplification beginning near the end of the reaction, but a Cq value was not recorded for
117 the corrected data. When the sample was re-tested in duplicate, Cq values of 33.40 and 35.39 were obtained,
118 suggesting some issues with reproducibility may exist for samples with amplification late in the run.

119 Instrument costs ranged from under US$5,000 (two3) to over US$50,000 (Cepheid). With the exception of the Open
120 qPCR system we did not observe any differences in the ability of the instruments to successfully identify Atlantic
121 salmon from any of the samples using the InstantLabs Atlantic Salmon Kit. The similarity in performance for
122 confirmation of the presence of the target species across instruments suggests that all instruments tested here may be
123 suitable options for use with pre-packaged real-time PCR assays. The Hunter instrument returned a “Positive” result
124 for all samples. The Cq values obtained with the other instruments were as follows: 19.10-31.55 (Smart Cycler II),
125 20.85-35.39 (Open qPCR), 17.11-33.21 (two3), 18.66-31.67 (Rotor-Gene Q), and 19.43-33.94 (CFX Connect). The
126 Cq values listed for the two3 instrument were calculated after a correction in the algorithm. At the time of the study,
127 this device and its software were in beta testing, and although the raw data clearly showed amplification for some
128 samples, a Cq value was not given. This has been corrected in a recent update of the software and we have reported
129 the corrected values here.

The assay was overall very rapid, with the time to detection ranging from 50 to 81 minutes depending on the instrument. This run time is typical for many real-time PCR assays and slightly shorter than conventional PCR, which often takes 60-120 min followed by an additional 30-90 min for gel electrophoresis. It also allows for a much faster turnaround time than DNA sequencing based approaches like DNA barcoding, which would require multiple PCR steps and sequencing at a specialized facility, usually taking at minimum a few days for results. However, certain instruments of those tested may be preferred due to shorter run times. DNA extraction took less than 40 minutes, with only a heating block and microcentrifuge required in addition to the kit components. This provides ample time savings, as typical DNA extractions often take several hours for lysis and require specialized equipment such as a high speed centrifuge. This and other rapid DNA extraction methods available help reduce the amount of sample preparation time, thereby increasing the ease with which to conduct screening for a specific target using real-time PCR. With limited steps, and no mixing of reagents, this presents a simple and effective tool for rapid identification of Atlantic salmon. This method for species identification was shown to be robust across many sample types as well as the various instruments tested. This is likely to also be the case for similar lyophilized kit preparations paired with simple extraction methods. Therefore, we conclude that the appropriate instrument for the testing facility/users can be chosen to meet existing needs for run speed, cost, portability and throughput, without sacrificing assay performance. However, test performance should always be evaluated on the specific instrument on which testing will occur using known reference standards, which can be generated using whole genome amplification (Bourque et al. 2017) or other means.

In the context of use for food testing, simple test kits like the example illustrated here could be run on benchtop research instrumentation, such as the Cepheid II, CFX Connect, or Rotor-Gene Q, or on the lower priced-portable instrumentation tested in this study, such as the Hunter or two3 instruments. Table 1 shows a comparison of instruments used in this study. The flexibility in choice of instrument should allow easier adoption of this method as there does not appear to be an instrument-dependent effect on assay performance. While low-cost, handheld, options like the two3 device performed well, and may extend real-time PCR testing into the field, the low throughput (3 samples) may not be appropriate for large-scale operations. The Open qPCR system was a low-cost option that performed well, however its portability is limited due to the lid design. To accommodate multiple tube sizes, the lid is adjustable, however the recommendation is therefore to re-calibrate the instrument (>1 h process) each time the machine is moved. This may limit the use of the machine where more rigour is required for results, or where

158 portability is desirable. Benchtop research instruments allow more flexibility with protocols and data analysis, but
159 for non-expert users this can provide unnecessary complications. More portable instruments, like the two3 and
160 Hunter systems tested in this study, focus on pre-programmed assays. The two3 device operates via an included
161 iPhone and app, giving some familiarity to the system for non-expert users. The Hunter has a barcode scanner to
162 confirm that the correct kit is used with the selected program, reducing the likelihood of human error.

163 Real-time PCR in general represents a powerful tool for species identification in food. It allows targeted screening to
164 take place rapidly and accurately, in multiple product types, including highly processed or mixed products, and
165 potentially in field settings. This approach may help to address some of the key issues within the seafood supply
166 chain related to species identification, particularly when done in a more standardized way with pre-packaged testing
167 options. Simple species identification kits like the one evaluated in this study have the potential to facilitate rapid
168 screening of seafood for species authenticity in a wide range of settings without the need for expert analysis or
169 sending samples to specialized facilities for testing. While the InstantLabs InstantID kit was used in this study, our
170 findings illustrate the potential for any lyophilized real-time PCR kit to provide more accessibility to real-time PCR
171 testing by simplifying and standardizing the approach. From a cost perspective, this particular kit is ~$20/sample.
172 When compared to DNA sequencing (often $150+ for commercial testing), this provides a means for screening
173 target species at a lower cost per sample. These, and other, commercial qPCR kits are a premium price compared to
174 traditional PCR (<$1/sample, plus the cost of gel electrophoresis), or even running in-house qPCR (~$3/sample).
175 However, the pre-packaged and standardized components, along with extensively simplified protocols for DNA
176 extraction, allow easier use with minimal training, and may produce better consistency in results.

177 In addition to validating the performance of a specific kit for Atlantic salmon identification, we have confirmed
178 flexibility in instrument choice to run kits of this type, which is likely to extend to other assays. This can provide a
179 tool to many interested stakeholders in the seafood supply chain and can help to combat food fraud related to species
180 authenticity, which in addition to economic and environmental impacts has been determined to impact human health
181 and consumer choice (Naaum and Hanner 2016). While we tested a commercially available assay from one
182 company, there are other sources for these kits, including options for customization in some cases. Real-time PCR
183 assays could in theory be developed for any species of interest, provided there is reliable sequence data from which
184 to design the assay. This would include sufficient sequence data for a target and close relatives that might be present

in the same samples or be confused for the target. Using DNA barcode sequences as a starting point allows the relatively straightforward generation of assays by using the millions of sequences available for this target marker (Naaum and Hanner 2016).

Simplified and/or portable methods can facilitate scientific field research to continue to identify and document incidences of food mislabelling and extend the ability of citizen scientists to participate. These methods also expand the available tools for regulators to combat this problem. Increased regulatory testing has been shown to reduce cases of seafood mislabelling (Mariani et al. 2015). Better labelling practices and proper tracking of seafood products is necessary to ensure the continued sustainability of this critical industry (Cawthorn and Mariani 2017), and simpler, more cost-effective testing options can help support the enforcement of new regulations as well as bring access to testing to developing countries, small-scale fisheries, and remote areas.

Commercially available test kits and instruments that allow simple and rapid species identification also provide a means for industry to access this testing without the need for a large amount of specialized equipment or personnel. While some training would certainly still be required, the simplified protocols reduce this requirement to a minimum, allowing more people to have the ability to confidently perform qPCR testing. Voluntary testing by industry represents one of the ways to discourage species mislabelling throughout the supply chain. Traditional methods for testing require adaption of protocols from scientific studies, which requires a high level of expertise, or shipment of samples to testing facilities. These methods are often time consuming and/or costly. Therefore, the increased accessibility to screening afforded by straightforward commercially available analytical kits for qPCR may allow easier implementation of this method into existing QA/QC processes and facilitate use of the technology for brand protection and supply chain management.

Conclusions

DNA testing provides a means for assuring the authenticity of food products. However, many existing methods may be too slow and costly for large-scale implementation to combat food fraud. Commercial kits for real-time PCR of target species offer one potential solution. These kits may allow rapid, on-site screening of target species. While not universally applicable to any target or type of adulteration, customized screening for species substitution can be achieved using this approach. As a case study, one such kit for the identification of Atlantic salmon was evaluated. It was shown to be robust across multiple instruments, users and sample types, and illustrates the potential of similar

kits as tools in food authenticity testing. Access to simpler and faster testing, as offered by this and other real-time PCR species identification kits, may increase implementation into the food supply chain by reducing the expertise level required to run a successful qPCR test without compromising the integrity of the results.

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Conflict of Interest Statement

These kits were manufactured using primers and a probe designed at the University of Guelph for use by InstantLabs. The authors currently have no financial or personal investment with InstantLabs. No input from InstantLabs was made into the design, outcome or reporting of results for this study and no financial gain was made by any of the parties involved in this study. A. Naaum and R. Hanner are involved with a separate commercial company offering authenticity testing services for food and natural health products, but which also had no oversight of this study, made no financial gain, and provided no funding. R. Hellberg and T. Okuma declare no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Informed consent was not applicable in this study.

Table 1. Comparison of real-time PCR instruments used in this study. Instrument costs are approximate and subject to change. Data analysis was considered automatic if an indication of presence or absence of the target was given automatically during data output. Portability values are relative, and based on the ease with which the instrument can be transported and used in different settings.

Instrument	Cost of Instrument (USD)	Run Time (min)[a]	Portability	Data Analysis	Sample Throughput	Fluorescence Channels	Plastics
Smart Cycler II*	High (>$40K)	55	Medium-low	Manual	16	4	Custom tubes
Hunter	Low (<$10K)	72	High	Automatic	6**	1	Custom cartridges
two3	Low (<$10K)	72	Very high (handheld)	Automatic	3	2	Custom tube strips
Open qPCR	Low (<$10K)	50	Medium	Manual	16	1-2	Universal tubes
Roter-Gene Q	Medium ($10-$40K)	81	Medium-low	Manual	36[b]	2-6	Universal tubes
CFX Connect	Medium ($10-$40K)	72	Medium-low	Manual	96	2	Universal tubes/ 96-well plates

[a] Based on the assay used in the current study

[b] Throughput settings used in the current study. The Rotor-Gene Q has the capability to run up to 100 samples at a time using custom tubes and rotor discs.

* The Smart Cycler II has since been discontinued by Cepheid.

** The Hunter instrument has since been updated for a 12-well standard cartridge rather than 6 as used in this study.

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