

Seafood Authenticity Testing System Using PCR-RFLP and Bioanalyzer Technology

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Introduction

BACKGROUND: As demand for seafood increases and regulations regarding product labeling become stricter, suppliers are under greater pressure to authenticate the species used in their products. While protein-based methods such as isoelectric focusing are in common use, DNA methods provide more objective, specific, and robust results, even in processed or mixed samples.

APPROACH: Agilent Technologies, in collaboration with Campden BRI, has developed a PCR-RFLP method to identify fish species in fresh and processed seafood:

STEP 1: DNA is rapidly isolated from small amounts of tissue using a spin column method, resulting in pure DNA ready for analysis.

STEP 2: A *cytochrome b* target fragment, common to most fish species, is PCR-amplified using a master mix format for ease of setup.

STEP 3: The PCR product is treated with 3 different restriction enzymes that differentially cut the DNA based on polymorphisms between species.

STEP 4: The digestion products are separated by size using the Agilent Bioanalyzer lab-on-a-chip system.

STEP 5: The digestion pattern is decoded using the RFLP Matcher software by comparison to validated profiles in an integrated database.

Experimental Methods

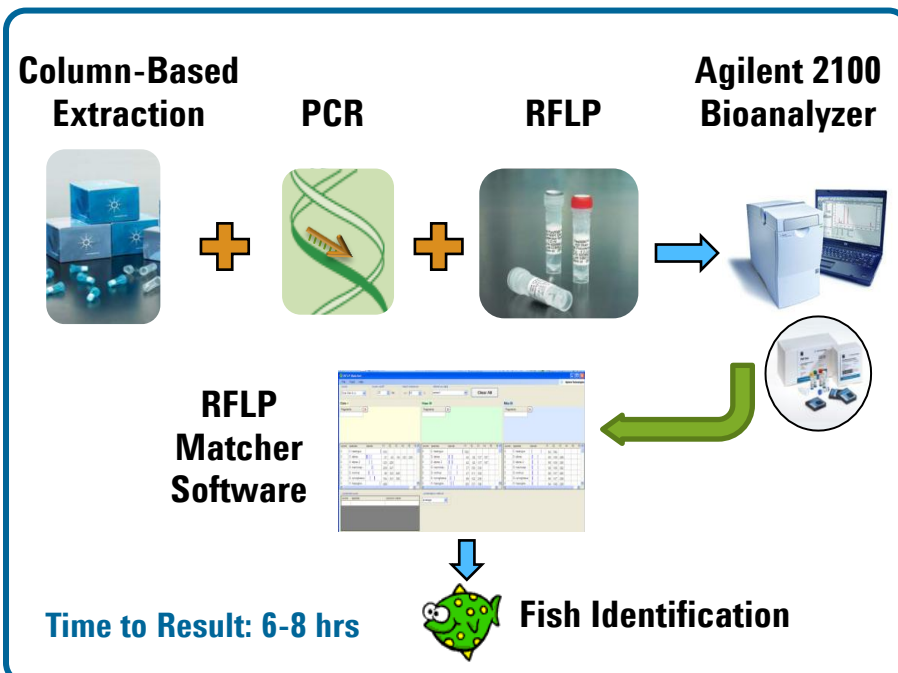


Figure 1. Agilent Seafood Authenticity Testing System Workflow.

Extraction: DNA was extracted from 40 mg – 1 µg of tissue by incubation with Proteinase K for 10 minutes at 65°C. The supernatant was applied to a silica-based spin column, washed, and the DNA eluted in TE. The concentration was measured by a Nanodrop spectrophotometer.

Polymerase Chain Reaction (PCR): Positive control salmon DNA or sample extract was added to a PCR reaction containing Agilent's Stratagene-brand *Taq* DNA polymerase and PCR primers that anneal to conserved sequences of the fish mitochondrial *cytochrome b* (*cytb*) region. Reactions were cycled using a 72 min protocol.

Restriction Fragment Length Polymorphism (RFLP): PCR product was directly digested with Ddel, NlaIII, or HaeIII restriction enzymes in the appropriate buffer for 2 hrs at 37°C followed by inactivation at 65°C for 15 min and addition of EDTA.

Capillary Electrophoresis on the 2100 Bioanalyzer: Each restriction digest product was loaded into wells of a DNA 1000 Bioanalyzer chip according to standard protocols.

RFLP Matcher Analysis: The .xad files generated from the Bioanalyzer run were imported into the RFLP Matcher software. Profiles were automatically compared to the fish database to generate rank scores that identify the fish species.

Results and Discussion

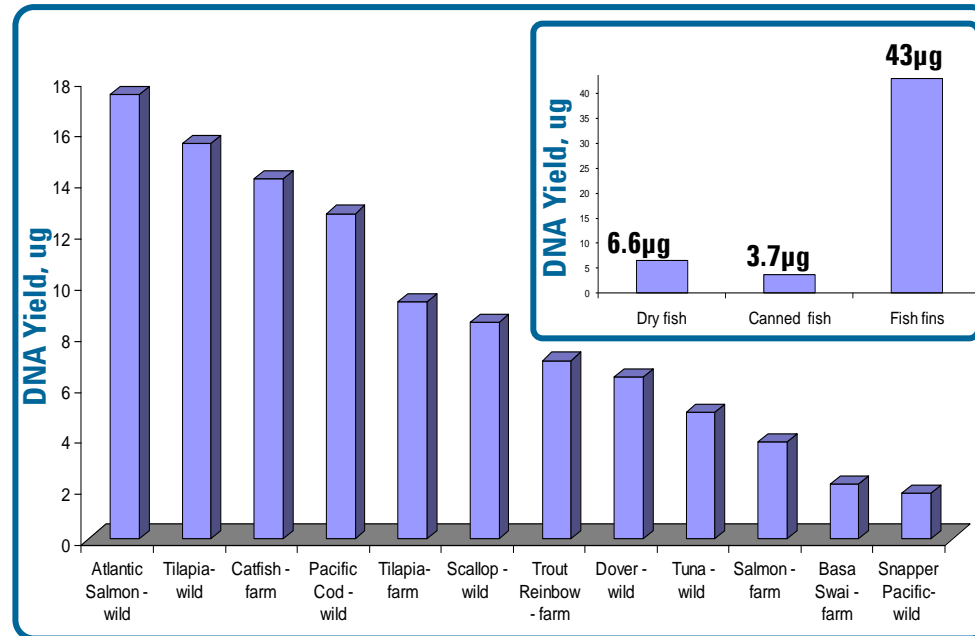


Figure 2. Efficient Extraction of DNA from a Variety of Fish Species and Sample Types.

Yields ranged from 2-43 µg with an average A_{260}/A_{280} of 2.18. Only 0.5 – 500 ng gDNA is required for subsequent PCR. The Agilent DNA extraction routinely produced 2.5-8X greater DNA yield than competitors' kits and significantly greater purity (not shown).

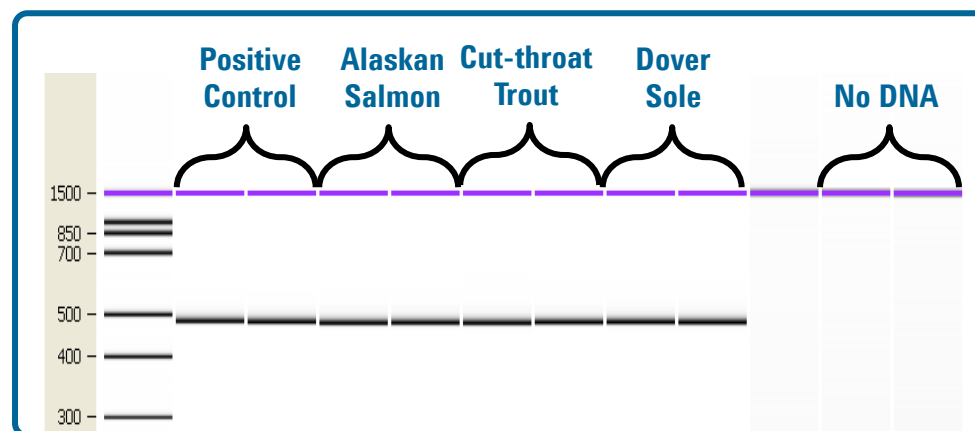


Figure 3. Similar PCR Products from Different Fish Species.

Cytb PCR products from positive control DNA, sample extracts, or no-template controls were analyzed on a Bioanalyzer DNA 1000 chip, showing a product of 480 ± 20 bp. Polymorphisms between species produce slightly different apparent mobility.

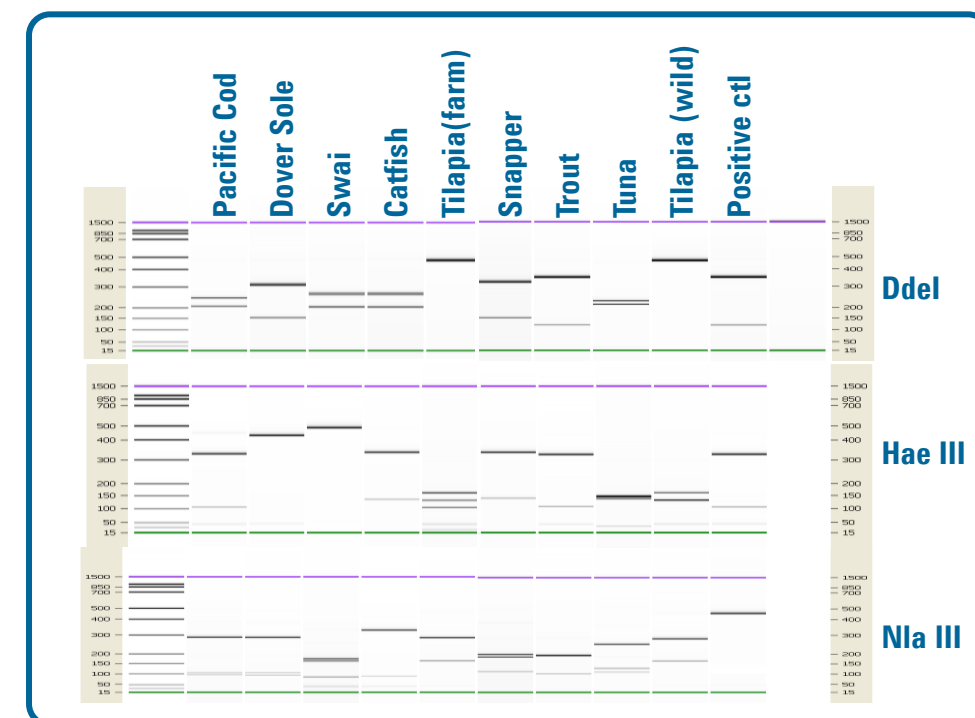


Figure 4. RFLP Profiles Reveal Identity of Different Fish Species. A variety of fish species, including gadoids, salmonids, flatfish, and others, are distinguishable by the RFLP patterns generated with Ddel, HaeIII, and NlaIII.

Alaska pollock	Coley	John Dory	Sardine
Albacore tuna	Cut-throat trout	Lemon sole	Sea bass
Arctic Char	Dab	Ling	Skate
Argentinian hake	Dolly varden	Mackerel	Skipjack tuna
Atlantic cod	Dover sole	Monkfish	South African hake
Atlantic salmon	Emperor	Monkfish substitute	Sword fish
Bigeye tuna	European anchovy	Pacific cod	Theadfin bream
Blacksea bream	European hake	Pacific Halibut	Tilapia
Bluefin tuna	European plaice	Pangasius	Turbot
Bonito	Flounder	Pink salmon	Whiting
Catfish	Gilthead bream	Pollock	Witch
Cherry salmon	Greenland halibut	Rainbow trout	Yellowfin sole
Chinook salmon	Halibut	Red Mullet	Yellowfin tuna
Chum salmon	Herring	Red salmon	Yellowtail snapper
Coho salmon	Hoki		

Table I. Common Names of over 50 Species for Which Validated Profiles Have Been Produced from Authenticated Fish Samples. In addition to validated samples, hundreds of theoretical profiles have been derived from publically-available *cytb* sequences.

Results and Discussion

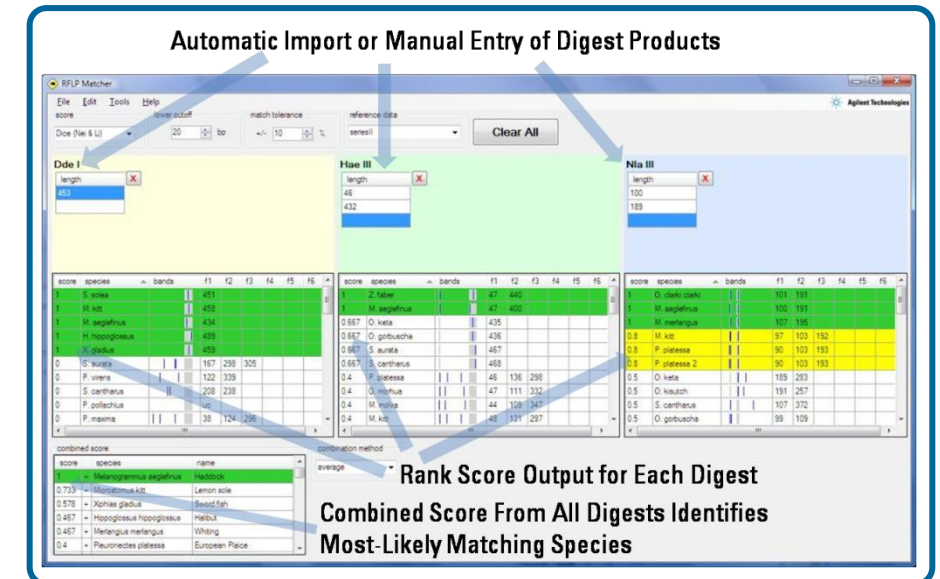


Figure 5. RFLP Matcher Software Automates Identification of Fish Species.

Demonstration of RFLP Matcher Software features:
 (1) Automatic import or manual input of digest product sizes;
 (2) Rank score (1.0 = perfect match) identifies fish species;
 (3) Selectable match algorithms including sample mixtures;
 (4) User-defined threshold and match tolerance settings;
 (5) Selection of source database (validated or theoretical).

Fish species / Test Site	Ddel	Hae III	Nla III	RFLP matcher score	RFLP matcher species
Trout	121,353,360	38,108,327	100,194	0.867	<i>Oncorhynchus clarki clarki</i>
Test Site A	121,354,361	39,109,328	100,194	0.867	
Trout	119,351,357	108,327	99,192	1	<i>Oncorhynchus clarki clarki</i>
Test Site B	119,351,357	109,327	99,192	1	
Trout	122,356,363	39,109,331	100,195	1	<i>Oncorhynchus clarki clarki</i>
Test Site C	122,356,363	39,110,330	101,196	1	
Trout	123,357,364	39,110,332	100,194	1	<i>Oncorhynchus clarki clarki</i>
Test Site D	122,356,363	39,111,331	101,195	1	
Trout QC specs	120,350,358	40,108,327	101,194	0.867	<i>Oncorhynchus clarki clarki</i> (Cut-throat trout)
Mean	121,354,361	39,109,329	100,194		Correct call RFLP Call: 8/8
SD	1.5, 2.4, 2.8	0.4, 1.0, 2.1	0.8, 1.4		

Table II. Multi-site Validation Study of the Seafood Authenticity Testing System and RFLP Matcher Accuracy. 4 sites processed identical tissue samples in duplicate. The tissue sample was identified as trout at a local supermarket. The results demonstrate precision and reproducibility of the method. Of over 12 additional fish species tested (in triplicate) at Agilent to date, 100% produced the expected pattern with the RFLP matcher score >0.8.

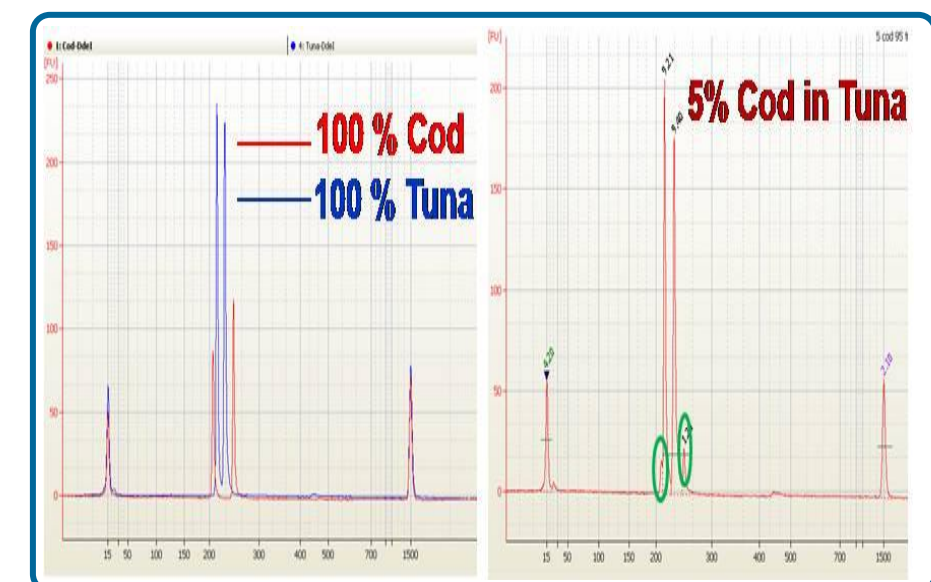


Figure 6. Minor Fish Species Can Be Detected In DNA Admixtures. In this example, cod DNA extract was blended with tuna DNA in a 1:20 ratio, PCR amplified, and digested with Ddel. Cod peaks are circled in green, and can be identified by RFLP Matcher using a Mixture algorithm.

Summary

The Agilent Seafood Authenticity Testing System:

- Produces reproducible, precise results in 1 working day
- Generates objective data that is easy to interpret using RFLP Matcher software
- Can be implemented in commercial testing facilities due to streamlined protocols and simple set-up procedures
- Offers flexibility with the ability to expand the database with other authenticated fish species
- Is sensitive enough to detect as little as 5% of a minor fish species in a mixture