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Short Technical Communication
Enhanced fish species identification by PCR-RFLP using the 2100 Bioanalyzer system

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Abstract: In the seafood market new regulations and an increasing number of cases involving substitution and fraud drive the need of stakeholders for a robust, easy to use and well accepted method of fish species identification. PCR-RFLP of mitochondrial target sequences has been used successfully in the past for the purpose of species identification. Issues with assay robustness due to the use of individually prepared and non-optimized components and the manual nature of analysis potentially affect the reliability of the results. In this technical communication an optimized solution using the Agilent 2100 Bioanalyzer is presented. The improved method allows analysis from sample to result in one working day and facilitates analysis by introducing a dedicated analysis software for pattern recognition and species identification.

Keywords: Species identification, mitochondrial DNA, PCR-RFLP, Agilent 2100 Bioanalyzer

Introduction

The global demand for seafood has grown considerably. Fish is valued and advertised as healthy food and consumption has increased significantly. Limitations of the resource and the potential for increased profits lead to the problem of substitution and mislabeling for a substantial part of the market (Jacquet and Pauly, 2008; von der Heyden *et al*., 2010; Miller and Mariani, 2010). In order to validate shipments along the supply chain and to protect the consumer, tests to identify the species are required. In addition new regulations to fight illegal, unregulated and unreported fishing activities have been established and need to be monitored (EC Council Regulation No. 1005/2008 and EC Commission Regulation 1010/2009, US Department of Commerce, Proposed Rules 50 CFR Part 300, Docket No.: 080228336-9133-01).

Protein pattern based methods are still commonly used for fish species identification. Analysis by isoelectric focusing is listed as one of the AOAC official methods (AOAC official method 980.16) for this purpose. However these methods tend to be less reliable with processed food or mixed samples and can be very subjective and difficult to analyze. DNA based testing methods on the other hand allow sensitive detection and identification from almost all but the most heavily processed food samples. The use of DNA based methods for fish or seafood species identification has been described multiple times (Rasmussen and Morrissey, 2009).

Commonly, mitochondrial target sequences like the *Cyt b* or the *Cox-1* gene in combination with restriction analysis or sequencing have been used for identification of fish species (Russel *et al*., 2000; Espiñeira *et al*., 2008; Yancy *et al*., 2008). Dooley and co-workers successfully adopted and validated an earlier PCR-RFLP method using a *Cyt b* PCR target sequence and analysis of restriction fragment patterns on the Agilent 2100 Bioanalyzer (Dooley *et al*., 2005). Based on this method a solution has been developed which combines an optimized set of reagents and protocols with sensitive, reproducible and high resolution analysis using the Bioanalyzer and dedicated RFLP pattern analysis software. The solution introduced here enables a fast time from sample to result with minimal impact on the sample. The set of DNA purification, PCR target amplification, restriction digest and instrumental analysis, using the RFLP Decoder software for pattern matching, allows the end user to obtain a species identification within a working day. The workflow using the kit is shown in Figure 1.

Materials and Methods

Fish and meat samples

Authenticated fish samples were obtained through Campden BRI (UK), University of Kansas and

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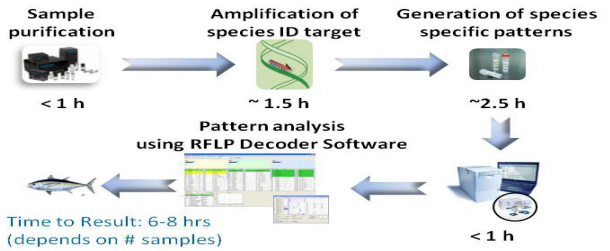


Figure 1. Schematic representation of the FishID workflow. A schematic representation showing the individual steps of the kit workflow and the approximate time for every step.

University of Washington (both USA). Additional samples including meat samples were obtained from the local supermarkets and fishmongers.

Isolation of DNA from fish or meat samples

Ten mg to 200 mg of tissue (muscle tissue, fin clippings, eggs) was lysed using 20 µl Proteinase K in 200 µl Proteinase K Digestion Buffer prewarmed at 65°C per sample. The samples were incubated for 15 min. at 65°C on a shaking thermoblock at 800 rpm. After centrifugation for 5 min. according to the protocol, 150 µl of the clear supernatant were transferred to 500 µl of Nucleic Acid Binding Buffer avoiding any undigested material. The sample lysate and Nucleic Acid Binding Buffer mixture was transferred to the spin column and treated according to protocol. The DNA was eluted from the column after 2 washes with 80% Ethanol and a dry spin using 100 µl Elution Buffer. DNA concentration and 260/280, 260/230 ratios were checked using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific).

Amplification of Cyt b *target sequence*

One µl of the purified DNA was used in a 20 µl PCR reaction using 10 µl of 2x mastermix, 2 µl of 10x primer mix and 7 µl water per sample. The PCR was run using an Mx3005 real-time PCR cycler (Agilent Technologies) not measuring fluorescence with the thermoprofile described in the protocol. The samples used for PCR amplification include the kit supplied salmon positive control DNA and a no template control (NTC). Successful amplification of the positive control and samples as well as a negative NTC was monitored using the Bioanalyzer and a DNA1000 assay.

Restriction digestion of the PCR products

An aliquot of 2.5 µl of the PCR reaction was used in the restriction digestion using the 3 enzymes *Dde* I, *Hae* III and *Nla* III supplied with the kit according to the protocol. The restriction digestion was carried out for 2 h as described in the protocol using the Mx3005 thermocycler. Inactivation of the enzymes was also performed according to protocol, heating the reactions to 65°C for 15 min. and adding 1 µl of 60 mM EDTA per sample.

Analysis of restriction patterns using Bioanalyzer and the RFLP Decoder software

The digested samples and the positive control salmon DNA were run on a DNA 1000 chip according to protocol. For each sample the three digests were loaded in consecutive wells, allowing the analysis of 4 samples per chip. The XAD file created by the 2100 Bioanalyzer Expert software was loaded directly into the RFLP Decoder software for analysis. Parameters used in the software were: Scoring method Dice for single species samples or Mixture for samples indicated as a potential mixture, Lower Cutoff 30 bp and Match Tolerance 10%. In the XAD window the following parameters were set: Ignore Molarity < 1.0 nmol/l, Detect and Remove Primer Dimers selected, Integrator was set to a Minimum Peak Height of 10%. The database used was the Agilent Reference Database 1.0 containing 49 experimental profiles derived from authenticated species.

Analysis of intra- and interlab variability

To assess interlab variability a set of 10 authenticated samples provided with the help of Steve Garrett (Campden BRI, UK) was sent to five independent labs and analyzed in our own lab. Five samples were labeled with the correct species name and five samples were only labeled with an identification number. The size of the fragments obtained by each lab including our own as well as the accuracy and the score of identification obtained with the RFLP Decoder software were analyzed. Intralab variability was assessed within our own labs by at least 3 independent experiments starting from the tissue material.

Results

Authenticated fish samples were analyzed using the optimized protocol and the kit reagents. To validate detection of non-fish species that can appear in food preparations either as contamination or as component of the food preparation a number of non-fish meat samples were also analyzed.

Successful DNA isolation was monitored on a spectrophotometer and PCR amplification was verified by running the PCR products on a DNA 1000 chip using the Bioanalyzer (data not shown). Time spent on tissue lysis and DNA purification varied depending on the number of samples. In our hands, DNA was obtained from 10 samples in about 45 minutes using the spin columns provided with the kit.

Amplification and restriction digestion according to the protocol took roughly 1.5 h and 2.5 h respectively. Including positive control, 3 DNA 1000 chips were required to analyze the restriction profiles for all 10 samples, resulting in a runtime of about 1.5 h. Final analysis of the patterns with the RFLP Matcher software was simple and did not add significant time to the overall workflow. This meant that the total time from sample to result for the 10 samples used was 6 hours, or less than one working day.

Profiles obtained on the Bioanalyzer for a number of species are shown in Figure 2. The RFLP pattern combinations generated from the *Cyt b* PCR product using the 3 enzymes supplied with the kit allowed straightforward determination of the species shown. Highly related species and common substitution species like Atlantic cod, Pacific cod and Blue whiting are easily distinguished.

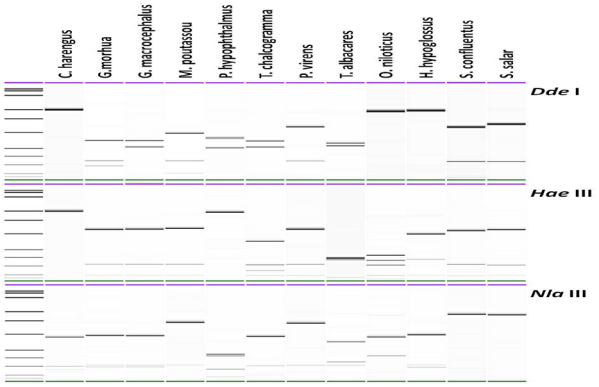


Figure 2. Restriction digest profiles of different fish species analyzed on the Bioanalyzer. Profiles for *Dde* I, *Hae* III and *Nla* III for a number of different fish species shown as virtual gel image on the Bioanalyzer.The samples include gadoids, salmonids and other commonly sold fish. All of them are clearly distinguishable by their generated unique sets of profiles. From left to right: Atlantic herring, Atlantic cod, Pacific cod, Blue Whiting, Pangasius, Alaskan pollock, Coley, Yellowfin tuna, Tilapia, Halibut, Bull trout, Salmon.

A major advantage of using PCR-RFLP over some other DNA based methods is its applicability with mixed samples. Our method was tested using DNA admixtures of two species from 50% for each species down to 95% of a major and 5% of a minor species. Similar tests were performed using weight by weight tissue admixtures. For both types of mixtures the species in dual species mixtures were detected down to a level of 5% for the minor species. Figure 3 shows the example of a 95:5 weight by weight mixture of *Thunnus alalunga* (Albacore tuna) and *Oncorhynchus nerka* (Sockeye salmon).

Intra-laboratory and inter-laboratory variability was assessed using the kit and performing the analysis using the Bioanalyzer and the RFLP Decoder software. Intra-lab variability was tested on independent samples for the same fish and exemplary results for Alaskan pollock and Coley are shown in Figure 4a.

To analyze inter-lab variability 10 different samples were used in a ring trial with 5 labs participating in the test. All labs were using the kit and performing the analysis on the Bioanalyzer with identification of species by the RFLP Decoder software. Exemplary results for *Gadus morhua* (Atlantic cod) are shown in Figure 4b.

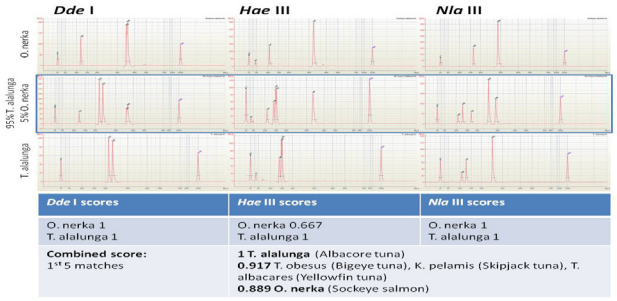
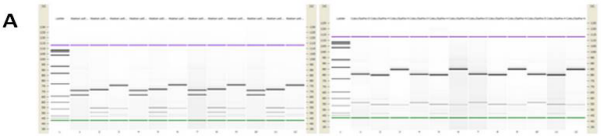


Figure 3. Analysis of DNA or tissue admixtures. Electropherogram of *Dde* I, *Hae* III and *Nla* III fragment patterns for *Oncorhynchus nerka* (Sockeye salmon), *Thunnus albacares* (Yellowfin tuna) and a weight-by-weight (5:95) tissue mixture of both. RFLP Decoder scores using the Mixture scoring are shown in the table. The two species in the mixture were within the 5 top scoring matches in the database.



B

Test Site	Sample	*Dde* I Pattern	*Hae* III Pattern	*Nla* III Pattern	RFLP Decoder score	RFLP Decoder match
A	Atlantic Cod	93, 122, 246	45, 109,328	96, 107, 289	1	Gadus morhua
B	Atlantic Cod	94, 124,250	45, 109, 334	97, 107, 295	1	Gadus morhua
C	Atlantic Cod	93, 122, 245	44, 108, 327	95, 108, 289	1	Gadus morhua
D	Atlantic Cod	94, 122, 246	47, 108, 327	95, 105, 288	1	Gadus morhua
E	Atlantic Cod	93, 122,248	45, 108, 331	95, 106, 292	1	Gadus morhua
Agilent	Atlantic Cod	95, 122, 246	43, 109, 324	96, 108, 288	1	Gadus morhua
	Mean	94, 122, 247	45, 109, 328	96, 107, 290		
	SD	0.8, 0.8, 1.8	1.3, 0.5, 3.8	0.8, 1.2, 2.8		
	%CV	0.9, 0.7, 0.7	3.0, 0.5, 1.1	0.9, 1.1, 1.0		

Figure 4. Intra- and Interlab variability using the Agilent FishID solution. A: Intralab variability of restrictions profiles for *Theragra chalcogramma* (Alaskan pollock, left) and *Pollachius virens* (Coley, right). Virtual gel image shows the patterns for 4 independent samples. The individual patterns show perfect correlation with each other. No significant variability is observed. B: 5 labs analyzed 10 supplied samples using the kit. A typical example of the observed interlab variability is shown for *Gadus morhua* (Atlantic cod). The observed variability is very low.

Finally, the ability of the kit to detect potential meat contaminations was tested. Meat from pork, wild boar, beef, lamb, turkey and chicken was tested according to the kit protocol. Figure 5a shows that pork, wild boar, beef and lamb gave rise to a single PCR product, whereas turkey resulted in two products being formed and chicken having multiple products

after PCR. Similarly the RFLP patterns shown in Figure 5b for porcine, beef and lamb PCR products are well defined and unique for the species. Turkey, despite its two PCR products, also produced a clear RFLP profile whereas the chicken profile was non-conclusive.

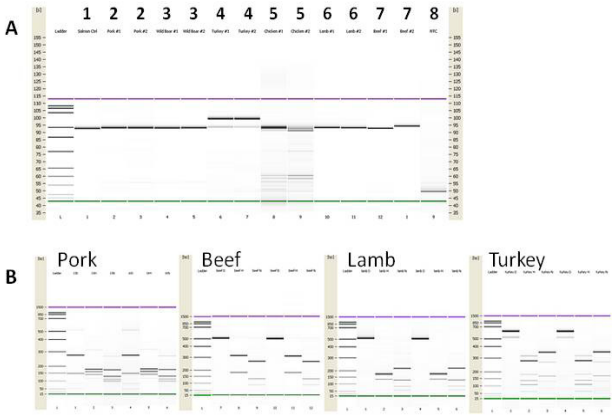


Figure 5. Detection of meat contamination by the Agilent FishID solution. A: Validation of successful PCR on the Bioanalyzer: 1 - kit positive control (Atlantic salmon), 2 – pork, 3 – wild boar, 4 – turkey, 5 – chicken, 6 – lamb, 7 – beef and 8 – NTC. All samples but the poultry samples give rise to one specific PCR product. Turkey gives rise to a larger main product and a minor product of the expected size. Chicken doesn't produce a specific product. B: RFLP profiles run on the Bioanalyzer. The lanes correspond to the *Dde* I, *Hae* III and *Nla* III patterns done in duplicate from independent samples for pork, beef, lamb and turkey. All of them result in unique pattern combinations which can be used for identification.

Discussion

The usage of PCR-RFLP for species identification is a well established method. Compared to other methods of species identification one of its major benefits is the possibility to perform identification even from mixed samples. Reproducible and accurate sizing with high resolution to resolve fragments only having small size differences can be critical to assign the right species to an unknown sample. The method established by Dooley *et al*. (2005) on the Bioanalyzer enables identification of a large number of fish species. It is widely used by public analyst labs in the United Kingdom (S. Garrett, personal communication). Unlike traditional gel electrophoresis the Bioanalyzer allows the analysis of complex patterns with high resolution following a simple workflow.

We optimized individual steps in the workflow for increased ease of use and a rapid protocol. This allowed analysis of a set of samples within one working day. The use of standardized industry grade reagents adapted optimally to every step contributed to the overall reproducibility of the analysis.

A major improvement provided by the enhanced fish species identification solution is the RFLP Decoder software. Traditionally manual pattern analysis was performed which is time consuming and error prone especially when working with mixed samples. In addition, it is required to validate the species by running an authenticated sample in parallel. Oftentimes access to authenticated samples is limited. The database supplied with the software built from experimentally derived profiles of authenticated samples streamlines that process. The software provides a simple means of analysis using standard scoring techniques to identify the most likely matches in the database as well as indicating potentially mixed samples. The analysis can be modified using a mixture specific scoring method to allow species identification for such a sample. The flexibility of the software allows easy addition of user-generated profiles to extend the number of species that can be identified on the base of experimental patterns.

Results also support the kit's ability to detect the presence of pork, beef, lamb and turkey which might be present in the same food preparation. Further work is under way to adapt the kit to specialized fish species identification tasks.

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