

Agilent DNA Fish ID Ensemble

Part Number 5500-0100

Protocol

Version B, August 2012

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Procedures.**



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In this Guide...

This document describes how to use the Agilent DNA Fish ID Ensemble to identify the species of a fish by extracting DNA and performing PCR and RFLP analysis.

1 Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

2 Procedures

This chapter contains instructions on how to extract genomic DNA from fish samples and perform restriction fragment length polymorphism (RFLP) analysis using PCR amplification and restriction enzyme digestion.

3 Quick Reference Protocols

This chapter contains Quick Reference protocols with streamlined instructions covering the preparation of the fish samples through analysis of restriction digest patterns.

4 Appendix A: Homogenized Fish Samples as the Starting Material

This chapter describes how to use homogenized fish samples in the fish identification protocol and instructions on fish sample homogenization.

5 Appendix B: Agilent DNA 1000 Kit Quick Start Guide

This chapter contains a copy of the Agilent DNA 1000 Kit Quick Start Guide with instructions on preparing the DNA 1000 chips.

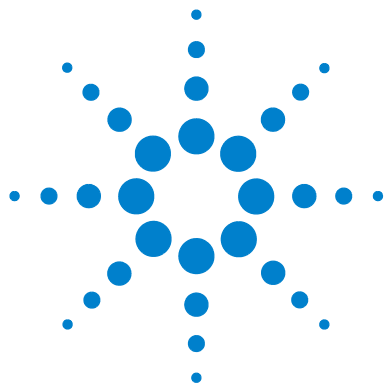
6 Appendix C: Bioanalyzer Electrode Cleaning Procedure

This chapter contains instructions on how to clean the electrodes of the Agilent 2100 Bioanalyzer.

7 References

Contents

1	Before You Begin	7
	Materials Provided with the Agilent DNA Fish ID Ensemble	8
	Storage Conditions	9
	Required Equipment, Supplies and Reagents	9
	Overview of the Agilent DNA Fish ID Solution	10
	Recommended Controls	10
2	Procedures	13
	To prepare the reagents	14
	To prepare the fish samples for DNA extraction	14
	To extract genomic DNA	15
	To set up the PCR reactions	16
	To run the PCR protocol	18
	To analyze the PCR products	18
	To digest PCR products with restriction enzymes	21
	To analyze the restriction digest patterns	23
	To identify the test sample species using the RFLP Decoder Software	28
	Troubleshooting Guide	30
3	Quick Reference Protocols	33
4	Appendix A: Homogenized Fish Samples as the Starting Material	39
5	Appendix B: Agilent DNA 1000 Kit Quick Start Guide	41
6	Appendix C: Bioanalyzer Electrode Cleaning Procedure	49
7	References	51



1

Before You Begin

Materials Provided with the Agilent DNA Fish ID Ensemble 8

Storage Conditions 9

Required Equipment, Supplies and Reagents 9

Overview of the Agilent DNA Fish ID Solution 10

Recommended Controls 10

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.



1 Before You Begin
Materials Provided with the Agilent DNA Fish ID Ensemble

Materials Provided with the Agilent DNA Fish ID Ensemble

Agilent DNA Isolation Kit, Part Number 5500-0051 (50 preparations)	
Nucleic Acid Binding Buffer	25 ml
High Salt Wash Buffer	24 ml
Elution Buffer (10 mM Tris-HCl, pH 7.5)	12 ml
DNA-Binding Spin Cups and 2-ml Receptacle Tubes	50 each
1.5-ml Collection Tubes	50 each
Proteinase K	2 × 0.5 ml
Proteinase K Digestion Buffer	2 × 5 ml
Agilent PCR-RFLP Fish Species ID Kit, Part Number 5500-0001 (100 reactions)	
Positive Control Salmon DNA (250 ng/μl)	10 μl
2× PCR Master Mix	1.25 ml
Nuclease-Free Water	2 ml
10× Fish Species ID Primer Mix	250 μl
10× NlaIII Restriction Enzyme *	50 μl
10× DdeI Restriction Enzyme	50 μl
10× HaeIII Restriction Enzyme**	50 μl
10× NlaIII/HaeIII Buffer	100 μl
10× DdeI Buffer	50 μl
BSA (100× solution)	100 μl
60 mM EDTA	300 μl
Agilent DNA 1000 Kit, Part Number 5067-1504 (25 chips)	
DNA Chips	25 each
Electrode Cleaner	1 each
Syringe	1 each
Spin Filters	3 each
DNA 1000 Ladder (yellow-capped tube)	35 μl
DNA 1000 Markers 15/1500 bp (green-capped tube)	2 × 1.2 ml
DNA Dye Concentrate*** (blue-capped tube)	90 μl
DNA Gel Matrix (red-capped tube)	3 × 500 μl

* Nla III is licensed under U.S. Patent No. 5,278,060.

** HaeIII is licensed under U.S. Patent No. 5,179,015.

*** See "Notice to Purchaser: DNA Dye Concentrate" on page 2 for licensing information.

Storage Conditions

Store the proteinase K and proteinase K digestion buffer at 4°C. Store the remaining DNA isolation kit components at room temperature.

Store the components of the PCR-RFLP reagent kit at –20°C upon receipt. Once thawed, store the 10× Primer Mix at 4°C. Continue storing the remaining PCR-RFLP components at –20°C.

Store all components of the DNA 1000 Kit at 4°C. Protect dye and dye mixtures from light; remove light covers only when pipetting.

Required Equipment, Supplies and Reagents

Additional Equipment, Supplies and Reagents Required
100% ethanol, 200 proof (USP grade or equivalent)
Sterile, nuclease-free water
Pipettes (10 µl, 100 µl and 1000 µl) with compatible tips
Incubator or water bath set to 65°C
Sterile glass bottle or polypropylene tube (e.g. 14-ml BD Falcon polypropylene round-bottom tubes or 50-ml BD Falcon polypropylene conical tubes)
Vortex mixer
Thermal cycler
Thin-walled PCR tubes
Microcentrifuge
Microcentrifuge tubes
Agilent 2100 Bioanalyzer (with PC, chip priming station and IKA vortex mixer)
Agilent RFLP Decoder Software

Overview of the Agilent DNA Fish ID Solution

The Agilent DNA Fish ID solution is a simple, fast, and accurate DNA-based method to identify the fish species present in a seafood sample. The method consists of purification of genomic DNA from fish samples and amplification of this DNA in a PCR reaction using primers that recognize a specific region of the fish genome. The PCR products are then used in restriction fragment length polymorphism (RFLP) analysis to identify the sample.

Protocol Summary

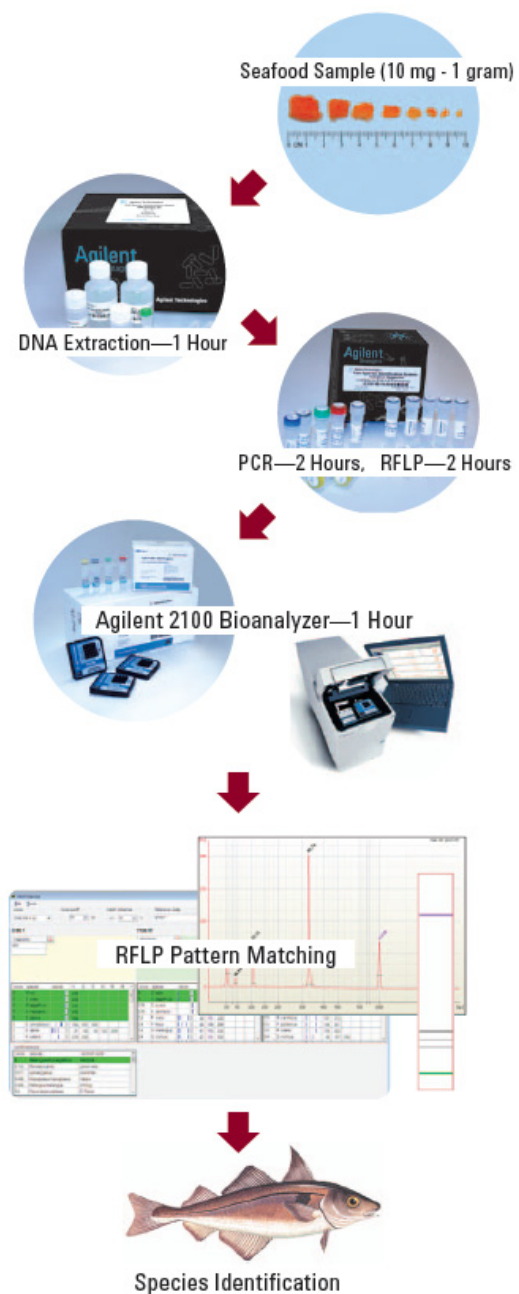
The samples are first treated with proteinase K to release the nucleic acids into solution. DNA is then isolated by suspending the sample in binding buffer and loading onto a micro-spin cup containing a silica-based fiber matrix. The nucleic acids in the sample bind to the fiber matrix. The immobilized nucleic acids are washed to remove contaminants, and total DNA is recovered in a final volume of 100 µl. The isolated DNA is ready for PCR amplification with the provided primers that bind to sequences found in all fish genomes. The PCR products are then digested with three different restriction enzymes. The fragment lengths produced in these digestion reactions can be resolved on the Agilent 2100 Bioanalyzer and analyzed with the RFLP Decoder software to determine the species of fish from which the DNA sample was prepared. [Figure 1](#) summarizes the protocol for the DNA fish ID system.

Recommended Controls

Once the DNA has been extracted from the samples of fish, it is subjected to PCR amplification followed by restriction enzyme digestion. We recommend including a positive control reaction and a negative control reaction alongside the test DNA samples during these steps. The kit includes a genomic DNA sample isolated from Atlantic salmon that can be used as a positive control. You can also use your own fish samples of known species as positive controls in the experiment. DNase-free water can be used in place of a DNA sample to provide a negative control.

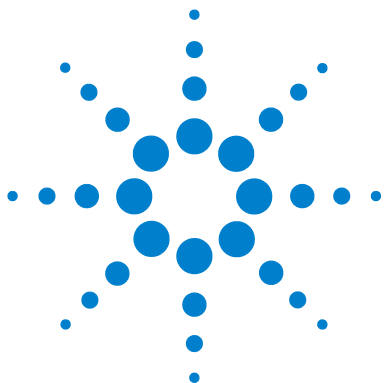
Additionally, when preparing the PCR reactions with the test samples, we recommend setting up the reactions in duplicate.

Figure 1 Overview of the DNA fish ID protocol.



1 Before You Begin

Recommended Controls



2 Procedures

- To prepare the reagents 14
- To prepare the fish samples for DNA extraction 14
- To extract genomic DNA 15
- To set up the PCR reactions 16
- To run the PCR protocol 18
- To analyze the PCR products 18
- To digest PCR products with restriction enzymes 21
- To analyze the restriction digest patterns 23
- To identify the test sample species using the RFLP Decoder Software 28
- Troubleshooting Guide 30

This chapter contains instructions on how to extract genomic DNA from fish samples and perform restriction fragment length polymorphism (RFLP) analysis using PCR amplification and restriction enzyme digestion.

NOTE

The procedures in this chapter include detailed instructions. [Chapter 3](#) has Quick Reference protocols with streamlined instructions covering the steps in “[To extract genomic DNA](#)” through “[To analyze the restriction digest patterns](#)”.



To prepare the reagents

High Salt Wash Buffer

Prepare 1× High Salt Wash Buffer by adding 16 ml of 100% ethanol to the bottle of High-Salt Wash Buffer.

After adding the ethanol, affix the “EtOH Added” sticker (provided) to the cap. Close the cap of the container tightly and store at room temperature.

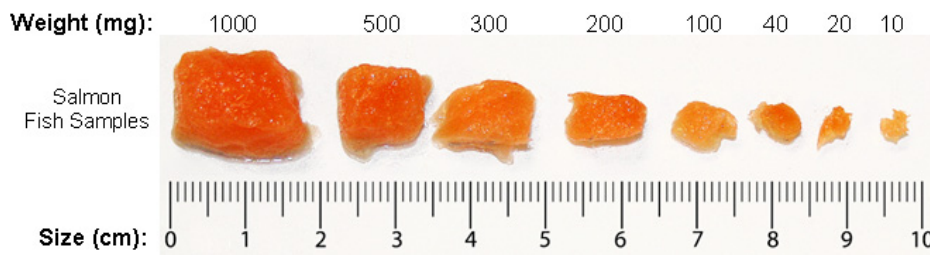
80% Ethanol

Prepare 80% ethanol by diluting 100% ethanol with DNase-free water.

To prepare 80 ml of 80% ethanol (enough for 50 fish samples), add 16 ml of DNase-free water to 64 ml of 100% ethanol.

To prepare the fish samples for DNA extraction

- 1 For each fish sample to be tested, place a piece of the fish tissue (raw or cooked) into a single 1.5-ml microcentrifuge tube. Samples ranging between 10 mg and 1 g have been used successfully, but 100–400 mg samples provide the optimal yield. Use the figure below as a guideline for estimating the weight of a raw fish sample based on sample size.



- 2 Pre-warm the Proteinase K Digestion Buffer to 65°C for 5 minutes in an incubator or water bath.
- 3 Prepare a working solution of Proteinase K by combining 200 µl of Proteinase K Digestion Buffer and 20 µl of Proteinase K per sample.
Prepare a fresh working solution of Proteinase K before each use.

- 4 Add 220 μl of the Proteinase K working solution to each 1.5-ml tube of fish sample. Incubate the tubes at 65°C for 10 minutes in an incubator or water bath.
- 5 Spin the tubes in a microcentrifuge for 3–5 minutes at 14,000 $\times g$ to pellet any undigested tissues.
- 6 Transfer 150 μl of each supernatant into a fresh 1.5-ml tube. **Avoid transferring any undigested material from the bottom of the tube or any oily material that may be present at the top of the tube** (see image below). These tubes of supernatant are the samples from which genomic DNA will be extracted.



To extract genomic DNA

- 1 Add 500 μl of Nucleic Acid Binding Buffer to each fish sample. Vortex or pipet the sample repeatedly until homogenized. The addition of this mixture will bring the total volume of each sample to 650 μl .
- 2 Transfer all 650 μl of each sample to a separate DNA Binding Spin Cup that has been seated within a 2-ml receptacle tube (provided) and snap the cap of the tube onto the top of the spin cup.
- 3 Spin the samples in a microcentrifuge for 1 minute at 14,000 $\times g$ to load the DNA onto the spin cup matrix.
- 4 Remove and **retain the spin cups** and discard the filtrates. For each sample, replace the spin cup in the receptacle tube, then add 500 μl of 1 \times High Salt Wash Buffer and cap the tube.
- 5 Spin the samples in a microcentrifuge at 14,000 $\times g$ for 1 minute.

2 Procedures

To set up the PCR reactions

- 6 Remove and **retain the spin cups** and discard the filtrates. For each sample, replace the spin cup in the receptacle tube, then add 500 μl of 80% ethanol and cap the tube.
- 7 Spin the samples in a microcentrifuge at $14,000 \times g$ for 1 minute.
- 8 Repeat steps 6 and 7 two more times for a total of 3 washes with 500 μl of 80% ethanol.
- 9 After the third wash in 80% ethanol, remove and **retain the spin cups** and discard the filtrates. Replace the spin cups in their receptacle tubes and spin in a microcentrifuge for 2 minutes at $14,000 \times g$ to dry the fiber matrix.
- 10 Transfer the spin cups to fresh 1.5-ml collection tubes. Add 100 μl of Elution Buffer to each spin cup directly on the fiber matrix inside the cup. Snap the caps of the collection tubes onto the spin cups and incubate at room temperature for 1 minute.
- 11 Spin the samples in a microcentrifuge at maximum speed for 1 minute.
- 12 The purified DNA is in the Elution Buffer in the microcentrifuge tube. Discard the spin cups and cap the tubes. The DNA may be stored at 4°C for up to one month. For long-term storage, store the DNA at -20°C or -80°C .
- 13 If desired, you may measure the concentration of the DNA samples in a spectrophotometer.

The genomic DNA extraction protocol typically yields samples with a concentration ranging from 5 ng/ μl to 500 ng/ μl . The PCR-RFLP protocol works wells with DNA samples ranging anywhere from 0.05 ng/ μl to 2000 ng/ μl .

To set up the PCR reactions

- 1 Prepare a 50 ng/ μl dilution of the positive control salmon DNA by combining 8 μl of the DNA stock with 32 μl of sterile, DNase-free water. Vortex briefly to mix. Keep the diluted sample on ice while setting up reactions.
The diluted sample may be stored at 4°C for future use.
- 2 Prepare the reactions (on ice) by combining the components in [Table 1](#) in order. Prepare a single reagent mixture for all PCR reactions that will be run simultaneously by scaling up the volumes listed in the table.

In addition to the test DNA samples, include a positive control reaction and a no-template control reaction. Preparing duplicate PCR reactions for each test DNA sample is recommended. Prepare enough reagent mixture for all your reactions plus one reaction volume excess.

*For example, if you have 5 test DNA samples, prepare enough reagent mixture for either **8 reactions** (5 test reactions, 1 positive control, 1 no-template control, and 1 excess) or **13 reactions** if you are including duplicates of the test reactions (10 duplicate test reactions, 1 positive control, 1 no-template control and 1 excess).*

Table 1 PCR Reagent Mixture

Component	Volume 1 Reaction	Volume 5 Reactions
Nuclease Free Water	9 µl	45 µl
2× PCR Master Mix	12.5 µl	62.5 µl
Primer Mix	2.5 µl	12.5 µl
Total volume	24 µl	120 µl

- 3** Vortex the reagent mixture well, then distribute 24 µl to each individual thin-walled PCR reaction tube.
- 4** Add 1 µl of the diluted positive control DNA to the positive control reaction tube. To the test sample tubes, add 1 µl of test DNA sample. For the no-template control reaction, add 1 µl of DNase-free water in place of the DNA.

To avoid cross-contamination, use a fresh pipet tip for each DNA sample. After adding the sample, mix the reaction by quickly pipetting the contents of the tube up and down.

- 5** Cap the reaction tubes, vortex the tubes to mix and centrifuge the tubes briefly.

To run the PCR protocol

- 1 Place the reactions in the thermal cycler and run the PCR program shown below.

Table 2 **PCR Cycling Protocol**

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	5 minutes
		95°C	30 seconds
2	40	50°C	30 seconds
		72°C	30 seconds
3	1	72°C	7 minutes

To analyze the PCR products

At this point, it is helpful to analyze the PCR products on a Bioanalyzer DNA 1000 Lab chip to ensure the product sizes are in the expected range.

- 1 Prepare the chip. Refer to [“Appendix B: Agilent DNA 1000 Kit Quick Start Guide”](#) on page 41 for complete instructions.

NOTE

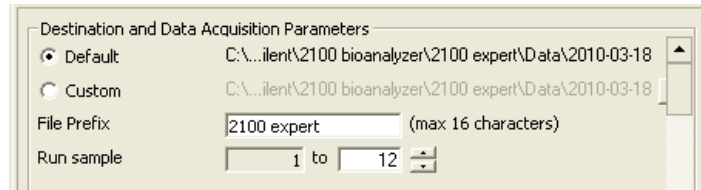
The steps for chip priming (see [“Loading the Gel-Dye Mix”](#) on page 46) are especially critical for a successful chip run. Perform these steps with care.

- 2 Once the chip is ready, start the 2100 Expert software. The software opens to the *Instrument* context.
- 3 Open the lid of the Agilent 2100 Bioanalyzer. Check that the electrode cartridge is inserted properly and the chip selector is in position.
- 4 Place the chip carefully into the receptacle and close the lid.

CAUTION

Forced closing of the lid may damage the electrodes and dropping the lid may cause liquid spills resulting in bad results. Do not use force to close the lid and do not drop the lid onto the inserted chip.

- 5 In the *Instrument* context of the software, select the appropriate assay from the **Assay** menu (**Assay > Electrophoresis > dsDNA > DNA 1000 Series II.xsy**).
- 6 Accept the current *File Prefix* or modify it. Data will be saved automatically to a file with a name using the prefix you entered. At this time, you can also customize the file storage location.



- 7 Click the **Start** button to start the chip run.

CAUTION

After each run, immediately remove the used chip from the Bioanalyzer and dispose of it according to good laboratory practice. Then perform the electrode cleaning procedure described in [“Appendix C: Bioanalyzer Electrode Cleaning Procedure”](#) on page 49.

An image of a Bioanalyzer gel with PCR samples from 4 different fish species is shown in [Figure 2](#). The expected results are summarized in [Table 3](#).

2 Procedures
To analyze the PCR products

Figure 2 Bioanalyzer gel image of PCR reaction samples. Each gel lane is labeled with the common name of the fish sample.

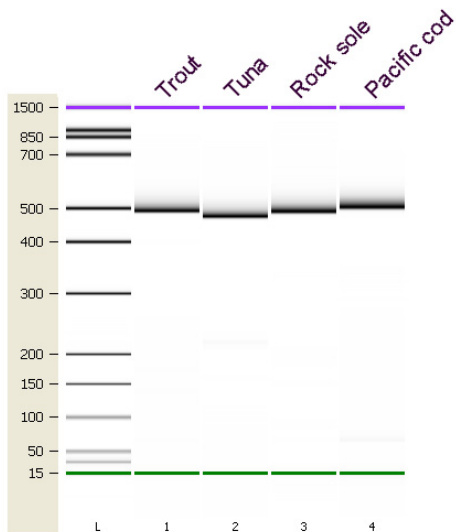


Table 3 Expected Sizes of PCR Products

Sample	Expected Product Size
Positive control salmon DNA	490 bp \pm 10%
Test samples	490 bp \pm 10%
No-template control	No product; the presence of product in the no-template control is an indication of contamination or primer dimer

To digest PCR products with restriction enzymes

Once the PCR is complete, the PCR reactions are treated with restriction enzymes for restriction fragment length polymorphism (RFLP) analysis.

- 1 Label the 0.5-ml tubes or 0.2-ml strip tubes that are to be used for the restriction digest reactions. Each PCR reaction will be digested with three different restriction enzymes: DdeI, HaeIII and NlaIII. Therefore, for each PCR reaction, label three separate tubes with the name of the PCR sample and the name of the restriction enzyme.
- 2 Prepare the reagent mixture for the DdeI digestions by combining the components in [Table 4](#) *in order*. Prepare a single reagent mixture for all DdeI digestion reactions (plus at least one reaction volume excess) using multiples of each component.

Table 4 DdeI Digestion Reagent Mixture

Component	Volume
Nuclease Free Water	1.5 µl
10× DdeI Buffer	0.5 µl
10× DdeI enzyme	0.5 µl

- 3 Vortex the reagent mixture well, then distribute 2.5 µl to the individual reaction tubes that were labeled for DdeI.
- 4 Prepare the reagent mixture for the HaeIII digestions by combining the components in [Table 5](#) *in order*. Prepare a single reagent mixture for all HaeIII digestion reactions (plus at least one reaction volume excess) using multiples of each component.

Table 5 HaeIII Digestion Reagent Mixture

Component	Volume
Nuclease Free Water	1.5 µl
10× NlaIII/HaeIII Buffer	0.5 µl
10× HaeIII enzyme	0.5 µl

2 Procedures

To digest PCR products with restriction enzymes

- 5 Vortex the reagent mixture well, then distribute 2.5 μ l to the individual reaction tubes that were labeled for HaeIII.
- 6 Prepare the reagent mixture for the NlaIII digestions by combining the components in [Table 6](#) *in order*. Prepare a single reagent mixture for all NlaIII digestion reactions (plus at least one reaction volume excess) using multiples of each component.

Table 6 NlaIII Digestion Reagent Mixture

Component	Volume
BSA (diluted to 3.33 \times)*	1.5 μ l
10 \times NlaIII/HaeIII Buffer	0.5 μ l
10 \times NlaIII enzyme	0.5 μ l

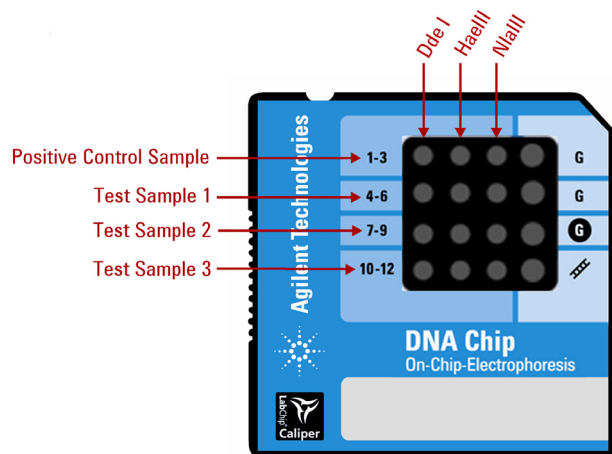
*Prepare the 3.33 \times BSA dilution by combining 1 μ l of the provided 100 \times BSA stock with 29 μ l of sterile, nuclease -free water. Diluted BSA may be stored at -20°C for future use.

- 7 Vortex the reagent mixture well, then distribute 2.5 μ l to the individual reaction tubes that were labeled for NlaIII.
- 8 For each digestion reaction, add 2.5 μ l of the appropriate PCR product to the labeled tubes. All of the test PCR reactions as well as the positive control reaction need to be digested with all three restriction enzymes.
- 9 Vortex the digestion reactions and then briefly centrifuge the tubes.
- 10 Incubate all the digestion reactions at 37°C for 2 hours. This incubation can be performed in the thermal cycler. If desired, reactions may be left at 37°C overnight.
- 11 Transfer the reactions to 80°C for 20 minutes. This incubation can be performed in the thermal cycler.
- 12 (Optional) Add 1 μ l of 60 mM EDTA to each reaction and vortex well. Store the reactions at 4°C until you are ready to proceed to the next step.

To analyze the restriction digest patterns

Analyze the restriction digest reactions on the Agilent 2100 Bioanalyzer to determine the fragment lengths produced during digestion.

- 1 Pipet 1 μ l of digest reaction into one of the 12 sample wells on a DNA 1000 Lab chip according to the guidelines in the figure below. Refer to “Appendix B: Agilent DNA 1000 Kit Quick Start Guide” on page 41 for complete instructions on preparing these chips.



NOTE

The steps for chip priming (see “Loading the Gel-Dye Mix” on page 46) are especially critical for a successful chip run. Perform these steps with care.

- 2 Once the chip is ready, start the 2100 Expert software. The software opens to the *Instrument* context.
- 3 Open the lid of the Agilent 2100 Bioanalyzer. Check that the electrode cartridge is inserted properly and the chip selector is in position.
- 4 Place the chip carefully into the receptacle and close the lid. The chip fits only one way. The electrodes in the cartridge fit into the wells of the chip.

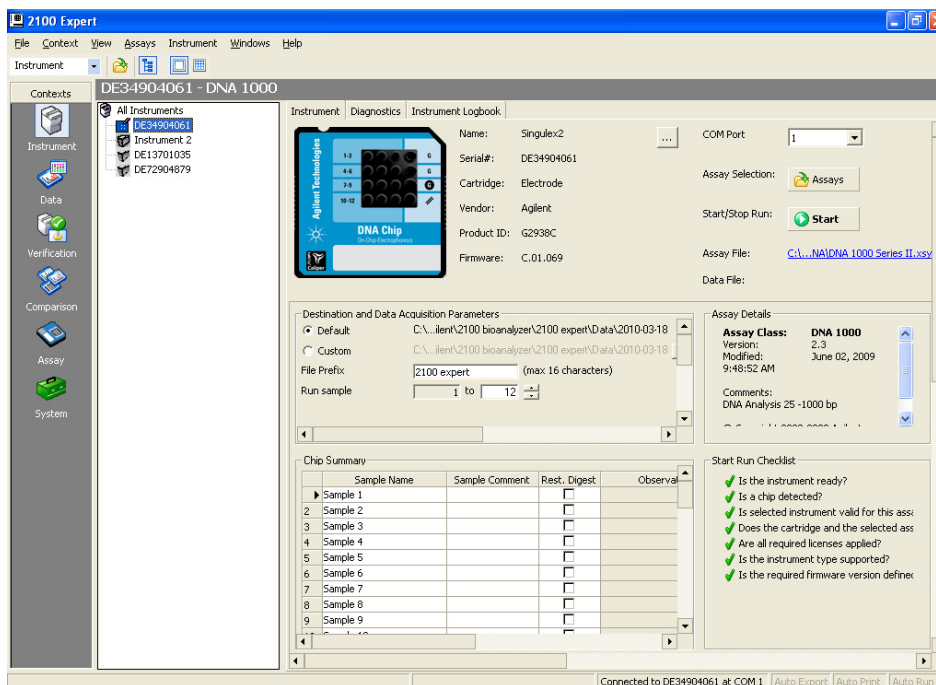
CAUTION

Forced closing of the lid may damage the electrodes and dropping the lid may cause liquid spills resulting in bad results. Do not use force to close the lid and do not drop the lid onto the inserted chip.

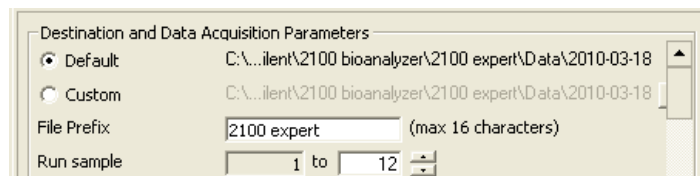
2 Procedures

To analyze the restriction digest patterns

The 2100 Expert software screen shows that you have inserted a chip and closed the lid by displaying the chip icon at the top left of *Instrument* context. An example screen is shown below. Note that the **Rest. Digest** check boxes in the *Chip Summary* panel do not need to be marked.



- 5 In the *Instrument* context of the software, select the appropriate assay from the **Assay** menu (**Assay > Electrophoresis > dsDNA > DNA 1000 Series II.xsy**).
- 6 Accept the current *File Prefix* or modify it. Data will be saved automatically to a file with a name using the prefix you entered. At this time, you can also customize the file storage location.



- 7 Click the **Start** button to start the chip run.

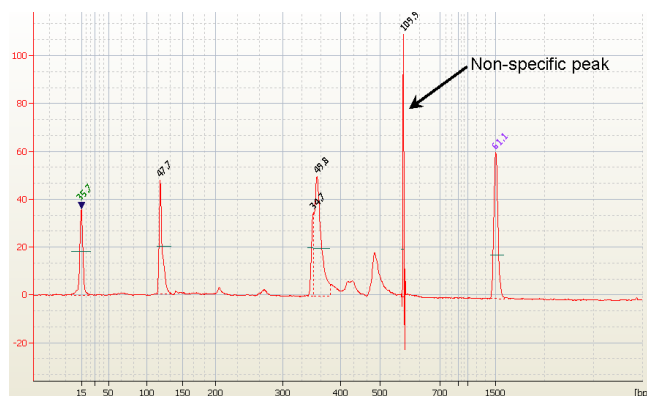
CAUTION

After each run, immediately remove the used chip from the Bioanalyzer and dispose of it according to good laboratory practice. Then perform the electrode cleaning procedure described in “[Appendix C: Bioanalyzer Electrode Cleaning Procedure](#)” on page 49.

- 8 When the run is complete, peaks are identified for all samples using the settings of the peak find algorithm. Review the electropherogram in the *Data* context to determine if any genuine peaks have been missed or if any non-specific peaks have been erroneously identified.
 - *If you believe the algorithm has failed to detect a genuine peak, you may lower the Height Threshold setpoint for that well to a value that allows the algorithm to identify the peak. To lower the threshold, click **View > Setpoints**, and with the **Local** tab selected, enter a new value into the **Height Threshold [FU]** field.*

Local	
Normal	Collapse
Integration Start Time [s]	30
Integration End Time [s]	128.95
Slope Threshold	0.5
Area Threshold	0.1
Height Threshold [FU]	20
Width Threshold [s]	0.5

- *Occasionally, non-specific peaks appear on the electropherogram as dramatic spikes (see below for an example). If the electropherogram includes a peak such as this, exclude the peak from analysis. To exclude a peak, turn on Manual Integration, select the peak on the electropherogram, then right-click and select Exclude Peak.*



2 Procedures

To analyze the restriction digest patterns

- 9 Go to the *Assay* context and select the *Chip Summary* tab. In the Sample Name field, enter a sample name for all 12 wells on the chip as shown in the figure below.

	Sample Name	Sample Comment	Status	Observation	Result Label	Result Color
1	► Positive Control, Ddel					
2	Positive Control, HaeIII					
3	Positive Control, NlaIII					
4	DNA Sample 1, Ddel					
5	DNA Sample 1, HaeIII					
6	DNA Sample 1, NlaIII					
7	DNA Sample 2, Ddel					
8	DNA Sample 2, HaeIII					
9	DNA Sample 2, NlaIII					
10	DNA Sample 3, Ddel					
11	DNA Sample 3, HaeIII					
12	DNA Sample 3, NlaIII					

Chip Lot #

Reagent Kit Lot #

Chip Comments :

Sample Information

Study Information

Import...

Export...

An image of a Bioanalyzer gel with restriction digest samples from four different fish species is shown in [Figure 3](#). The expected results for the positive DNA sample are summarized in [Table 7](#).

Figure 3 Bioanalyzer gel image of restriction digest reaction samples. Each gel lane is labeled with the restriction enzyme used in that reaction.

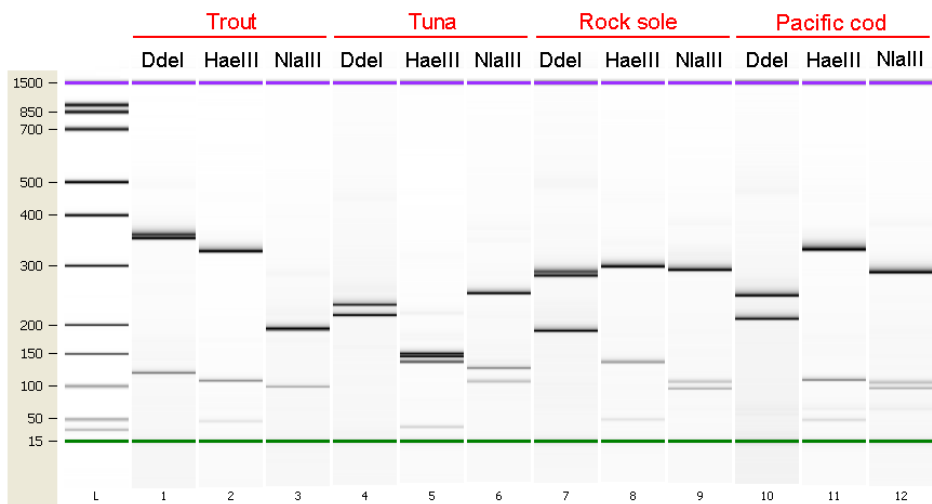


Table 7 Expected DNA Fragment Sizes in the Salmon Positive Control

Restriction Enzyme	Expected Product Size (bp)
DdeI	117, 332, 340
HaeIII	40, 105, 333
NlaIII	459

To identify the test sample species using the RFLP Decoder Software

The Agilent software application *RFLP Decoder* may be used to identify the fish species for the test DNA samples based on the fragment lengths produced in the digestion reactions.

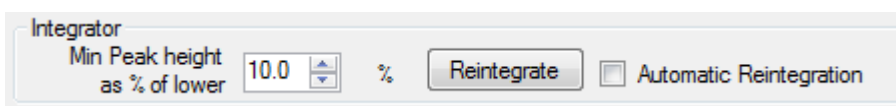
NOTE

These instructions provide the basics on use of the RFLP Decoder software. Refer to the software's help system for detailed information on operating the software and interpreting the display.

- 1 Launch the RFLP Decoder program.
- 2 Click **File > Open > XAD File**.
The **Open** dialog box will open.
- 3 Select the XAD file for the DNA chip that included the restriction digest reactions. Click **Open**.

The data from the XAD file are loaded into the Bioanalyzer import table on the right side of the screen.

- 4 In the field labeled "Min Peak height as % of lower", the recommended value is 10.0%. If needed, you may use a lower value to identify small peaks that were missed, or a higher value to discard peaks resulting from non-specific noise in the electropherogram. Click **Reintegrate** before proceeding, or mark the check box for Automatic Reintegration.



Integrator

Min Peak height as % of lower: 10.0 %

[Reintegrate] ☐ Automatic Reintegration

- 5 In the Bioanalyzer import table, select three digestion reactions corresponding to one DNA sample and specify the appropriate restriction enzyme for each well using the drop-down lists under the **Enzyme** column. In the figure below, the Enzyme column has been filled out for the Atlantic salmon sample.

	Enzyme	F1	F2	F3
Atlantic salmon-D	DdeI	121	332	338
Atlantic salmon-H	HaeIII	105	332	
Atlantic salmon-N	NlaIII	465		

- 6 Near the bottom right corner of the screen, click **Calculate**.

The fragment length data obtained from the Bioanalyzer import table will populate the fields in the fragment data panes/tabs on the left side of the screen.

- 7 In the *Score* drop-down list near the top left corner of the screen, select the appropriate algorithm for analysis of the data. If the fish sample being analyzed consists of a single fish species, select **Dice (Nei Li)** in the *Score* drop-down list. If the samples may consist of a mixture of species, select **Mixture**.

The table labeled *Combined score* lists the best species matches based on the results of all three digestion reactions.

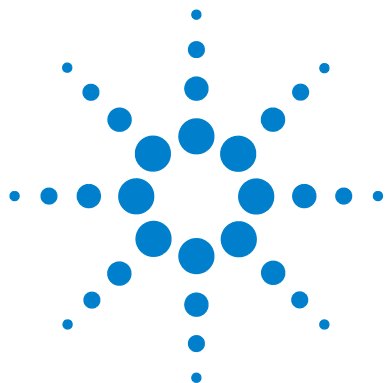
- 8 In the *Lower Cutoff* and *Match Tolerance* fields in the analysis parameters at the top of the screen, you may adjust the settings to improve the score of the best species match.
- The *Lower Cutoff* value is used to discard any fragments shorter than the length designated in the field. The default is 30 bp.
 - The *Match Tolerance* value determines how close in length a fragment must be to the predicted fragment to be considered a match. The default is +/- 10%
- 9 Repeat steps 5 through 8 for the remaining DNA samples that were included on this same chip.

Troubleshooting Guide

The following table lists troubleshooting suggestions for possible scenarios you may encounter.

Observation	Suggestion
The PCR reaction did not amplify a product	The extraction protocol may not have yielded any intact DNA. Analyze a sample of the DNA on a spectrophotometer. If no DNA is detected, repeat the extraction with a piece of fish tissue between 100 and 400 mg. For some samples, a smaller amount of tissue yields better results.
The PCR reaction amplified multiple products	If all products are around 490 bp ($\pm 10\%$), the fish sample may consist of multiple fish species. The RFLP Decoder can identify multiple species using the Mixture score method.
	If the Bioanalyzer gel of the PCR reactions shows signs of DNA degradation (smeared bands), the DNA sample or PCR reactions may have become contaminated. Ensure the DNA is being stored at -20°C . When setting up the PCR reactions, use separate tips for each pipetting step.
If you see a note that the sum of the fragment lengths is greater than the expected amplicon size, but you do not believe the sample contains multiple fish species.	If the note only appears for only one of the restriction enzymes, it is unlikely that the sample is a mixture of species. If the note appears for two enzymes, the sample could be a mixture. If all three enzymes have a note, the same is very likely a mixture.
	The sample may be contaminated with non-fish DNA. Contamination with bovine DNA, for example, is common in fish samples that had been mixed with milk or cheese. Try using the Mixture score method to prevent mis-matched fragments from impacting the combined score. You may also need to repeat the DNA extraction protocol using a tissue sample with less contaminating material (e.g. sauce, breading, etc.).
	The enzyme may not have completely digested the PCR product, creating partially-digested fragments that are longer than the expected length. Examine the fragment length to see if any may be the result of incomplete enzyme digestion.
	Review the Bioanalyzer electropherogram for the PCR reaction (pre-digest) to see if any non-specific peaks were amplified. If so, try excluding any fragments in the digest reaction that may be attributed to the non-specific PCR product.

Observation	Suggestion
If the software identified multiple species as a perfect match or close match for your fish sample	If all the species identified as a potential match are highly related (e.g. albacore tuna and yellowfin tuna), the software is likely identifying all the species together because of their similar fragment patterns. You can try lowering the Match Tolerance setting until only one species is a close or perfect match, but also consider what you know about the sample (such as where the fish was caught) to determine which of the potential matches is the correct one.
	If several of the fragments included in analysis are potentially non-specific, increase the minimum peak height threshold to exclude these fragments.
The software did not identify any species that are a perfect match or close match for your fish sample	Review the Bioanalyzer electropherograms for the digest reactions. Could any of the peaks being used for fragment analysis be background noise? If so, try excluding those peaks from analysis. Does the electropherogram show any peaks that are below the minimum peak height threshold but may actually be real products of the digest? Try lowering the threshold to include these smaller peaks.
	Review the Bioanalyzer electropherogram for the PCR reaction (pre-digest) to see if any non-specific peaks were amplified. If so, try excluding any fragments in the digest reaction that may be attributed to the non-specific PCR product.
	Exclude any fragments that may be primer dimers. These fragments will generally be around 68 bp long.
	Is it possible that your fish sample contains more than one species or is contaminated with non-fish DNA (e.g. from milk or cheese present in the sample)? If so, be sure the Mixture score method is selected.
	The sample could be from a fish species that is not in the reference database. Try using the theoretical database to see if a match is found. If you have an idea of what the species may be, run a positive control sample with a fish of that species. Then, add an entry for this species to the database and include the fragment data you obtained with the positive control sample.
	If the fragment data was imported from an XAD file, be sure you clicked Reintegrate on the XAD import window.



3 Quick Reference Protocols

To prepare the fish samples for DNA extraction 34

To extract genomic DNA 34

To set up and run the PCR reactions 35

To digest PCR products with restriction enzymes 36

To analyze the restriction digest patterns on a Bioanalyzer DNA 1000 Lab
Chip 37

This chapter contains Quick Reference protocols with streamlined instructions covering the preparation of the fish samples through analysis of restriction digest patterns.



To prepare the fish samples for DNA extraction

- 1 For each test sample, put a piece of fish tissue (10 mg to 1 g) into a 1.5-ml microcentrifuge tube.
- 2 Combine 200 μ l of Proteinase K Digestion Buffer (pre-warmed to 65°C) and 20 μ l of Proteinase K for each sample to create a working solution of Proteinase K.
- 3 Add 220 μ l of the Proteinase K working solution to each fish sample. Incubate the tubes at 65°C for 10 minutes.
- 4 Spin the tubes in a microcentrifuge for 3–5 minutes at 14,000 $\times g$.
- 5 Transfer 150 μ l of each supernatant into a fresh 1.5-ml tube. **Avoid transferring any undigested material or any oily material.**

To extract genomic DNA

- 1 Add 500 μ l of Nucleic Acid Binding Buffer to each sample. Vortex or pipet the sample repeatedly until homogenized.
- 2 Transfer each sample to a DNA Binding Spin Cup seated within a 2-ml receptacle tube (provided) and snap the tube caps onto the cups.
- 3 Spin the samples in a microcentrifuge for 1 minute at 14,000 $\times g$.
- 4 For each sample, discard the filtrate and replace the spin cup in the receptacle tube, then add 500 μ l of 1 \times High Salt Wash Buffer and cap the tube.
- 5 Spin the samples at 14,000 $\times g$ for 1 minute.
- 6 For each sample, discard the filtrate and replace the spin cup in the receptacle tube, then add 500 μ l of 80% ethanol and cap the tube.
- 7 Spin the samples at 14,000 $\times g$ for 1 minute.
- 8 Repeat steps 6 and 7 two more times for a total of 3 washes in 80% ethanol.
- 9 After the third wash, discard the filtrates and replace the spin cups in their receptacle tubes. Spin the samples for 2 minutes at 14,000 $\times g$ to dry the fiber matrix.
- 10 Transfer the spin cups to 1.5-ml collection tubes. Add 100 μ l of Elution Buffer to each cup directly on the fiber matrix. Snap the tube caps onto the cups and incubate at room temperature for 1 minute.
- 11 Spin the samples at maximum speed for 1 minute. The DNA is in the collection tube.

To set up and run the PCR reactions

- 1 Prepare a 50 ng/μl dilution of the positive control salmon DNA.
- 2 Prepare the PCR reagent mixture (below). Prepare enough for all your reactions plus one reaction volume excess. Include a positive control reaction and a no-template control reaction.

Component	Volume (1 Reaction)	Volume (5 Reactions)
Nuclease Free Water	9 μl	45 μl
2× PCR Master Mix	12.5 μl	62.5 μl
Primer Mix	2.5 μl	12.5 μl

- 3 Vortex the mixture, then distribute 24 μl to each PCR reaction tube.
- 4 Add 1 μl of the diluted positive control DNA to the positive control reaction tube. To the test sample reactions, add 1 μl of DNA sample. For the no-template control reaction, add 1 μl of DNase-free water.
- 5 Cap the reaction tubes, vortex to mix and then centrifuge briefly.
- 6 Place the reactions in the thermal cycler and run the program below.

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	5 minutes
		95°C	30 seconds
2	40	50°C	30 seconds
		72°C	30 seconds
3	1	72°C	7 minutes

- 7 To analyze the PCR results on the Bioanalyzer, load 1 μl of each PCR reaction on a DNA 1000 lab chip. Refer to [“Appendix B: Agilent DNA 1000 Kit Quick Start Guide”](#) on page 41 for complete instructions on preparing and running these chips on the Bioanalyzer.

To digest PCR products with restriction enzymes

- 1 Label the tubes for the restriction digest reactions. Each PCR product needs to be digested with 3 enzymes: DdeI, HaeIII and NlaIII.
- 2 Prepare the reagent mixture (below) for the DdeI digestions, and distribute 2.5 µl to the individual reaction tubes labeled for DdeI.

Component	Volume per reaction
Nuclease Free Water	1.5 µl
10× DdeI Buffer	0.5 µl
10× DdeI enzyme	0.5 µl

- 3 Prepare the reagent mixture (below) for the HaeIII digestions, and distribute 2.5 µl to the individual reaction tubes labeled for HaeIII.

Component	Volume per reaction
Nuclease Free Water	1.5 µl
10× NlaIII/HaeIII Buffer	0.5 µl
10× HaeIII enzyme	0.5 µl

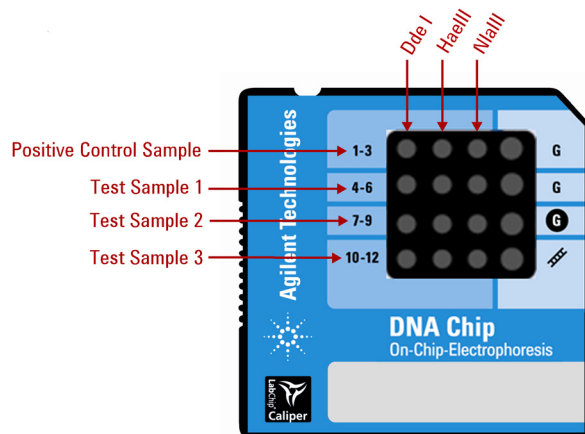
- 4 Prepare the reagent mixture (below) for the NlaIII digestions, and distribute 2.5 µl to the individual reaction tubes labeled for NlaIII.

Component	Volume per reaction
BSA (diluted to 3.33× with water)	1.5 µl
10× NlaIII/HaeIII Buffer	0.5 µl
10× NlaIII enzyme	0.5 µl

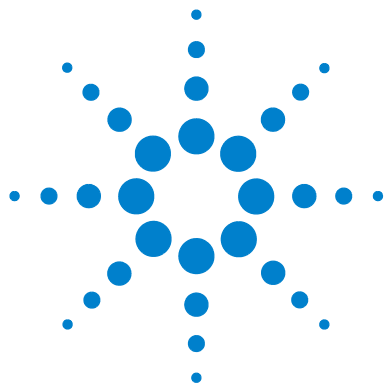
- 5 Add 2.5 µl of the appropriate PCR product to the digestion reactions. Vortex the reactions to mix and then briefly centrifuge the tubes.
- 6 Incubate all the digestion reactions at 37°C for 2 hours to overnight.
- 7 Transfer the reactions to 80°C for 20 minutes.
- 8 (Optional) Add 1 µl of 60 mM EDTA to each reaction and vortex well.

To analyze the restriction digest patterns on a Bioanalyzer DNA 1000 Lab Chip

- 1 For each digest reaction, pipet 1 μ l of the reaction into one of the 12 sample wells on a DNA 1000 Lab chip according to the guidelines in the figure below. Refer to [“Appendix B: Agilent DNA 1000 Kit Quick Start Guide”](#) on page 41 for complete instructions on preparing and running these chips on the Bioanalyzer.



- 2 During the run, the incoming raw signals are displayed in the *Instrument* context. If necessary, adjust the *Peak Height Threshold* setpoint.
- 3 Import the XAD file into the *RFLP Decoder* application to identify the test sample fish species. See [“To identify the test sample species using the RFLP Decoder Software”](#) on page 28 for more information, or refer to the application’s help system.



4

Appendix A: Homogenized Fish Samples as the Starting Material

This chapter describes how to use homogenized fish samples in the fish identification protocol and instructions on fish sample homogenization.



The use of homogenized fish samples

In place of using a piece of intact fish tissue, the fish identification protocol can be used with a homogenized sample of fish. For homogenized samples, use 40 to 200 mg of homogenized fish as the starting material for sample preparation (see [step 1](#) of “[To prepare the fish samples for DNA extraction](#)” on page 14).

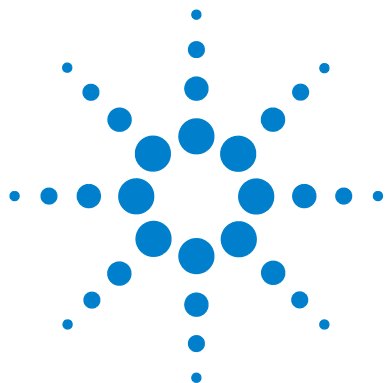
To homogenize fish samples

The homogenization protocol below was developed using the Omni Tissue Homogenizer, but other homogenizers may also be used.

- 1** In a container suitable for homogenization, combine 1 part fish tissue with 2 parts water.

For example, mix 5 g of fish with 10 ml of water.

- 2** Homogenize the sample until it is of a uniform consistency.
- 3** Measure out an aliquot weighing between 40 and 200 mg. This aliquot may be used in place of a fish tissue sample as the starting material in the fish identification procedures outlined in [Chapter 2](#).



5

Appendix B: Agilent DNA 1000 Kit Quick Start Guide

This chapter contains a copy of the Agilent DNA 1000 Kit Quick Start Guide with instructions on preparing the DNA 1000 chips.



Assay Principles

Agilent DNA kits contain chips and reagents designed for sizing and analysis of DNA fragments. Each Agilent DNA chip contains an interconnected set of microchannels that is used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. Agilent DNA kits are designed for use with the Agilent 2100 Bioanalyzer only.

Assay Kits

The Agilent DNA 1000 kit provides higher resolution of smaller fragments in comparison of our other DNA sizing kits. It can be used for the following applications:

- Analysis of PCR and RT-PCR products
- RFLP analyses
- Heteroduplex analysis using mismatch cleavage enzymes
- QC of sequencing templates

The complete DNA 1000 kit guide can be found in the online help of the 2100 Expert software.

Storage Conditions

- Keep all reagents and reagent mixes refrigerated at 4°C when not in use to avoid poor results caused by reagent decomposition.
- Protect dye and dye mixtures from light. Remove light covers only when pipetting. Dye decomposes when exposed to light.

Equipment Supplied with the Agilent 2100 Bioanalyzer

- Chip priming station (reorder number 5065-4401)
- IKA vortex mixer

Additional Material Required (Not Supplied)

- Pipettes (10 µl, 100 µl and 1000 µl) with compatible tips
- 0.5-ml microcentrifuge tubes for sample preparation
- Microcentrifuge

Sample Preparation

- PCR samples: For accurate determination of DNA concentration, the total DNA in sample must be between 0.1–50 ng/μl. If concentration of your particular PCR reaction is excessively high, dilute to 0.1–50 ng/μl in water.
- Restriction digests: Final concentration of DNA should not exceed 50 ng/μl. Add EDTA and/or heat inactivate the restriction enzyme according to the manufacturer instructions. Restriction endonucleases in combination with non-chelated metal ions may degrade internal DNA markers used in assay kit.

Physical Specifications	
Type	Specifications
Analysis run time	35 minutes
Number of samples	12 samples/chip
Sample volume	1 μl
Kit stability	4 months (Storage temperature: see individual box!)

Analytical Specifications	
Type	Agilent DNA 1000 Assay
Sizing range	25–1000 bp
Typical sizing resolution	± 5 bp 25–100 bp ± 5% 100–500 bp ± 10% 500–1000 bp
Sizing accuracy	±10%* (for ladder as sample)
Sizing reproducibility	5% CV (for ladder as sample)
Quantitation accuracy	20%* CV (for ladder as sample)
Quant. reproducibility	25–500 bp: 15% CV; 500–1000 bp: 5% CV (for ladder as sample)
Quantitative range	0.1–50 ng/μl
Maximum salt	250 mM for KCl or NaCl, 15 mM for MgCl ₂

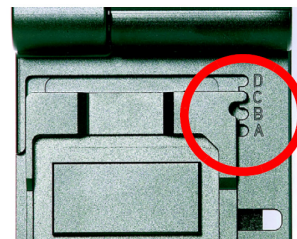
* Some fragments below 70 bp may deviate from the above specifications.

Setting up the Chip Priming Station

- 1 Replace the syringe:
 - a Unscrew the old syringe from the lid of the chip priming station.
 - b Release the old syringe from the clip. Discard the old syringe.
 - c Remove the plastic cap of the new syringe and insert it into the clip.
 - d Slide it into the hole of the luer lock adapter and screw it tightly to the priming station.



- 2 Adjust the base plate:
 - a Open the chip priming station by pulling the latch.
 - b Using a screwdriver, open the screw at the underside of the base plate.
 - c Lift the base plate and insert it again in position C. Retighten the screw.

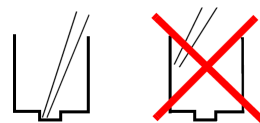


- 3 Adjust the syringe clip:
 - a Release the lever of the clip and slide it down to the lowest position.



Essential Measurement Practices

- Handle and store all reagents according to the instructions on the label of the individual box.
- Avoid sources of dust or other contaminants. Foreign matter in reagents and samples or in the wells of the chip will interfere with assay results.
- Keep all reagent and reagent mixes refrigerated at 4°C when not in use.
- Allow all reagents and samples to equilibrate to room temperature for 30 minutes before use.
- Protect dye and dye mixtures from light. Remove light covers only when pipetting. The dye decomposes when exposed to light and this reduces the signal intensity.
- Always insert the pipette tip to the bottom of the well when dispensing the liquid. Placing the pipette at the edge of the well may lead to poor results.
- Use a new syringe and electrode cleaners with each new Kit.
- Use loaded chips within 5 minutes after preparation. Reagents might evaporate, leading to poor results.
- Do not touch the Agilent 2100 Bioanalyzer during analysis and never place it on a vibrating surface.



Agilent DNA 1000 Assay Protocol - Edition April 2007

WARNING

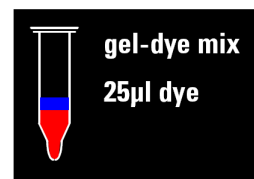
Handling DMSO

Kit components contain DMSO. Because the dye binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care.

Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples. Handle the DMSO stock solutions with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

Preparing the Gel-Dye Mix

- 1 Allow DNA dye concentrate (blue-capped tube) and DNA gel matrix (red-capped tube) to equilibrate to room temperature for 30 min.
- 2 Vortex DNA dye concentrate (blue) and add 25 μ l of the dye to a DNA gel matrix vial (red).
- 3 Vortex solution well and spin down. Transfer to spin filter.
- 4 Centrifuge at 2240 g \pm 20% for 15 min. Protect solution from light. Store at 4°C.



Loading the Gel-Dye Mix

- 1 Allow the gel-dye mix equilibrate to room temperature for 30 min before use.
- 2 Put a new DNA chip on the chip priming station.
- 3 Pipette 9.0 μ l of gel-dye mix in the well marked **G**.
- 4 Make sure that the plunger is positioned at 1-ml and then close the chip priming station.
- 5 Press plunger until it is held by the clip.
- 6 Wait for exactly 60 s then release clip.
- 7 Wait for 5 s. Slowly pull back plunger to 1-ml position.
- 8 Open the chip priming station and pipette 9.0 μ l of gel-dye mix in the wells marked **G**.




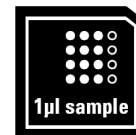
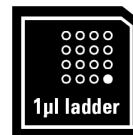
Loading the Markers

- 1 Pipette 5 μ l of marker (green-capped tube) in all 12 sample wells and ladder well. Do not leave any wells empty.



Loading the Ladder and the Samples

- 1 Pipette 1 μ l of DNA ladder (yellow-capped tube) in the well marked .
- 2 In each of the 12 sample wells pipette 1 μ l of sample (used wells) or 1 μ l of de-ionized water (unused wells).
- 3 Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting (2400 rpm).
- 4 Run the chip in the Agilent 2100 Bioanalyzer within 5 min.



Technical Support

In the US/Canada: 1-800-227-9770 (toll free); lsca-ibs-support@agilent.com.

In Europe: call your local Customer Care Center; bio_solutions@agilent.com.

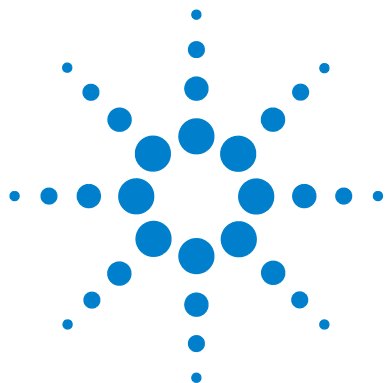
In Japan: 0120 477 111; yan_ccr@agilent.com

In Asia Pacific: call your local Customer Care Center; Bioanalyzer_ap@agilent.com

Further Information

Visit Agilent Technologies' unique Lab-on-a-Chip web site. It is offering useful information, support and current developments about the products and the technology:

<http://www.agilent.com/chem/labonachip>.



6 Appendix C: Bioanalyzer Electrode Cleaning Procedure

This chapter contains instructions on how to clean the electrodes of the Agilent 2100 Bioanalyzer.



To Clean Electrodes after a DNA 1000 Chip Run

NOTE

Use a new electrode cleaner with each new DNA 1000 kit.

CAUTION

Liquid spill might cause leak currents between the electrodes. Never fill too much water in the electrode cleaner.

- 1** Slowly fill one of the wells of the electrode cleaner with 350 µl deionized analysis-grade water.
- 2** Open the lid and place the electrode cleaner in the Agilent 2100 Bioanalyzer.
- 3** Close the lid and leave it closed for about 10 seconds.
- 4** Open the lid and remove the electrode cleaner.
- 5** Wait another 10 seconds to allow the water on the electrodes to evaporate before closing the lid.

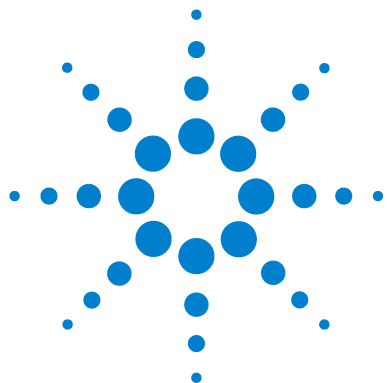
NOTE

After 5 assays, empty and refill the electrode cleaner.

After 25 assays, replace the used electrode cleaner with a new one.

NOTE

When switching between different assays, a more thorough cleaning may be required. Refer to the Maintenance and Troubleshooting chapter in the 2100 Expert help system.



7 References

References and Publications

- 1 Dooley, John J., Sage, Helen D., Brown, Helen M., Garrett, Stephen D. Improved fish species identification by use of lab-on-a-chip technology. *Food Control* **16** (7), 601-607 (2004).
- 2 Dooley, John J., Sage, Helen D., Clarke, Marie-Anne L., Brown, Helen M., Garrett, Stephen D. Fish Species Identification Using PCR-RFLP Analysis and Lab-on-a-Chip Capillary Electrophoresis: Application to Detect White Fish Species in Food Products and an Interlaboratory Study. *J. Agric. Food Chem.* **53**, 3348-3357 (2005).
- 3 Dooley, J., Garrett, S. Determination of PCR-RFLP Profiles for Fish Species Using the Agilent 2100 Bioanalyzer. Agilent Technologies Application Note (2005).
- 4 Dooley, J.J., Clarke, M.L., Sage, H.D., Brown, H.M., Garrett, S.D. Application of a chip-based capillary electrophoresis system to enable simple PCR-RFLP identification of fish species. FSA Final Report Q01069 (2004).



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In This Book

This document describes how to use the Agilent DNA Fish ID Ensemble to identify the species of a fish by extracting DNA and performing PCR and RFLP analysis.

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