FOOD STANDARDS AGENCY

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STANDARD OPERATING PROCEDURE FOR THE DISCRIMINATION OF SALMON SPECIES IN CANNED PRODUCTS BY PCR-RFLP ANALYSIS USING THE AGILENT 2100 BIOANALYZER

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CONTENTS

1. HISTORY / BACKGROUND

Differences in DNA sequences allow species to be discriminated. The mitochondrial cytochrome *b* (cyt *b*) gene, which contains regions of sequence variation, has proved a popular choice for taxonomists. It is not highly conserved and has a rapid mutation rate and is useful in the study of evolutionary relationships between species. There are also 1000s of copies of the mitochondrial genome in each cell making it an ideal target for DNA amplification in samples where DNA degradation has occurred.

The polymerase chain reaction (PCR) can be used to amplify DNA sequences. By combining PCR with restriction fragment length polymorphisms (RFLP) analysis it is possible to produce DNA fragment profiles that discriminate a large number of fish species.

Large cyt *b* DNA targets such as the 464bp target used in other PCR-RFLP methods cannot be amplified from heavily processed canned fish products. Therefore smaller targets must be used.

Detection of the small DNA fragments generated by PCR-RFLP is performed on a lab-on-a-chip capillary electrophoresis system, the Agilent 2100 Bioanalyzer.

This method was developed by Campden BRI for Food Standards Agency in project Q01099: "Extending the Lab-on-a-Chip Capillary Electrophoresis PCR-RFLP Database for a wider range of commercial fish species".

2. PURPOSE

The purpose of this SOP is to enable discrimination of five salmon species found in commercial canned products.

3. SCOPE

The method describes amplification of a 168bp cyt *b* amplicon and RFLP profiling to discriminate five Pacific salmon species found in commercial canned products. These species include; red salmon (*Oncorhynchus nerka*), chum salmon (*Oncorhynchus keta*), pink salmon (*Oncorhynchus gorbuscha*), coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tschawytscha*). Other fish species not subject to this study could, in theory, generate the same DNA profile as the salmon species present in the sample as they may share the same genetic profile. However, the method can successfully be used to establish consistency between the sample under investigation and the known reference salmon species.

4. DEFINITIONS AND ABBREVIATIONS

DNA : Deoxy-ribonucleic acid. This molecule comprises strings of the four bases (Guanine, Adenosine, Thymine, Cytosine) forming genes.

dNTP : deoxy-nucleotide-triphosphates. An abbreviation for any of the four bases forming DNA.

NTC : No template control.

PCR : Polymerase chain reaction – a method of amplifying a single DNA fragment to produce millions of copies, which can be detected.

Primer : A short oligonucleotide designed to anneal to specific regions of DNA in order to facilitate the PCR. Primers are designed to complement regions of DNA bounding the gene of interest.

RFLP : Restriction fragment length polymorphism. Different sizes of DNA fragment produced by cutting DNA with restriction enzymes.

SDW : Sterile distilled water of molecular biology grade.

***Taq* polymerase :** A specific, heat-stable DNA polymerase used to replicate DNA targets during PCR

5. PRINCIPLE OF THE METHOD

This method describes the production of PCR-RFLP fingerprints for the discrimination of canned salmon. Species-specific profiles are produced using DNA extracted from fish samples. The method used for DNA extraction is not part of this method; however, DNA should be extracted using a method suited for use with canned food samples. One suitable method is the CTAB method, details of which can be found in the final report for FSA project Q01084 "Final optimisation and evaluation of DNA based methods for the authentication and quantification of meat species"

The polymerase chain reaction (PCR) is used to detect DNA sequences in living organisms and in materials derived from living organisms. It relies on the binding of single-stranded DNA fragments (primers) to a specific DNA target sequence and the copying of this target in the presence of excess amounts of DNA subunits (nucleotides) and a DNA polymerase (Taq). Multiple cycles at specific temperatures result in the million-fold copying of the target sequence.

Restriction enzymes are naturally produced by bacterial strains to degrade DNA at sequence specific sites, e.g. EcoR1 only cuts the six base-pair pattern G↓AATTC between the G and

first A as shown. By selecting the correct enzymes it is possible to digest DNA from different species to produce species-specific DNA fragments. These fragments can be separated by electrophoretic methods to produce species-specific patterns know as restriction fragment length polymorphism (RFLP) fingerprints.

PCR-RFLP techniques combine DNA amplification and RFLPs to produce limited fragment fingerprints which are easier to interpret. They also have the advantage that only small amounts of DNA are required as the PCR step increases the amount of template DNA for restriction digests. PCR primers are used to amplify mitochondrial cyt *b* gene sequences from all fish species.

Restriction enzymes are used to digest amplified DNA to produce species-specific PCR-RFLP fingerprints. Species discrimination is achieved by separating DNA fragments by capillary electrophoresis using the Agilent 2100 Bioanalyzer and a DNA1000 LabChip. Individual species are identified by their unique fingerprint patterns.

6. MATERIALS AND EQUIPMENT

All reagents should be of a suitable purity defined for molecular biology analysis (e.g. Sigma molecular biology products). Reagents for PCR are stored in a dedicated PCR reagent freezer at -15^{o}C to -22^{o}C for up to six months, unless otherwise stated.

Note: Solutions 6.2.1 – 6.2.6 should be prepared in a laminar flow cabinet. The cabinet should be decontaminated using UV irradiation. Latex gloves should be worn throughout the procedure.

6.1 Water

The water used should be ultra-pure water, molecular grade or equivalent purity.

6.2 Solutions, standards and reference materials

6.2.1 4mM dNTP mixture

dCTP, dGTP, dATP, dTTP, bought as individual 100mM solutions.

Using a P100 Gilson pipette, add 38µl of each 100mM stock dNTP to a sterile labelled 1.5ml Eppendorf. Use a P1000 Gilson pipette to add 800µl of ultrapure water and aspirate gently to mix.

Use a P200 Gilson pipette to aliquot into labelled portions of 200µl in sterile 1.5ml Eppendorf tubes.
Store at -15°C to -22°C for up to six months.

6.2.2 5µM primer solutions (Cyt B5-F and Cyt B5-R)

Table 1: Primer Specifications

Target Sequence	Mitochondrial cyt *b* gene from fish species
PCR product size	~168bp
Cyt B5-F primer sequence	5' AAA ATC GCT AAT GAC GCA CTA GTC GA-3'
Cyt B5-R primer sequence	5' GCA GAC AGA GGA AAA AGC TGT TGA-3'

Use a P1000 Gilson pipette to add sufficient ultrapure water to dissolve the primers specified in Table 1 to produce a primer concentration of 100µM (100pmol per µl). Vortex thoroughly and leave overnight at 4°C until dissolved.

Note: If primers are required urgently, dissolve at 60°C for 1 hour.

Vortex primer solution to thoroughly mix. Centrifuge at 16,000g for 30 seconds to recover solution. Using a P1000 pipette remove about half the solution and place into a sterile, labelled 1.5ml Eppendorf tube. The solution may be stored at this point at -15°C to -22°C for up to two years.

Using a P1000 Gilson pipette, add 950µl of ultrapure water to a labelled sterile 1.5ml Eppendorf tube. Using a P100 Gilson add 50µl of the primer solution (100µM) to the water to give a primer solution of 5µM.

Vortex this solution to ensure it is thoroughly mixed and recover by centrifuging at 16,000g for 30 seconds. Using a P1000 pipette divide the solution between two sterile, labelled 1.5ml Eppendorf tubes. The solution may be stored at this point at -15°C to -22°C for up to two years.

6.2.3 AmpliTaq Gold® Polymerase

Enzyme kit from Applied Biosystems containing;

10 x PCR Buffer

25mM Magnesium Chloride

AmpliTaq Gold Polymerase

Store in a dedicated PCR freezer at -15°C to -22°C for up to six months.

6.2.4 PCR Mastermix

A PCR mastermix is prepared for the analysis of a batch of several samples

Remove aliquots of each reagent from the freezer and allow to thaw in the laminar flow cabinet.

Prepare the mastermix using the reagents and volumes detailed in the Table 2. Add the reagents to a sterile 2ml Eppendorf tube and mix thoroughly by gentle pipette aspiration prior to use.

Table 2: Preparation of PCR Mastermix

Reagents	Initial Concentration	Final Concentration in PCR Reaction (20µl reaction vol.)	Equivalent in a Single Reaction (µl)
PCR buffer	10x	1x	2
$MgCl_2$	25mM	5mM	4
dNTPs	4mM	200µM	1.2
CtyB5-F primer	5µM	0.3µM	1.2
CtyB5-R primer	5µM	0.3µM	1.2
Water	-	-	5.15
Volume			**14.8**
TaqGold	5U/µl	0.05U/µl	0.2
Volume	**(before addition of DNA extract)**		**15**
	5µl Template DNA		
Final Volume			**20**

6.2.5 EDTA Solution

0.5M Stock Solution

This is a standard lab stock solution of EDTA.
Weigh out 18.61g ± 0.01g EDTA (Ethylenediaminetetraacetic acid, Disodium Salt Dihydrate) into a 200ml beaker.

Add approximately 80ml ultrapure to dissolve. Adjust pH to 8.0 with NaOH.

Note: EDTA will not dissolve until pH is adjusted.

Once dissolved, make up to 100ml in a volumetric flask. Transfer to a labelled Schott bottle and autoclave.

Solution can be stored for one year.

60mM Working Solution

Dilute 0.5M stock solution with ultrapure water 3 in 25 to produce a 60mM working solution.

To prepare 10ml of 60mM EDTA solution, add 1.2ml 0.5M EDTA to 8.8ml ultrapure water. Mix well before use.

Solution can be stored for up to one month at ambient (room) temperature.

6.2.6 Restriction Enzymes

Restriction enzymes, as shown in Table 3, are obtained from New England Biolabs unless otherwise stated. All enzymes come with optimal buffers as shown.

Enzymes should be stored at -15˚C to -22˚C until the expiry date for each particular enzyme batch is reached.

Table 3: Details of Restriction Enzymes Used During this Method

Enzyme	Catalogue No.	Optimal Buffer	Incubation Temperature (°C)
Dde I	R0175L	NEBuffer 3	37
Bfa I	R0568S	NEBuffer 4	37

6.2.7 Positive and Negative DNA Controls

DNA extracts from single species fish are used as positive controls. After DNA extraction prepare DNA solutions at 10ng/µl using the formula in 6.2.8. This is a 10ng/µl working solution.

Use a P200 Gilson pipette to aliquot 50µl volumes of the DNA working solution into sterile, labelled 1.5ml Eppendorf tubes. Store aliquots at –15ºC to –22ºC for up to two years.

A negative extraction control should be prepared with every batch of DNA extracts. This control is prepared using water and can be used for PCR amplification in an undiluted form.

6.2.8 Template DNA Solutions of Samples

The concentration of DNA within unknown sample extracts is determined using spectrophotometry

Dilute extracts of sample DNA to 10ng/µl, for PCR amplification, using the formula below:

$$\left[\left(\frac{\text{Concentration (ng/µl)}*}{\text{Nominal concentration (10ng/µl)}}\right)-1\right] \text{x } 5 =$$

Volume of water to be added to 5µl sample extract for a final concentration of 10ng/µl (to the nearest 5µl)

- Value obtained from spectrophotometer

Use a P200 Gilson pipette to add Milli-Q water to a 1.5ml Eppendorf tube. Use a P10 Gilson pipette to add 5µl of sample DNA extract to the water. Vortex for 20 seconds to mix and centrifuge 30 seconds at 16,000g to recover solution. This is a 10ng/µl DNA working solution of the test sample for analysis.

DNA samples can be stored at 4ºC to 8ºC for up to 1 week or at –15ºC to –22ºC for longer periods up to 2 years.

6.2.9 Ethanol 80% (v/v)

Use a measuring cylinder to add 80ml Ethanol to 20ml ultra-pure water in a clean labelled Schott bottle. Store at ambient temperature for up to 3 months

6.2.10 Haz Tab

Chlorine disinfection tablets made with NaDCC (Sodium Dichloroisocyanurate). (Guest Medical, Edenbridge, Kent, UK). Follow manufacturer's instuctions to make up to appropriate concentration.

6.3 Equipment

6.3.1 Thermocycler.

6.3.2 Laminar flow hood (PCR hood).

6.3.3 Sets of Gilson precision pipettes (including, P10, P20, P100, P200, P1000).

6.3.4 Benchtop whirlimixer.

6.3.5 Benchtop centrifuge for microtubes.

6.3.6 Sterile filter pipette tips.

6.3.7 Agilent 2100 Bioanalyzer.

6.3.8 Vortex mixer – IKA model MS2-S8/S9.

The following items are sterilised by autoclaving (see note):

6.3.9 1.5 ml Eppendorf tubes

6.3.10 2.0 ml Eppendorf tubes.

Note: All equipment and reagents required to be autoclaved are sterilised using the following conditions: 121°C±2.5°C for 15 min±2min at 1.0 Bar.

The following items are UV sterilised for 5 minutes using the UV light source in a laminar flow cabinet:

6.3.11 PCR tube strips with attached caps (eight reactions each).

6.3.12 PCR tube storage block for preparation of PCRs.

6.3.13 96 well PCR plates and lids or seals.

7. PROCEDURES

7.1 Sample preparation

Note: DNA should be extracted using either a CTAB extraction or other suitable commercial kit method (Tepnel, Promega, R-Biopharm, Qiagen etc.)

Perform replica DNA extractions on at least two individual salmon flakes or pieces from different regions within the can. Do not homogenise the fish material from the whole can.

Perform DNA extraction from samples following the CTAB method or other appropriate method. Record method use in laboratory note book.

Perform DNA extraction in duplicate for the first sample and for every 10 samples thereafter.

Include a suitable DNA extraction positive control and a DNA extraction negative control with every batch of samples. Record the positive control sample code in your laboratory notebook.

Note: The positive control should be chosen to match one of the species being tested for. If no species are declared for analysis, a sample of known species should be used.

For the negative control, use water in place of the sample.

Quantify the DNA extractions using a spectrophotometer.

Dilute sample DNA to 10ng/µl using the formula shown in 6.2.8. Diluted DNA is now known as template DNA.

7.2 Amplification of cyt *b* Sequences

This method is suitable for the analysis of extracts of fish products with template DNA of between 50ng and 100ng for each reaction. For example, 5µl of an extract with a DNA concentration of 10ng/µl gives 50ng of DNA for the amplification reaction.

However if the concentration of extracted DNA is below 10ng/µl, the method must be performed on undiluted extract.

In the laminar flow hood

Note: Use sterile filter tip pipette tips and wear disposable gloves during the procedure.

Wipe laminar flow hood with tissue dampened with sterilising solution (Haz tab). Dry flow hood with tissue then wipe hood with tissue dampened with 80% ethanol.

Remove the reagents and primer working stocks from the freezer and allow to completely thaw to room temperature in the laminar flow cabinet. Once thawed vortex for 20 seconds and recover solutions by centrifuging at 16,000g for 20 seconds.

Label enough PCR tubes for reactions allowing two tubes per unknown sample and three additional tubes for the positive, negative and no template control (NTC) (water blank) controls. Label a 1.5ml Eppendorf tube for mastermix preparation.

Place all tubes in suitable rack and place into PCR hood. UV sterilise tubes for 5 minutes.

Safety Note: Ensure cabinet is closed before switching on UV lights. Do not tamper with safety micro switches on cabinet door.

Using a P1000 or P200 Gilson pipette, as appropriate, prepare mastermixes using the reagents and volumes detailed in Table 2 in 2ml Eppendorf tubes.

Using an appropriate (P10 or P20) Gilson pipette, add Amplitaq Gold to the mastermix and mix thoroughly by vortexing for 20 seconds.

Centrifuge tubes at 16,000g for 30 seconds to recover solution.

Using a P20 Gilson pipette, aliquot 15µl of mastermix into two replicate Eppendorf tubes for each sample to be tested. A PCR negative control (NTC), a positive control and an extraction negative control should also be prepared by aliquoting 15µl of mastermix into an Eppendorf tube for each control.

Use a P10 Gilson to pipette 5µl of diluted template DNA solution into the two replicate wells for each sample. Use a fresh tip for each replicate. Cap each tube after adding the DNA solution. Repeat for each unknown sample.

Use a P10 Gilson to pipette 5µl of positive control DNA (from DNA extraction positive) solution and negative control DNA (from DNA extraction negative) solution into their respective reaction wells. Use a fresh tip for each control. Cap each tube after adding the DNA solution.

Use a P10 Gilson pipette to add 5µl of ultrapure water to the NTC wells. Cap the tubes after adding the water.

Remove all used tips and tubes, replace tube holders and wipe the laminar flow hood with sterilising solution.

Transfer Eppendorf tubes prepared for PCR to the thermocycler laboratory.

In post-PCR laboratory

Place PCR tubes into a thermocycler and use the programme found in Table 4.

Safety Note: Avoid touching heating block and heated lid, as they achieve temperatures of over 95°C.

After the PCR programme is complete, remove the tube strips from thermocycler and store samples at 1ºC to 6ºC for up to 2 days. Alternatively, PCR products can be stored for up to three months at between -15ºC and -22ºC.

Note: Do not remove PCR products from the thermocycler laboratory.

Table 4: PCR Amplification Conditions

PCR program	95ºC/5 min 95ºC/40 sec 50ºC/80 sec 72ºC/80 sec 72ºC/5 min 4°C/hold	**45 cycles**

7.3 Confirmation of cyt *b* Gene Amplification

Run the purified DNA on an Agilent DNA1000 LabChip to confirm fragment has been amplified, and to determine the concentration of DNA, before proceeding with a restriction enzyme digest.

Print out results of chip analysis. The purpose of this is to confirm PCR amplification occurred in all samples. Fix this securely into laboratory notebook.

7.4 Restriction Digestion of PCR Products

Parts of the following stage are performed in the PCR set-up laboratory and parts in the post-PCR laboratory.

Note: Take care not to move samples from the post-PCR lab to the PCR set-up lab.

The preparation of restriction digest reactions should be performed on ice. Restriction enzymes should only be taken from the freezer (-20°C) for as short a time as possible and handled as little as possible.

In the PCR set-up, label 0.2ml PCR tubes with sample name and restriction enzyme and arrange in a suitable rack. Include tubes for restriction digest positive controls for each enzyme. Place all tubes on ice.

For each enzyme reaction prepare a mastermix as shown in Table 5 in a 0.5ml Eppendorf tube. To prepare enough reaction mastermix count the number of samples to test. Add 1 for the PCR positive control, which has now become the restriction digest positive control, and multiply the total number of reactions by the values in column 3 of Table 5 (‘Volume for 1 Digest’). Finally add an extra 10% for pipetting errors.

Table 5: Preparation Volumes for Restriction Digests Mastermix

Component	Final Concentration	Volume for 1 Digest (µl)	Volume for 10 Digests[1] (µl)
10x Buffer[2]	1x	0.5	5.5
Enzyme		0.5	5.5
SDW	~	1.5	16.5
Volume	~	2.5	27.5

1. An extra 10% has been added to values shown to allow for pipetting errors.
2. See Table 3 for correct buffer to use with each enzyme.

Vortex the enzyme mastermix thoroughly to mix. Centrifuge at 16,000g for 15 seconds to recover the solution.

Leave enzyme mastermixes on ice and transfer to the Post-PCR laboratories for the next step

Use a P10 Gilson pipette to add 2.5µl of the mastermix to the respective labelled PCR tubes.

Leave the tubes on ice and take them to the Post-PCR laboratory for the next steps.

Use a P10 Gilson pipette to add 2.5µl of PCR product (from 7.3) to the respective labelled PCR tubes.

Use a P10 Gilson pipette to add 2.5µl of PCR positive control DNA to the restriction digest positive control tube.

Place the tubes in the thermocycler and incubate samples for at least 4 hours (or overnight) at 37°C ± 1.0°C. Terminate reactions by heating samples to 65°C ± 1°C for 10-15 minutes.

Digests can be stored at +3°C to +6°C for up to 2 days. For longer times store samples at -15°C to -25°C.

7.5 Fingerprinting samples on the Agilent 2100 Bioanalyzer

The Agilent 2100 Bioanalyser is a capillary electrophoretic system which is used to separate, size and quantify DNA products according to size. Different sized DNA products require different chip assays according to expected size range. For the analysis of fish PCR-RFLP products the DNA1000 LabChip should be used.

Note: Before loading samples on the 2100 Bioanalyzer, use a P10 Gilson pipette to add 1µl of 60mM EDTA to each 5µl digest and mix to achieve a final concentration of 10mM EDTA.

Remove tubes containing prepared matrix, DNA size ladder (yellow cap) and upper and lower size markers (green cap) from fridge (+1°C to +6°C) and leave to warm to room temperature for 1 hour.

Prime DNA1000 LabChip according to the manufacturer's instructions using prepared gel matrix.

Safety Note: Gel matrix contains a DNA binding dye. Avoid contact with skin. Wear gloves and goggles when handling.

Use a P10 Gilson to load 5µl of size markers into all sample wells and ladder well, ensuring marker settles onto bottom of well and does not remain on sides.

Using P10 Gilson pipette to load 1µl of ladder into the well labelled with a ladder symbol, ensuring ladder settles onto bottom of well and does not remain on sides.

Using P10 Gilson pipette to load 1µl of digested DNA sample into one of the 12 sample wells, 1 – 12. Ensure samples have settled onto bottom of well and have not remained on sides of well. Fill any spare wells with 1µl of size marker.

Use the IKA vortex to vortex the chip for 1 minute at 2,400 rpm, then load into slot in 2100 Bioanalyser.

Safety Note: The 2100 Bioanalyzer contains a laser. Do not interfere with the normal operation of this instrument.

Select chip assay type as DNA1000 assay. Press start when chip is ready and wait for 1-2 minutes to ensure analyser starts and there are no problems with chip. If chip error is reported

- Stop run and remove chip.
- Check chip wells to ensure samples are in bottom of wells and are not adhering to sides. If sample is on sides use a pipette to move it into base of well. Reload chip into analyser and restart run.
- If all samples are in bottom of wells invert chip and examine chip wells for bubbles. If chip contains bubbles discard chip and reload samples into fresh chip.

- If problems persist and there are no obvious problems consult your line manager. You may need to run a full instrument diagnostics test.

After run is complete save file into appropriate folder. Remove DNA chip from analyser and clean analyser pins with cleaning chip containing approximately 350µl SDW.

7.6 Quality Assurance

7.6.1 Negative Controls

The purpose of the negative controls are to identify if contamination has occurred during the extraction or PCR procedures.

An extraction negative control must be prepared with every batch of DNA extracts. The extraction negative is usually manipulated last at each stage of the process, to pick up any possible source of contamination.

A PCR negative control must be used as a method control with every set of samples amplified at the same time. For a PCR negative, 5µl ultrapure water replaces the sample DNA extract, when setting up the PCR. The PCR negative is usually manipulated last at each stage of the process, to pick up any possible source of contamination.

The PCR negative control should show no PCR product present. Presence of the PCR product indicates contamination has occurred and the PCR batch is invalid and all samples must be re-amplified. If this occurs, consult your line manager.

A DNA extraction negative control showing a strong visible band of equivalent size to the positive PCR control means the extraction batch is invalid and all DNA from samples must be re-extracted.

7.6.2 Positive PCR Controls

A positive PCR control, relating to the sample type*, is amplified with every batch of samples. The positive must be treated as an unknown sample during the DNA extraction and amplification stages.

* Where a particular sample type is expected (from information supplied by the client) use this species as the positive PCR control where possible.

7.6.3 PCR Amplification

PCR products resulting from the amplification of DNA extracted from samples and controls are separated according to size using a DNA1000 LabChip.

The presence of a PCR product is indicated by a DNA fragment of 168bp ± 5%.

7.6.4 Restriction Digest Quality Control

Restriction digestion positive controls should produce bands of sizes shown in Appendix 1 ± 5% for fragments >100bp or ± 15% for fragments <100bp. If digestion is incomplete, i.e. some undigested DNA remains, digestion assays with that enzyme should be repeated.

Complete digestion of PCR products from samples is assumed based on comparisons to digestion of positive controls.

Positive controls are completely digested with a specific enzyme if only the DNA fragments of sizes shown in Appendix 1 are detected by the 2100 Bioanalyzer.

If the expected fragments are not observed, or additional larger fragments are also observed, consult your line manager. It is likely that complete digestion has not occurred and samples may require re-analysis.

7.6.5 2100 Bioanalyzer Quality Control

A ladder with eleven DNA fragments ranging in size from 15bp to 1500bp should be fully resolved and detected by the bioanalyzer using default settings.

The internal size markers (15bp and 1500bp) should be clear of other DNA fragments. Check that the bioanalyzer has identified the markers correctly. Consult your line manager if other fragments appear to be co-migrating near the markers.

7.6.6 Presence of Species

If the restriction digest positive control DNA shows complete digestion with a specific enzyme, it is assumed that enzymatic digestion of sample DNA has also proceeded to completion with that enzyme

8. EXPRESSION OF RESULTS

Compare the sample profiles to those profiles of the salmon species found in Appendix 1.

Results are expressed in the report as the following:

For sample X, the PCR-RFLP profiles are consistent with the presence of <....> species.

The method cannot identify the species present in a sample as other related salmon species may have the same PCR-RFLP profile.

9. PRECISION AND ACCURACY

All DNA extracts are analysed in duplicate. The results are valid if the DNA fragments obtained in each duplicate are the same, i.e. identical fingerprint patterns ± 5%. If duplicates do not give the same result, consult your line manager and repeat if appropriate.

10. APPENDICES

10.1 APPENDIX ONE

168bp CYT B PCR-RFLP PROFILES FOR PACIFIC SALMON SPECIES

Species	DNA Fragments (bp) produced using a DNA1000 Labchip following Digestion with the Enzyme			
	***Bfa* I**		***Dde* I**	
	Expected	**Observed**	**Expected**	**Observed**
Chinook salmon	19, 57, 92	(21), 61, 83, 99	51, 117	(21), 60, 123
Coho salmon	19, 57, 92	(22), 61, 84, 99	48, 51, 69	(21), 60, 74
Red salmon	19, 42, 50, 57	(21), 44, 60, 85, 99	48, 120	(21), 59, 124
Chum salmon	19, 30, 50, 69	(21), 23, 59, 74, 95	48, 120	(22), 58, 122
Pink salmon	12, 19, 30, 50, 57	(21), 24, 62, 84	48, 120	(21), 58, 124

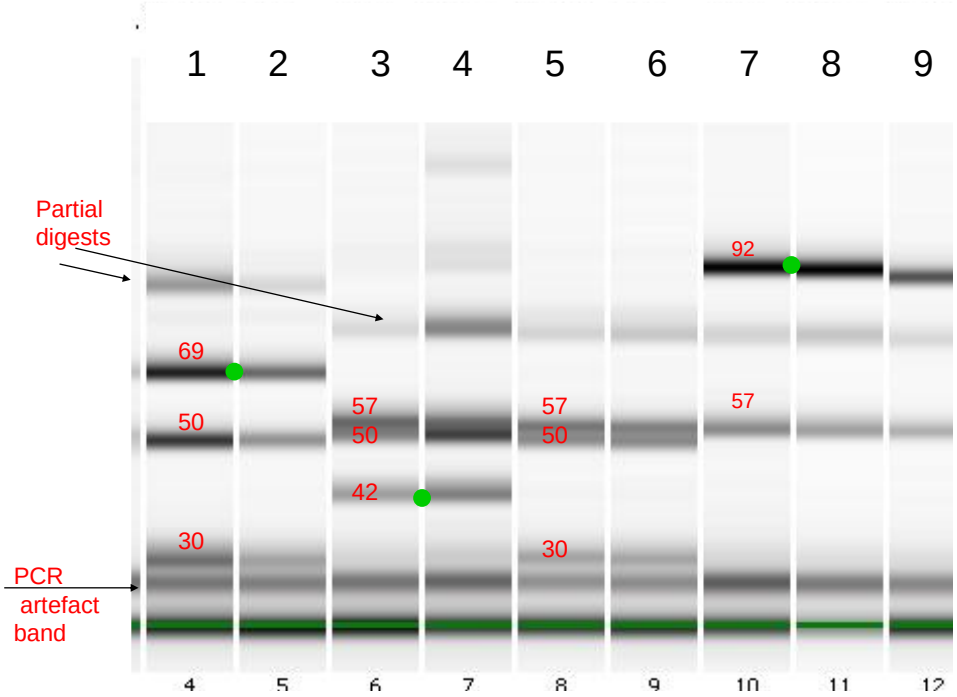
Red fragments= Possible artefact due to incomplete digestion of PCR product

() possible primer dimer formed during PCR

19bp *Bfa*I fragments co-migrate with the primer dimer.

The theoretical 50 & 57 bp fragments produced with *Bfa* I in pink and red salmon often run as a double headed band at approximately 60bp

***Bfa* I PCR-RFLP fragments viewed on Bioanalyzer generated gel image**
(zoom setting at below 200bp)



Red number are the theoretical DNA fragment sizes in bp
Green spots indicate individual DNA fragments that are unique to the species

Lanes	Salmon species
1&2	chum
3&4	red
5&6	pink
7&8	chinook
9	coho