AFLP™ Microbial Fingerprinting

Protocol

Applied Biosystems

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Contents

Introduction 1
- What is AFLP? 1
- Advantages of AFLP. 1
- Applications of AFLP. 1

The AFLP Technique. 3
- Template Preparation and Adaptor Ligation. 3
- Preselective Amplification 3
- Selective Amplification. 5
- Simplifying Complex Patterns 5
- Testing New Genomes 6
- Primer Selection Guidelines 7
- Genome Analysis Guide 8
- Fluorescent Dye-labeling and Marker Detection 9

Materials Needed to Perform AFLP 10
- AFLP Kit Modules 10
- AFLP EcoRI Ligation/ Amplification Module. 10
- AFLP MseI Ligation/ Amplification Module. 11
- AFLP Amplification Core Mix Module 11
- Storage and Stability of Kit Components 11
- Materials Required But Not Supplied. 12

Sample Preparation 14
- Before Starting an AFLP Experiment. 14
- Preparing Samples for PCR Amplification. 14
- Anneal Adaptor Pairs 15
- Prepare Enzyme Master Mix 15

Prepare Restriction-Ligation Reactions 16
Dilute Restriction-Ligation Reactions 16
Amplification of Target Sequences 17
Overview 17
Preselective Amplification 17
Verify Successful Amplification 18
Prepare Template 19
Selective Amplification 19
Evaluating Results 21
Overview 21
Preparing the Loading Buffer for the ABI 373 and ABI PRISM 377 . . 21
Loading and Electrophoresis on the ABI 373 and ABI PRISM 377 . . . 22
Preparing the Loading Buffer for the ABI PRISM 310 23
Loading and Electrophoresis on the ABI PRISM 310 23
Using GeneScan to Analyze Results 24
Evaluating ABI 373 DNA Sequencer Results 29
Evaluating ABI PRISM 377 DNA Sequencer Results 30
Evaluating ABI PRISM 310 Genetic Analyzer Results 31
Appendix A. Troubleshooting 32
Appendix B. References 35
Appendix C. Related Reagents, Consumables, and Accessories 38
Appendix D. Technical Support 42
Contacting Technical Support 42
To Contact Technical Support by E-Mail 42
Hours for Telephone Technical Support 42
To Contact Technical Support by Telephone or Fax 43
To Reach Technical Support Through the Internet 46
To Obtain Documents on Demand 47
. 47

Introduction

What is AFLP?

The AFLP™ amplified fragment polymorphism technique is used to visualize hundreds of amplified DNA restriction fragments simultaneously. The AFLP band patterns, or fingerprints, can be used for many purposes, such as monitoring the identity of an isolate or the degree of similarity among isolates. Polymorphisms in band patterns map to specific loci, allowing the individuals to be genotyped or differentiated based on the alleles they carry.

AFLP technology combines the power of restriction fragment length polymorphism (RFLP) with the flexibility of PCR-based technology by ligating primer-recognition sequences (adaptors) to the restricted DNA.

Advantages of AFLP

Some of the advantages of the AFLP technique are the following:

- Only small amounts of DNA are needed.
- Unlike randomly amplified polymorphic DNAs (RAPDs) that use multiple, arbitrary primers and lead to unreliable results, the AFLP technique uses only two primers and gives reproducible results.
- Many restriction fragment subsets can be amplified by changing the nucleotide extensions on the adaptor sequences. Hundreds of markers can be generated reliably.
- High resolution is obtained because of the stringent PCR conditions.
- The AFLP technique works on a variety of genomic DNA samples.
- No prior knowledge of the genomic sequence is required.

Applications of AFLP

Applications for AFLP in microbial fingerprinting include the following:

- differentiation and tracking of highly related microbes at the species or strain level
- high-resolution genotyping for taxonomic applications
- detection of DNA polymorphisms in genome evolution studies
- determining the relatedness of pathogenic organisms in epidemiological studies
- mapping of cloned fragments in bacterial and yeast artificial chromosomes (BACs and YACs)

An example of AFLP fingerprints is shown in Figure 1. The first 24 lanes show six samples each of four different *Escherichia coli* strains (each of the six samples represents a different growth phase of the organism). The final 11 lanes show different growth phases of a single strain of *Legionella pneumophila*. Note that the *E. coli* fingerprints are similar to each other and different from the *Legionella* fingerprint. Within a strain, all of the bands are reproducible.

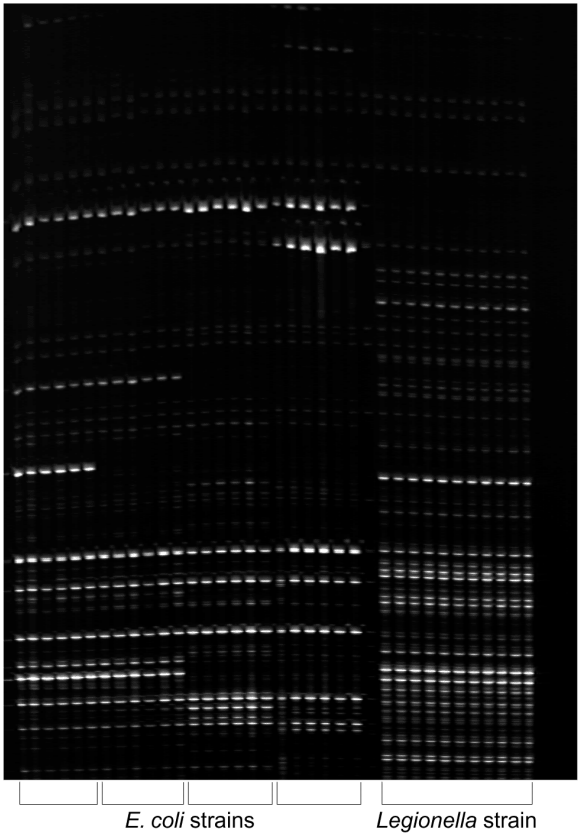

Figure 1 AFLP fingerprints of four *E. coli* strains and one *Legionella* strain

Large population studies provide data for the linkage of a band with a given phenotype, such as pathogenicity. For examples of other applications, refer to the literature cited in Appendix B on page 35.

The AFLP Technique

Template Preparation and Adaptor Ligation

The first step of the AFLP technique is to generate restriction fragments by using two restriction endonucleases (EcoRI and MseI in the AFLP Microbial Fingerprinting Kit). Double-stranded adaptors supplied with each kit are ligated to the ends of the DNA fragments, generating template DNA for subsequent polymerase chain reaction (PCR) amplification.

Restriction and ligation may take place in a single reaction if the buffers are compatible (Figure 2). Adaptor sequences have been designed such that ligation of the adaptor oligonucleotide to the restricted DNA does not regenerate the recognition site. If the buffers are not compatible, the reactions must be run sequentially.

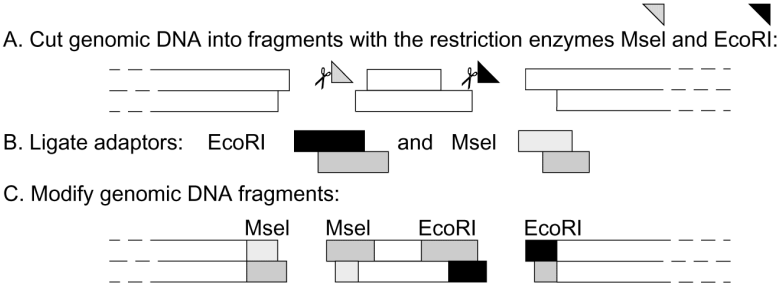

Figure 2 Example of template preparation and AFLP adaptor ligation

Preselective Amplification

The sequences of the adaptors and the restriction site serve as primer binding sites for a subsequent low-level selection or “preselective” amplification of the restriction fragments.

Only those genomic fragments that have an adaptor on each end amplify exponentially during PCR amplification (Figure 3 on page 4). This step effectively “purifies” the target away from sequences that amplify only linearly, *i.e.*, those with one modified end.

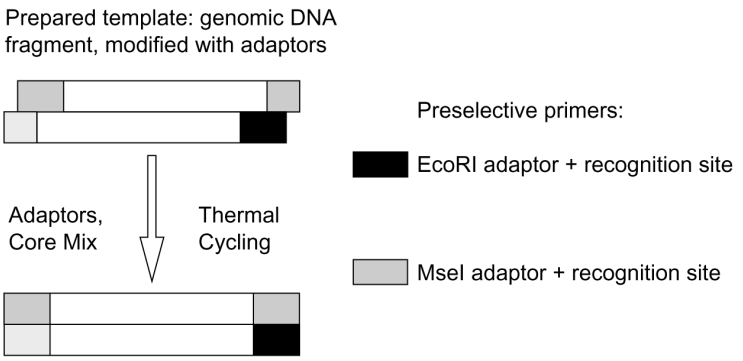

Figure 3 Preselective amplification of the prepared template

In the microbial genomes targeted by this kit, the core primer sequence is used. In larger genomes, such as plants and some fungi, this amplification would create too many fragments. In those cases, the preselective amplification is performed with additional nucleotides on the end of each primer. Each added nucleotide reduces the number of sequences by a factor of four.

The thermal cycling conditions of the preselective amplification step have been optimized to generate a constant final mass of fragments. Band intensity in subsequent reactions can therefore be correlated with relative differences in representation of the fragments within the genome, and not to the overall amount of genomic DNA that went into the initial restriction-ligation mix.

It is not necessary to perform this step if:

- relative peak height information is not desired
- methods are available to normalize the final signal
- very accurate quantitation of the input DNA is performed routinely

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Selective Amplification

Additional PCR amplifications are run to reduce the complexity of the mixture further so that the fragments can be resolved on a polyacrylamide gel. These amplifications use primers chosen from the 18 available AFLP Microbial Fingerprinting Kit Selective Primers (nine EcoRI fluorescent dye-labeled primers and nine unlabeled MseI primers). After PCR amplification with these primers, a portion of the samples is analyzed on a Applied Biosystems DNA Sequencer.

Selective amplification with an EcoRI and an MseI primer amplifies primarily EcoRI-MseI-ended fragments. The EcoRI-EcoRI fragments do not amplify well. The MseI-MseI fragments are not visualized because they do not contain fluorescent dye labels. Only the EcoRI-containing strands are detected (Figure 4).

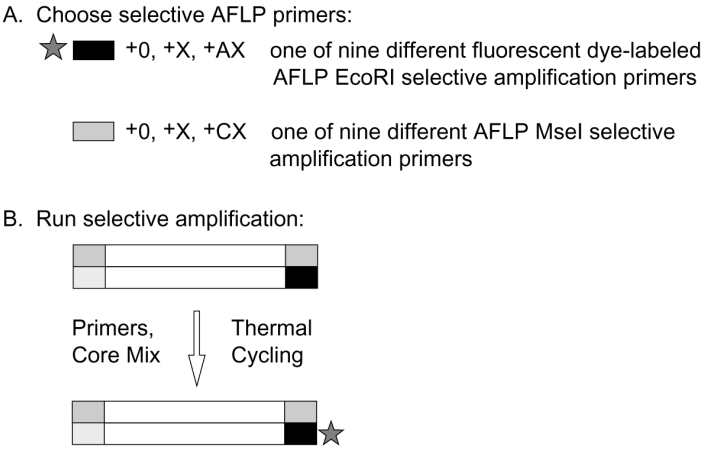

Figure 4 Selective amplification with fluorescent dye-labeled primers

Simplifying Complex Patterns

Figure 5 on page 6 shows examples of AFLP fingerprint patterns that were prepared using different selective primers. Note that the EcoRI selective primers with one-nucleotide extensions (EcoRI-A, EcoRI-T, and EcoRI-G) give simpler patterns than that obtained using the primer with no extra nucleotide (EcoRI-0).

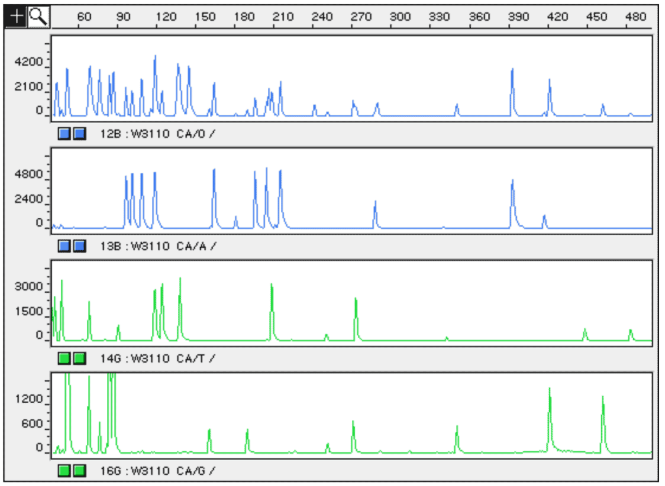

Figure 5 AFLP fingerprints of *E. coli* W3110 Reference DNA. The MseI-CA and fluorescent dye-labeled EcoRI-0, EcoRI-A, EcoRI-T, and EcoRI-G selective primers (shown here top to bottom, respectively) were used.

If the complexity of the AFLP pattern is still too high at the +2/+2 level, we recommend reamplifying the preselective amplification sample with the preselective primers from the AFLP Ligation and Preselective Amplification Modules of the AFLP Regular and Small Plant Genome Mapping Kits (P/N 402004 and 402273, respectively).

Testing New Genomes

When testing novel genomes, you must be sure that the DNA restriction digest with EcoRI and MseI generates enough fragments for comparison of samples. There is a large variability in the number of restriction sites within microbial genomes. No assurances of kit performance are made for organisms not listed.

Empirical guidelines suggest that if the G-C content of the genome is >65%, MseI will not give a significant number of fragments. Optimal results are obtained with MseI when the G-C content is <50%. EcoRI also tends to produce more fragments in G-C-poor genomes. In cases where an organism's G-C content is unknown, the effectiveness of the restriction enzymes must be determined empirically.

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Primer Selection Guidelines

For genomes that restrict well with the EcoRI and MseI restriction endonuclease combination, some general recommendations can be made in terms of the genome size and the selective nucleotides to choose for subsequent amplification (Table 1).

Table 1. Guide to choosing selective primers

Application	Nucleotide Addition	EcoRI Primers	MseI Primers
Cosmids, BACs, P1 mapping	+0/+0	EcoRI-0 FAM	MseI-0
YACs, some larger BACs	+0/+1	EcoRI-0 FAM	MseI-A MseI-C MseI-G MseI-T
	+1/+0	EcoRI-A FAM EcoRI-C NED EcoRI-G JOE EcoRI-T JOE	MseI-0
Bacteria	+0/+2	EcoRI-0 FAM	MseI-CA MseI-CC MseI-CG MseI-CT
	+1/+1	EcoRI-A FAM EcoRI-C NED EcoRI-G JOE EcoRI-T JOE	MseI-A MseI-C MseI-G MseI-T
	+2/+0	EcoRI-AA JOE EcoRI-AC FAM EcoRI-AG JOE EcoRI-AT NED	MseI-0
Yeast, small fungi genomes	+2/+2	EcoRI-AA JOE EcoRI-AC FAM EcoRI-AG JOE EcoRI-AT NED	MseI-CA MseI-CC MseI-CG MseI-CT
Large fungi genomes	+2/+3 +3/+2	Use the primers from the AFLP Regular and Small Plant Genome Mapping Kits. See Appendix C on page 38 for the primers available.	

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Genome Analysis Guide

Some bacterial and fungal genomes that have been analyzed successfully using EcoRI, MseI, and the primers in this kit are shown in Table 2.

Table 2. Genomes analyzed with EcoRI and MseI primer pairs

Organism	Primer Pairs Used Successfully[a]	Primer Pairs to Avoid[b]
Acinetobacter sp.	EcoRI-C/MseI-T	–
Aeromonas sp.	EcoRI-A/MseI-T	–
Aspergillus sp.	EcoRI-A/MseI-G EcoRI-A/MseI-CA EcoRI-C/MseI-CA EcoRI-T/MseI-A	–
Bacillus sp.	EcoRI-0/MseI-A	–
Candida utilis	EcoRI-G/MseI-A	–
Clostridium sp.	EcoRI-C/MseI-C	–
Vancomycin-resistant *Enterobacter*	EcoRI-A/MseI-T EcoRI-G/MseI-A EcoRI-T/MseI-C	–
Escherichia coli	EcoRI-0/MseI-C EcoRI-A/MseI-C EcoRI-G/MseI-A EcoRI-T/MseI-C	EcoRI-0/MseI-A EcoRI-0/MseI-G
Eutypa sp.	EcoRI-A/MseI-CA EcoRI-AC/MseI-C	–
Legionella pneumophila	EcoRI-A/MseI-G EcoRI-AC/MseI-C	EcoRI-0/MseI-A
Nensenula anomola	EcoRI-A/MseI-T EcoRI-G/MseI-A	–
Paenibacillus larvae	EcoRI-C/MseI-A	–
Pichia membrefaciens	EcoRI-AC/MseI-C	
Saccharomyces sp.	EcoRI-A/MseI-CA EcoRI-AC/MseI-C	–
Schizosaccharomyces pombe	EcoRI-AC/MseI-C	–
Xanthomonas sp.	EcoRI-0/MseI-C	–

a. Producing 25–130 bands evenly dispersed from 50–500 bases with intensities of 100–2000 relative fluorescent units

b. Too few or too many bands or uneven size distribution

Note The list in Table 2 on page 8 is not exhaustive. Refer to the publications listed in Appendix B on page 35 for in-depth discussion of primer choices.

Fluorescent Dye-labeling and Marker Detection

Applied Biosystems has adapted the AFLP technique for use with its ABI PRISM™ fluorescent dye-labeling and detection technology. PCR products are dye-labeled during amplification using a 5´ dye-labeled primer.

For high throughput, you can co-load up to three different reactions labeled with different colored dyes in a single lane on the ABI 373 or ABI PRISM 377 DNA Sequencer or in a single injection on the ABI PRISM 310 Genetic Analyzer. Load an internal lane size standard with a fourth color in every lane to size all amplification fragments accurately.

You can automate the scoring of the large numbers of markers that are typically generated by analyzing your results with GeneScan® Analysis and Genotyper® software.

Materials Needed to Perform AFLP

AFLP Kit Modules

The AFLP Microbial Fingerprinting Kit (P/N 402948) is organized into three individual modules:

- AFLP EcoRI Ligation/Amplification Module (P/N 402941)
- AFLP MseI Ligation/Amplification Module (P/N 402942)
- AFLP Amplification Core Mix Module (P/N 402005)

The AFLP EcoRI and MseI Ligation/Amplification Modules provide sufficient reagents to modify and do preselective amplifications on 100 individual DNA samples (10 ng each). There are sufficient amounts of the 18 selective primers (nine EcoRI and nine MseI) to test two preselective samples with all 81 selective primer pair combinations and to test the remaining 98 samples with six selective primer pair combinations. The AFLP Amplification Core Mix Module contains all of the components necessary to amplify modified target sequences.

AFLP EcoRI Ligation/ Amplification Module

This module (P/N 402941) contains the following reagents:

- EcoRI adaptor (100 µL, 2 µM)
- EcoRI core sequence (50 µL, 10 µM)
- Nine selective primers (each 500 µL, 1 µM):

Selective primer	Designation
AFLP EcoRI-0, FAM (no additional nucleotide)	+0
AFLP EcoRI-A, FAM	+1
AFLP EcoRI-C, NED	+1
AFLP EcoRI-G, JOE	+1
AFLP EcoRI-T, JOE	+1
AFLP EcoRI-AA, JOE	+2
AFLP EcoRI-AC, FAM	+2
AFLP EcoRI-AG, JOE	+2
AFLP EcoRI-AT, NED	+2

- *E. coli* W3110 Reference DNA (25 µL, 10 ng/µL), sufficient for 25 restriction-ligation reactions

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AFLP MseI Ligation/ Amplification Module

This module (P/N 402942) contains the following reagents:

- MseI adaptor (100 µL, 20 µM)
- MseI core sequence (50 µL, 10 µM)
- Nine selective primers (each 500 µL, 5 µM primer):

Selective primer	Designation
AFLP MseI-0 (no additional nucleotide)	+0
AFLP MseI-A	+1
AFLP MseI-C	+1
AFLP MseI-G	+1
AFLP MseI-T	+1
AFLP MseI-CA	+2
AFLP MseI-CC	+2
AFLP MseI-CG	+2
AFLP MseI-CT	+2

- *E. coli* W3110 Reference DNA (25 µL, 10 ng/µL), sufficient for 25 restriction-ligation reactions

AFLP Amplification Core Mix Module

This module (P/N 402005) provides five tubes of Core Mix (total volume 7.5 mL) containing the following:

- buffer
- nucleotides
- AmpliTaq® DNA Polymerase

The AFLP Amplification Core Mix Module contains sufficient reagents for 1000 amplification reactions (10 µL each) of target genomic sequences. You determine how the selection occurs by choosing primer pairs from the AFLP EcoRI and MseI Ligation/Amplification Modules.

Note For information on ordering selective primers and other AFLP reagents individually, see Appendix C on page 38.

Storage and Stability of Kit Components

Store all kit components at –15 to –25 °C in a non-frost-free freezer. If stored properly, kit components will last up to one year.

continued on next page

Materials Required But Not Supplied

Reagents (see Appendix C on page 38 for more information)

! WARNING ! Chemical hazard. Before handling any of the chemicals listed below, familiarize yourself with the Materials Safety Data Sheet (MSDS). Always follow safety precautions and wear proper protective equipment (eye protection, gloves, lab coat). Dispose of waste in accordance with all local, state, and national regulations.

- Nuclease-free distilled deionized water
- EcoRI restriction endonuclease, 500 Units
- MseI restriction endonuclease, 100 Units
- T4 DNA Ligase, 100 Units
- 10X T4 DNA ligase buffer containing ATP (see page 16)
- NaCl, 0.5 M, nuclease-free (molecular biology grade)
- Bovine serum albumin (BSA), 1.0 mg/mL, nuclease-free
- 1X $TE_{0.1}$ buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), nuclease-free
- 6% denaturing polyacrylamide gel (for the ABI 373 DNA Sequencer)
- 5% Long Ranger gel (for the ABI PRISM 377 DNA Sequencer)
- Performance Optimized Polymer 4 (POP-4, Applied Biosystems P/N 402838, for the ABI PRISM 310 Genetic Analyzer)
- Deionized formamide (Applied Biosystems P/N 400596)
- GeneScan-500 ROX Size Standard (Applied Biosystems P/N 401734)
- DNA size markers *(e.g.*, Boehringer Mannheim set VI)
- Dye Primer Matrix Standard Kit (Applied Biosystems P/N 401114)
- NED Matrix Standard (Applied Biosystems P/N 402996, substitutes for TAMRA)

Equipment

- Microcentrifuge
- Pipettors, 2 µL, 20 µL and 200 µL, with sterile pipette tips
- Gel-loading pipette tips, 0.17-mm flat (ABI PRISM 377)
- Sterile 0.5-ml microcentrifuge tubes
- Thermal cycler (Applied Biosystems)
- Sterile 0.2-mL MicroAmp® Thin-Walled Reaction Tubes and caps (GeneAmp® PCR Instrument Systems 2400 and 9600)
- Sterile GeneAmp Thin-Walled 0.5-mL Reaction Tubes (DNA Thermal Cycler 480)

Sample Preparation

Before Starting an AFLP Experiment

Before setting up an AFLP experiment, determine whether or not your genomic DNA restricts properly with EcoRI and MseI.

Step	Action
1	Digest 1–3 µg of DNA with the enzymes MseI and EcoRI separately, then with both together, according to the manufacturer's instructions.
2	Load the digestion products in one lane on a 1.5% mini-agarose gel with size markers.
3	Stain with ethidium bromide. **! WARNING ! Ethidium bromide is a powerful mutagen and is moderately toxic. Wear gloves, a lab coat, and safety glasses when using this dye. After use, decontaminate ethidium bromide solutions before disposal.**
4	View on a UV transilluminator. For an example of what a successful digest looks like, see Figure 6 on page 18 (left half).

Preparing Samples for PCR Amplification

To prepare samples for the AFLP preselective and selective amplification reactions, you must:

- anneal the adaptor pairs
- prepare a restriction-ligation enzyme master mix
- prepare the restriction-ligation reactions
- dilute the restriction-ligation reactions

continued on next page

Anneal Adaptor Pairs

You must anneal the adaptor pairs supplied with the AFLP EcoRI and MseI Ligation/Amplification Modules before you can use them for the restriction-ligation reactions.

Step	Action
1	From the AFLP EcoRI and MseI Ligation/Amplification Modules, remove the tubes labeled MseI Adaptor Pair and EcoRI Adaptor Pair.
2	Heat tubes in a water bath at 95 °C for five minutes.
3	Allow tubes to cool to ambient temperature for ten minutes.
4	Spin in a microcentrifuge for ten seconds at 1400 x g (maximum).

Prepare Enzyme Master Mix

Prepare an Enzyme Master Mix to perform the restriction-ligation reactions for all 100 DNA samples, or a proportionate amount for fewer reactions.

Step	Action
1	Combine the following in a sterile 0.5 mL microcentrifuge tube: ♦ 10 µL 10X T4 DNA ligase buffer with ATP[a] ♦ 10 µL 0.5 M NaCl ♦ 100 Units MseI ♦ 500 Units EcoRI ♦ 100 Units T4 DNA Ligase
2	Add sterile distilled water to bring the total volume to 100 µL.
3	Mix gently.
4	Spin down in a microcentrifuge for 10 seconds.
5	Store on ice until ready to aliquot into individual reaction tubes. **IMPORTANT** For best results, use the Enzyme Master Mix within one to two hours. Do not store Master Mix beyond the day on which it is to be used!

a. 1X T4 DNA Ligase Buffer with ATP: 50mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml bovine serum albumin.

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Prepare Restriction-Ligation Reactions

The restriction-ligation reactions prepare the template for adaptors and then ligate adaptor pairs to the prepared template DNA.

Step	Action
1	Combine the following in a sterile 0.5 mL microcentrifuge tube: ♦ 0.01 µg genomic DNA in 5.5 µL sterile distilled water (or 1.0 µL of *E. coli* W3110 reference DNA plus 4.5 µL sterile distilled water) ♦ 1.0 µL 10X T4 DNA ligase buffer that includes ATP ♦ 1.0 µL 0.5M NaCl ♦ 0.5 µL 1.0 mg/mL BSA (dilute from 10 mg/mL if necessary) ♦ 1.0 µL MseI adaptor ♦ 1.0 µL EcoRI adaptor ♦ 1.0 µL Enzyme Master Mix
2	Mix thoroughly and place in a microcentrifuge for ten seconds.
3	Incubate at room temperature overnight or for two hours at 37 °C in a thermal cycler with a heated cover to prevent EcoRI* (star) activity. **Note** Be careful that the volume of enzyme added does not cause the amount of glycerol to be >5%, which also leads to EcoRI* activity.

Dilute Restriction-Ligation Reactions

Dilute the restriction-ligation samples to give the appropriate concentration for subsequent PCR.

Step	Action
1	Add 189 µL of $TE_{0.1}$ buffer to each restriction-ligation reaction.
2	Mix thoroughly.
3	Store the mixture at 2–6 °C for up to one month, or at –15 to –25 °C for longer than a month.

Note The mixture can be used as the template for selective AFLP reactions (see page 19), or the modified restriction fragments can be amplified in preselective AFLP reactions first (see page 17), then amplified selectively. How to choose what reactions to perform is discussed in “The AFLP Technique” on page 3.

Amplification of Target Sequences

Overview

This protocol has been optimized for the GeneAmp® PCR Systems 9600 and 2400 and the DNA Thermal Cycler 480. If you use a different thermal cycler, you may need to optimize the conditions. The temperature ramp times included in this protocol ensure identical products from any Applied Biosystems thermal cycler. Ramp time is crucial. If the temperature is increased too quickly, results may be inconsistent. See Appendix A on page 32 for troubleshooting tips.

Preselective Amplification

Sequences with adaptors ligated to both ends amplify exponentially and predominate in the final product.

Note Keep all reagents and tubes on ice until loaded into the thermal cycler.

Step	Action
1	Combine the following in a PCR reaction tube (0.2 mL for the GeneAmp PCR System 9600 or 2400, 0.5 mL for the DNA Thermal Cycler 480): ♦ 4.0 µL diluted DNA prepared by restriction-ligation ♦ 0.5 µL AFLP EcoRI preselective primer ♦ 0.5 µL AFLP MseI preselective primer ♦ 15.0 µL AFLP Amplification Core Mix **Note** If using the DNA Thermal Cycler 480, overlay your samples with 20 µL of light mineral oil.
2	Place the samples in a thermal cycler at ambient temperature.
3	Run the PCR method shown in Table 3, entering all ramp times as 0.01 (one second) on the GeneAmp PCR System 9600 and DNA Thermal Cycler 480 or 90% on the GeneAmp PCR System 2400.
4	Store at 2–6 °C.

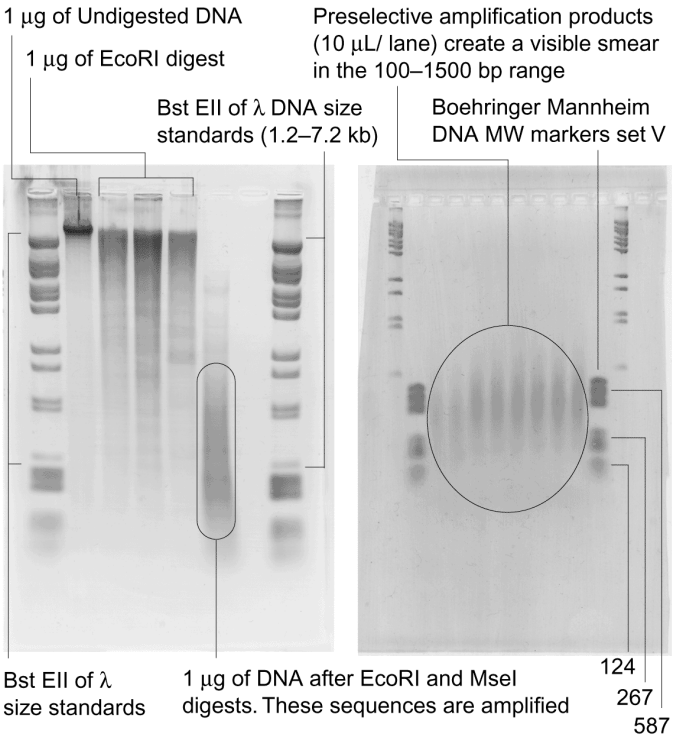
Table 3. Thermal cycler parameters for preselective amplification

HOLD	CYCLE			HOLD
	Each of 20 Cycles			
72 °C 2 min.	94 °C 20 sec.	56 °C 30 sec.	72 °C 2 min.	4 °C (forever)

continued on next page

Verify Successful Amplification

Run an agarose yield gel to check that amplification has occurred.

Step	Action
1	Run 10 µL of each reaction on a 1.5% agarose gel in 1X TBE buffer at 4V/cm for 3–4 hours.
2	Stain the gel with ethidium bromide. **! WARNING ! Ethidium bromide is a powerful mutagen and is moderately toxic. Wear gloves, a lab coat, and safety glasses when using this dye.**
3	View the gel on a UV transilluminator. A smear of product from 100–1500 bp should be clearly visible (Figure 6, right half).  **Figure 6** Gel results after restriction digestion of 1–3 µg of DNA (left) and after preselective amplification (right)

continued on next page

Prepare Template

Prepare the preselective amplification products for selective amplification.

Step	Action
1	Combine the following in a sterile 0.5 mL microcentrifuge tube: ♦ 10.0 µL preselective amplification product ♦ 190.0 µL TE $_{0.1}$ buffer
2	Mix thoroughly, then spin down in a microcentrifuge for ten seconds.
3	Store the diluted preselective amplification product at 2–6 °C if not used immediately.

Selective Amplification

Amplify the EcoRI- and MseI-modified fragments.

Step	Action
1	Combine the following in a PCR tube (0.2 mL for the GeneAmp PCR System 9600 or 2400, 0.5 mL for the DNA Thermal Cycler 480): ♦ 1.5 µL diluted preselective amplification product ♦ 0.5 µL MseI primer at 5 µM ♦ 0.5 µL dye-labeled EcoRI primer at 1 µM ♦ 7.5 µL AFLP Core Amplification Mix **Note** If using the DNA Thermal Cycler 480, add 20 µL of light mineral oil to the tube.
2	Run PCR using the thermal cycler parameters shown in Table 4 on page 20. **Note** For the GeneAmp PCR System 9600 and DNA Thermal Cycler 480, enter all ramp times as 0.01 (one second). For the GeneAmp PCR System 2400 enter all ramp times as 90%.
3	Store at 2–6 °C.

Table 4. Thermal cycler parameters for selective amplification

HOLD		CYCLE		Number of Cycles
94 °C 2 min.	94 °C 20 sec.	66 °C 30 sec.	72 °C 2 min.	1
–	94 °C 20 sec.	65 °C 30 sec.	72 °C 2 min.	1
–	94 °C 20 sec.	64 °C 30 sec.	72 °C 2 min.	1
–	94 °C 20 sec.	63 °C 30 sec.	72 °C 2 min.	1
–	94 °C 20 sec.	62 °C 30 sec.	72 °C 2 min.	1
–	94 °C 20 sec.	61 °C 30 sec.	72 °C 2 min.	1
–	94 °C 20 sec.	60 °C 30 sec.	72 °C 2 min.	1
–	94 °C 20 sec.	59 °C 30 sec.	72 °C 2 min	1
–	94 °C 20 sec.	58 °C 30 sec.	72 °C 2 min.	1
–	94 °C 20 sec.	57 °C 30 sec.	72 °C 2 min.	1
–	94 °C 20 sec.	56 °C 30 sec.	72 °C 2 min.	20
60 °C 30 min.		–		1
4 °C forever		–		1

Evaluating Results

Overview

You can evaluate the results of the AFLP reactions by using GeneScan software to analyze data from samples loaded and run on the ABI 373 or ABI PRISM 377 DNA Sequencer or on the ABI PRISM 310 Genetic Analyzer.

The following instructions describe step-by-step procedures for loading samples and performing electrophoresis on these instruments.

Preparing the Loading Buffer for the ABI 373 and ABI PRISM 377

Prepare a loading buffer mix of the following reagents in the proportions shown in sufficient quantity for each sample:

- 1.25 µL deionized formamide
- 0.25 µL blue dextran/50 mM EDTA loading solution (supplied with the size standard)
- 0.5 µL of GeneScan-500 [ROX] size standard

! WARNING ! Chemical hazard: formamide is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.

Note You can store any remaining loading buffer at 2–6 °C for a week.

continued on next page

Loading and Electrophoresis on the ABI 373 and ABI PRISM 377

For specific instructions about loading and running samples, refer to the ABI 373 DNA Sequencing System User's Manual or the ABI PRISM 377 DNA Sequencer User's Manual.

Step	Action	
1	Add 2.0 µL of the loading buffer mix to a 0.2-mL MicroAmp Reaction Tube. Use one tube for each sample.	
2	**On the ABI 373 DNA Sequencer:**	**On the ABI PRISM 377 DNA Sequencer:**
	Add 1.0 µL of the selective amplification product to the tubes.	Dilute the selective amplification product with two parts of TE buffer. Add 1.0 µL of the diluted product to the tubes.
3	Heat tubes to 95 °C for three minutes.	
4	Quick-chill on ice.	
5	**On the ABI 373 DNA Sequencer:**	**On the ABI PRISM 377 DNA Sequencer:**
	Load the entire sample onto a 6% denaturing polyacrylamide gel using 1X TBE running buffer.	Load 1.5–2 µL of the sample onto a 5% denaturing Long Ranger gel using 1X TBE running buffer.

IMPORTANT Use Filter Set A with AFLP Microbial Fingerprinting Kit modules on the ABI 373 or ABI PRISM 377 DNA Sequencer. Make the matrix with the Dye Primer Matrix Standards (P/N 401114), substituting the NED Matrix Standard (P/N 402996) for TAMRA.

Table 5. ABI 373 and ABI PRISM 377 Electrophoresis Parameters

Instrument	Well-to-read distance	Limiting parameter	Time
ABI 373	24 cm	1680 volts	11.0 hours
ABI PRISM 377	36 cm	2500 volts	4.0 hours

continued on next page

Preparing the Loading Buffer for the ABI PRISM 310

Prepare a loading buffer mix of the following reagents in the proportions shown in sufficient quantity for each sample:

- 24.0 µL deionized formamide
- 1.0 µL of GeneScan-500 [ROX] size standard

! WARNING ! Chemical hazard: formamide is a teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a well-ventilated area. Use chemical-resistant gloves and safety glasses when handling.

Note You can store any remaining loading buffer at 2–6 °C for a week.

Loading and Electrophoresis on the ABI PRISM 310

For specific instructions about loading and running samples, refer to the *ABI PRISM 310 Genetic Analyzer User's Manual*.

Step	Action
1	Add 25.0 µL of the loading buffer mix to a sample tube.[a] Use one tube for each sample.
2	Add 0.5 µL of the selective amplification product to the tubes.
3	Heat tubes to 95 °C for 3–5 minutes.
4	Quick-chill on ice.
5	Place the Genetic Analyzer sample tubes in the 48-well or 96-well sample tray.

a. Use 0.5-mL Genetic Analyzer sample tubes for the 48-well sample tray and 0.2-mL MicroAmp Reaction Tubes for the 96-well sample tray.

IMPORTANT Use the GS STR POP4 A run module and ABI PRISM 310 Genetic Analyzer Collection Software, version 1.0.2 or higher, with the AFLP Microbial Fingerprinting Kit. Make the matrix with the Dye Primer Matrix Standards (P/N 401114), substituting the NED Matrix Standard (P/N 402996) for TAMRA.

Table 6. ABI PRISM 310 Electrophoresis Parameters

Pattern Complexity	Injection Time (sec.)	Injection Voltage (kV)	Run Time (min.)	Run Voltage (kV)
Dense patterns[a]	12	15	30	13
Simple patterns	5	13	26	15

a. Use these conditions when running any sample for the first time.

continued on next page

Using GeneScan to Analyze Results

After your sample data is collected, you can use GeneScan Analysis software to analyze and display sizing results for all samples in any combination of tabular data and electropherograms (with or without legends). When you display electropherograms and tabular data together, the Results Display window is divided into upper and lower panes. The upper pane contains electropherogram panels and the corresponding legends; the lower pane contains the tabular data.

The following procedure describes how to set the GeneScan Analysis software parameters. For more complete information, refer to the *ABI PRISM GeneScan Analysis Software User's Manual*.

Setting GeneScan Analysis Software Parameters

Step	Action
1	Under the Settings menu, select Analysis Parameters. Set the parameters as shown in Figure 7 for the ABI 373 and ABI PRISM 377. On the ABI PRISM 310, use an analysis range of 2600–10000 data points and peak amplitude thresholds of 100.

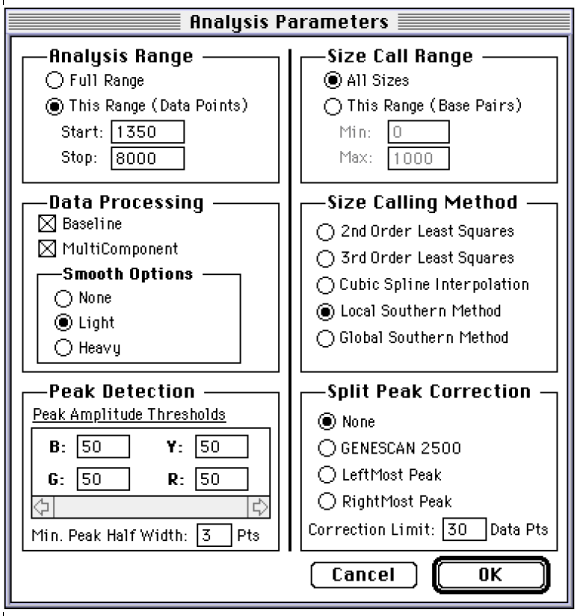


Figure 7 Analysis Parameters dialog box on the ABI PRISM 377

Setting GeneScan Analysis Software Parameters *(continued)*

Step	Action	
2	Click OK.	
3	In the Analysis Control Window, define a size standard as follows:	
	a.	Indicate the dye color of the Size Standard.
	b.	Choose Define New... from the pop-up window, and select a Sample File (data for one lane). The size standard peaks within the defined Analysis Range appear.
	c.	Assign a size value to each peak.
	d.	Close the window and enter a standard name when a prompt appears.
4	Highlight the sample(s) to be analyzed and click on the Analyze button.	
5	After a successful analysis, view your results in the Results Display window, and then save the project.	
6	Select Save As from the File menu to save the data to a file.	

GeneScan-500 Size Standard

The GeneScan-500 standard is made of double-stranded DNA fragments, but only one of the strands is labeled with an ABI PRISM dye. Consequently, under denaturing conditions, even if the two strands migrate at different rates, only the one labeled strand is detected. Because of this, you can avoid split peaks, which result when two strands move through a denaturing gel at different rates. Under denaturing conditions, you can achieve a linear range of separation for fragment sizes of up to 500 bases (Figure 8 on page 26).

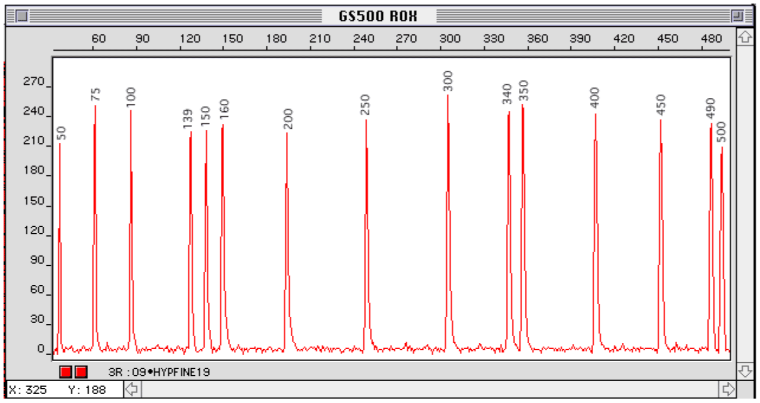


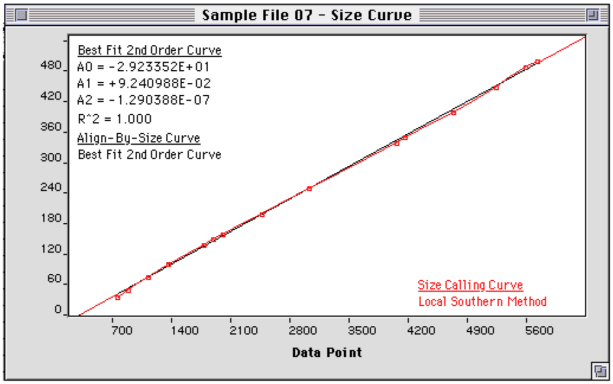
Figure 8 Electropherogram of GeneScan-500 run under denaturing conditions

Using the Standard Sizing Curve

The Standard Sizing Curve is a measure of how well the standard definition matches the GeneScan size standard, and whether or not it is linear.

To align the data by size, GeneScan calculates a best-fit least squares curve for all samples. This is a third-order curve when you use the Third Order Least Squares size calling method. For all other size calling methods it is a second-order curve.

Displaying the Standard Sizing Curve

Step	Action
1	Select a sample or multiple samples in the Analysis Control or Results Control window. To select several consecutive samples, shift-click the first and last sample in the group you wish to select.
2	Choose Standard Sizing Curve from the Project menu (Figure 9).  **Figure 9** Standard Sizing Curve window The R^2 value and the coefficients of the curve are provided. The R^2 value is a measure of the accuracy of fit of the best-fit second order curve. **Note** You can only display the sizing curve for a sample if a valid sizing curve exists for that sample.
3	Examine how the data points fit on the curve and look at the R^2 value to evaluate the size calling. The data points should fit close to the curve and the R^2 value should be between 0.99 and 1.00.
4	When you are finished, click the close box.

Defining Polymorphic Peaks for Genotyper Analysis

In addition to sizing AFLP fragments, GeneScan software enables you to prepare AFLP results data for downstream analysis by the Genotyper software application. Before starting Genotyper, define the polymorphic peaks to be scored.

Step	Action
1	In GeneScan, overlap the analyses of reactions from different samples to identify the polymorphic peaks.
2	Under the View menu, use the Custom Colors option to change the display color of one or more of the samples so that the electropherograms are in different colors.
3	Record the sizes of the polymorphic peaks and the samples that produced them.

Figure 10 shows the polymorphic peak patterns from a GeneScan analysis of two AFLP samples. Polymorphic peaks are labeled with size and origin.

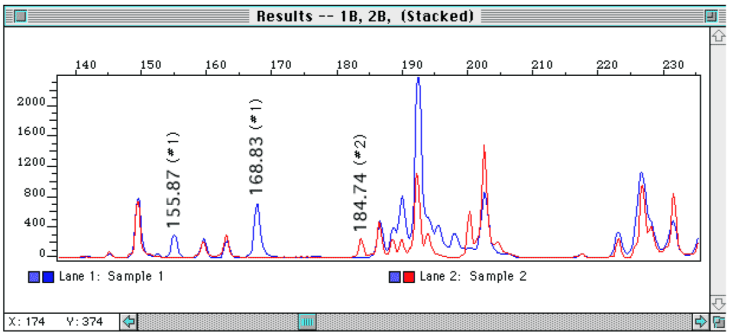


Figure 10 Overlapping electropherograms for two AFLP samples

You can import GeneScan results data into a Genotyper software template. Used together, GeneScan and Genotyper can automate segregation scoring of AFLP results.

For more information on how you can analyze polymorphic peaks using Genotyper, see the *Genotyper DNA Fragment Analysis Software User's* Manual.

continued on next page

Evaluating ABI 373 DNA Sequencer Results

If you run samples under the recommended electrophoresis conditions, and analyze them with GeneScan, resulting electropherogram data from the ABI 373 DNA Sequencer should look similar to data from samples run on the ABI PRISM 377 DNA Sequencer.

Figure 11 shows a representative electropherogram of fluorescent dye-labeled AFLP products run on an ABI 373 DNA Sequencer and analyzed using GeneScan analysis software. The analyzed products are DNA fragments modified with MseI and JOE dye-labeled EcoRI selective amplification primers. The JOE-labeled EcoRI fragments are displayed as peaks in the electropherogram.

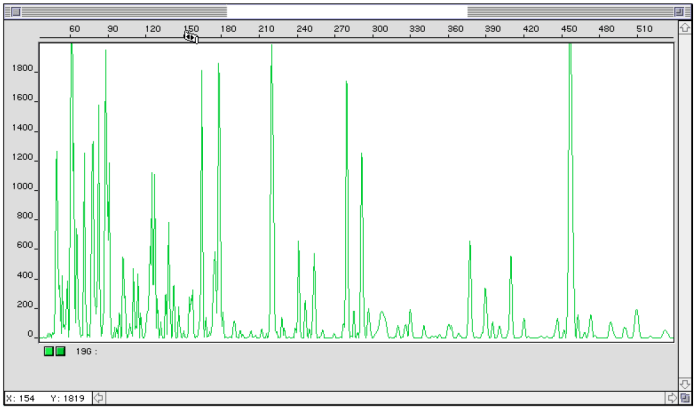


Figure 11 Electropherogram of AFLP samples run on an ABI 373

continued on next page

Evaluating ABI PRISM 377 DNA Sequencer Results

A representative electropherogram of fluorescent dye-labeled AFLP products run on an ABI PRISM 377 DNA Sequencer and analyzed using GeneScan analysis software is shown in Figure 12. The analyzed products are DNA fragments amplified with MseI and FAM dye-labeled EcoRI selective amplification primers. The FAM-labeled EcoRI fragments are displayed as peaks in the electropherogram.

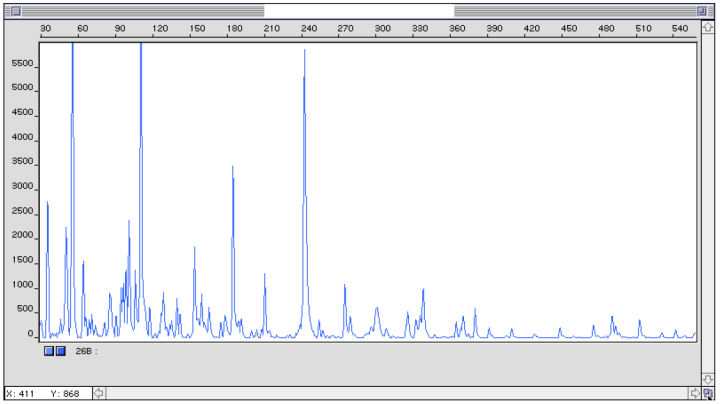
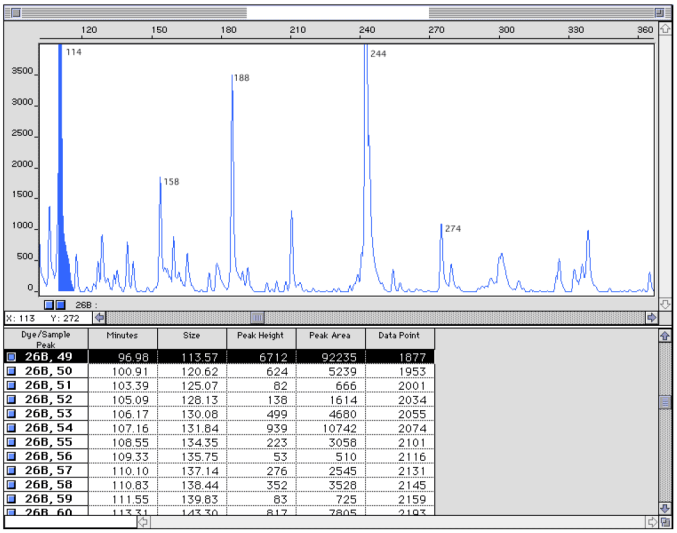


Figure 12 Electropherogram of AFLP sample run on an ABI PRISM 377

Figure 13 on page 31 shows an expanded electropherogram of select peaks from the same AFLP samples shown in Figure 12. Tabular data in Figure 13 shows the sizes of sample fragments in mobility units. All sample fragments were sized using the GeneScan-500 [ROX] size standard. Electropherogram data and tabular data were generated using GeneScan Analysis software version 2.0.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
26B, 49	96.98	113.57	6712	92235	1877
26B, 50	100.91	120.62	624	5239	1953
26B, 51	103.39	125.07	82	666	2001
26B, 52	105.09	128.13	138	1614	2034
26B, 53	106.17	130.08	499	4680	2055
26B, 54	107.16	131.84	939	10742	2074
26B, 55	108.55	134.35	223	3058	2101
26B, 56	109.33	135.75	53	510	2116
26B, 57	110.10	137.14	276	2545	2131
26B, 58	110.83	138.44	352	3528	2145
26B, 59	111.55	139.83	83	725	2159
26B, 60	113.31	143.30	817	7805	2193

Figure 13 Expanded electropherogram and size data for AFLP sample

Evaluating ABI PRISM 310 Genetic Analyzer Results

An electropherogram of *E. coli* W3110 Reference DNA run on an ABI PRISM 310 Genetic Analyzer is shown in Figure 14. The MseI-CA and FAM-labeled EcoRI-A selective primers were used. Note that the band pattern looks very similar to that shown in Figure 5 on page 6. There are slight differences because fragments size differently on the ABI PRISM 310 compared to the ABI 373 and ABI PRISM 377.

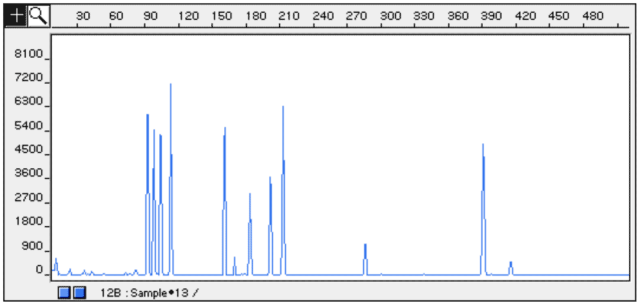


Figure 14 ABI PRISM 310 electropherogram of *E. coli* W3110 Reference DNA

Appendix A. Troubleshooting

Table 7. Troubleshooting AFLP Procedures

Observation	Possible Causes	Potential Solution
Unsuccessful amplification (faint or no peaks)	Incomplete restriction-ligation	Repeat restriction-ligation with fresh enzymes and buffer. Use an agarose gel to check.
	PCR inhibitors may exist in the DNA sample	Try different extraction procedures. Use an agarose gel to check.
	Insufficient or excess template DNA	Use recommended amount of template DNA. Use an agarose gel to check. If DNA is stored in water, check water purity.
	Insufficient enzyme activity	Use the recommended amount of restriction digestion enzyme, ligase, and AmpliTaq DNA Polymerase.
	$TE_{0.1}$ buffer not properly made, or contains too much EDTA	Add appropriate amount of $MgCl_2$ solution to amplification reaction. Remake the $TE_{0.1}$.
	Incorrect thermal cycling parameters	Check protocol for correct thermal cycling parameters.
	High salt concentrations of K^+, Na^+, or Mg^{2+}	Use correct amount of DNA and buffer. High salt and glycerol can inactivate restriction-ligation enzymes.
	Incorrect pH	Use correct amount of DNA and buffer.
	Tubes loose in the thermal cycler	Push reaction tubes firmly into contact with block before first cycle.
	Wrong style tube	Use Applied Biosystems GeneAmp Thin-Walled Reaction Tubes and DNA Thermal Cycler 480, or MicroAmp Reaction Tubes with Cap for the GeneAmp PCR System 9600 or System 2400.
	Primer concentration too low	Use recommended primer concentration.
	Ligase inactive	Check activity with control DNA.

Table 7. Troubleshooting AFLP Procedures *(continued)*

Observation	Possible Causes	Potential Solution
Inconsistent results with control DNA	Restriction incomplete	Repeat the restriction-ligation.
	Incorrect PCR thermal profile program	Choose correct temperature control parameters (refer to the *GeneAmp PCR System 9600 User's Manual*).
	GeneAmp PCR System 9600 misaligned lid	Align 9600 lid white stripes after twisting the top portion clockwise.
	For DNA Thermal Cycler 480, improper tube placement in block	Refer to the *DNA Thermal Cycler 480 User's Manual*.
	Pipetting errors	Calibrate pipettes, attach tips firmly, and check technique.
	Combined reagents not spun to bottom of tube	Place all reagents in apex of tube. Spin briefly after combining.
	Combined reagents left at room temperature or on ice for extended periods of time	Put tubes in block immediately after reagents are combined.
Extra peaks visible when sample is known to contain DNA from a single source	Contamination with exogenous DNA	Use appropriate techniques to avoid introducing foreign DNA during laboratory handling.
	Incomplete restriction or ligation	Extract the DNA again and repeat the restriction-ligation.
	Samples not denatured before loading in the autosampler	Make sure the samples are heated at 95 °C for three minutes prior to loading.
	Renaturation of denatured samples	Load sample immediately following denaturation, or store on ice until ready.
	Too much DNA in reaction, so that insufficient adaptor present	Use recommended amount of template DNA.
	Too much DNA amplified and/or loaded resulting in crossover between color channels	Re-run PCR using less DNA or load less sample during electrophoresis.

Table 7. Troubleshooting AFLP Procedures *(continued)*

Observation	Possible Causes	Potential Solution
Signal continually gets weaker	Outdated or mishandled reagents	Check expiration dates on reagents. Store and use according to manufacturers instructions. Compare with fresh reagents.
	Degraded primers	Store unused primers at –15 to –25 °C. Do not expose fluorescent dye-labeled primers to light for long periods of time.
Inconsistent sizing of known DNA sample	Inadvertent change in analysis parameters	Check settings for GeneScan analysis parameters.
	Change in size-calling method	Use same size-calling method.
	Incorrect internal standard	Use correct GeneScan size standard.
	Change in electrophoresis temperature	Check the Log for the record of the electrophoresis temperature.
Data was not automatically analyzed	Sample Sheet not completed	Complete Sample Sheet correctly.
Samples run faster than usual with decreased resolution	Incorrect buffer concentration	Check if buffer concentration matches protocol requirements.
	Incorrect run temperature	Check the Log for the record of the electrophoresis temperature.

Appendix B. References

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Appendix C. Related Reagents, Consumables, and Accessories

This appendix contains ordering information and descriptions of various reagents, kits, and consumables, which you can use to perform procedures described in this protocol.

Table 8. AFLP EcoRI selective amplification primers

Primer	Part Number	Primer	Part Number
EcoRI-0, FAM	402949, 500 µL	EcoRI-TC, FAM	402265, 250 µL
EcoRI-A, FAM	402950, 500 µL	EcoRI-TG, FAM	402264, 250 µL
EcoRI-C, NED	402952, 500 µL	EcoRI-TT, TAMRA	402266, 250 µL
EcoRI-G, JOE	402953, 500 µL	EcoRI-AAC, TAMRA	402039, 250 µL 402031, 500 µL
EcoRI-T, JOE	402951, 500 µL	EcoRI-AAG, JOE	402042, 250 µL 402034, 500 µL
EcoRI-AA, JOE	402271, 250 µL 402954, 500 µL	EcoRI-ACA, FAM	402038, 250 µL 402030, 500 µL
EcoRI-AC, FAM	402269, 250 µL 402956, 500 µL	EcoRI-ACC, TAMRA	402040, 250 µL 402032, 500 µL
EcoRI-AG, JOE	402268, 250 µL 402957, 500 µL	EcoRI-ACG, JOE	402044, 250 µL 402036, 500 µL
EcoRI-AT, NED	402955, 500 µL	EcoRI-ACT, FAM	402045, 250 µL 402037, 500 µL
EcoRI-AT, TAMRA	402270, 250 µL	EcoRI-AGC, TAMRA	402041, 250 µL 402033, 500 µL
EcoRI-TA, JOE	402267, 250 µL	EcoRI-AGG, JOE	402043, 250 µL 402035, 500 µL

Table 9. AFLP MseI selective amplification primers

Primer	Part Number	Primer	Part Number
MseI-0	402958, 500 µL	MseI-CAA	402021, 250 µL 402029, 500 µL
MseI-A	402959, 500 µL	MseI-CAC	402020, 250 µL 402028, 500 µL
MseI-C	402961, 500 µL	MseI-CAG	402019, 250 µL 402027, 500 µL
MseI-G	402962, 500 µL	MseI-CAT	402018, 250 µL 402026, 500 µL
MseI-T	402960, 500 µL	MseI-CTA	402017, 250 µL 402025, 500 µL
MseI-CA	402963, 500 µL	MseI-CTC	402016, 250 µL 402024, 500 µL
MseI-CC	402965, 500 µL	MseI-CTG	402015, 250 µL 402023, 500 µL
MseI-CG	402966, 500 µL	MseI-CTT	402014, 250 µL 402022, 500 µL
MseI-CT	402964, 500 µL		

Table 10. AFLP Plant Mapping Kit Modules

Module	Regular Plant Genomes (500–6000 Mb)	Small Plant Genomes (50–500 Mb)
Ligation and Preselective Amplification	P/N 402004	P/N 402273
Selective Amplification Start-Up	P/N 402006	P/N 402272

Table 11. Related consumables and accessories

Name	Description	Vendor
AFLP Protocol Reagents and Equipment		
AFLP Microbial Adaptor/Core Sequences	Consists of AFLP EcoRI and MseI adaptor pairs and core sequences	Applied Biosystems P/N 402943
E. coli W3110 DNA	Reference DNA	Applied Biosystems P/N 402990
T4 DNA ligase		New England Biolabs
T4 DNA ligase buffer		New England Biolabs
EcoRI restriction enzymes	Use higher concentration formulations of vendor-supplied enzymes	New England Biolabs
MseI restriction enzymes	Use higher concentration formulations of vendor-supplied enzymes	New England Biolabs
Bovine serum albumin (BSA)	Nuclease-free. Dilute 10 mg/mL solution supplied by vendor to 1.0 mg/mL	New England Biolabs
6% Pre-mixed polyacrylamide with 7.5 M urea in TBE buffer	Gel matrices for the ABI 373 DNA Sequencer	Amresco
LongRanger gel solutions	AT Biochem formulations. Used for the ABI PRISM 377 DNA Sequencer at 5% or 6% in TBE buffer	JT Baker P/N 4730-02 for 250 mL
Performance Optimized Polymer 4 (POP-4)	Polymer solution used with the ABI PRISM 310	Applied Biosystems P/N 402838
10X TBE buffer stock		Gibco
Deionized formamide		Applied Biosystems P/N 400596
Gel-loading pipette tips, 0.17 mm flat, for the ABI PRISM 377		Rainin P/N GT-1514

Table 11. Related consumables and accessories *(continued)*

Name	Description	Vendor
	Standards	
GeneScan-500 ROX size standard	Internal lane size standard labeled on a single strand with ROX NHS-ester dye. Shipped in two tubes containing 200 µL of material each. Sizes fragments between 35 and 500 bases	Applied Biosystems P/N 401734
Dye Primer Matrix Standard Kit	Although FAM, JOE, and ROX fluoresce at different wavelengths, there is some overlap in the emission spectra. To correct for this overlap (filter cross-talk), a mathematical matrix needs to be created and stored as a matrix file. When data is analyzed, the appropriate matrix is applied to the data to subtract out any emission overlap	Applied Biosystems P/N 401114
NED Matrix Standard	See above. NED substitutes for TAMRA as the yellow dye in the AFLP Microbial Fingerprinting Kit	Applied Biosystems P/N 402996

Appendix D. Technical Support

Contacting Technical Support

You can contact Applied Biosystems for technical support by telephone or fax, by e-mail, or through the Internet. You can order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents 24 hours a day. In addition, you can download documents in PDF format from the Applied Biosystems Web site (please see the section "To Obtain Documents on Demand" following the telephone information below).

To Contact Technical Support by E-Mail

Contact technical support by e-mail for help in the following product areas:

Product Area	E-mail address
Genetic Analysis (DNA Sequencing)	galab@appliedbiosystems.com
Sequence Detection Systems and PCR	pcrlab@appliedbiosystems.com
Protein Sequencing, Peptide and DNA Synthesis	corelab@appliedbiosystems.com
Biochromatography, PerSeptive DNA, PNA and Peptide Synthesis systems, CytoFluor®, FMAT™, Voyager™, and Mariner™ Mass Spectrometers	tsupport@appliedbiosystems.com
LC/MS (Applied Biosystems/MDS Sciex)	apisupport@sciex.com or api3-support@sciex.com
Chemiluminescence (Tropix)	tropix@appliedbiosystems.com

Hours for Telephone Technical Support

In the United States and Canada, technical support is available at the following times:

Product	Hours
Chemiluminescence	8:30 a.m. to 5:30 p.m. Eastern Time
Framingham support	8:00 a.m. to 6:00 p.m. Eastern Time
All Other Products	5:30 a.m. to 5:00 p.m. Pacific Time

To Contact Technical Support by Telephone or Fax

In North America

To contact Applied Biosystems Technical Support, use the telephone or fax numbers given below. (To open a service call for other support needs, or in case of an emergency, dial **1-800-831-6844** and press **1**.)

Product or Product Area	Telephone Dial...	Fax Dial...
ABI PRISM® 3700 DNA Analyzer	**1-800-831-6844,** then press **8**	**1-650-638-5981**
DNA Synthesis	**1-800-831-6844**, then press **21**	**1-650-638-5981**
Fluorescent DNA Sequencing	**1-800-831-6844**, then press **22**	**1-650-638-5981**
Fluorescent Fragment Analysis (includes GeneScan® applications)	**1-800-831-6844**, then press **23**	**1-650-638-5981**
Integrated Thermal Cyclers (ABI PRISM®877 and Catalyst 800 instruments)	**1-800-831-6844**, then press **24**	**1-650-638-5981**
ABI PRISM® 3100 Genetic Analyzer	**1-800-831-6844**, then press **26**	**1-650-638-5981**
BioInformatics (includes BioLIMS®, BioMerge™, and SQL GT™ applications)	**1-800-831-6844**, then press **25**	**1-505-982-7690**
Peptide Synthesis (433 and 43X Systems)	**1-800-831-6844**, then press **31**	**1-650-638-5981**
Protein Sequencing (Procise® Protein Sequencing Systems)	**1-800-831-6844**, then press **32**	**1-650-638-5981**
PCR and Sequence Detection	**1-800-762-4001**, then press **1** for PCR, **2** for the 7700 or 5700, **6** for the 6700 or dial **1-800-831-6844**, then press **5**	**1-240-453-4613**

Product or Product Area	Telephone Dial...	Fax Dial...
Voyager™ MALDI-TOF Biospectrometry and Mariner™ ESI-TOF Mass Spectrometry Workstations	**1-800-899-5858**, then press **13**	**1-508-383-7855**
Biochromatography (BioCAD® Workstations and Poros® Perfusion Chromatography Products)	**1-800-899-5858**, then press **14**	**1-508-383-7855**
Expedite™ Nucleic acid Synthesis Systems	**1-800-899-5858**, then press **15**	**1-508-383-7855**
Peptide Synthesis (Pioneer™ and 9050 Plus Peptide Synthesizers)	**1-800-899-5858**, then press **15**	**1-508-383-7855**
PNA Custom and Synthesis	**1-800-899-5858,** then press **15**	**1-508-383-7855**
FMAT™ 8100 HTS System and Cytofluor® 4000 Fluorescence Plate Reader	**1-800-899-5858**, then press **16**	**1-508-383-7855**
Chemiluminescence (Tropix)	**1-800-542-2369** (U.S. only), or **1-781-271-0045**	**1-781-275-8581**
Applied Biosystems/MDS Sciex	**1-800-952-4716**	**1-650-638-6223**

Outside North America

Region	Telephone Dial...	Fax Dial...
Africa and the Middle East		
Africa (English Speaking) and West Asia (Fairlands, South Africa)	**27 11 478 0411**	**27 11 478 0349**
South Africa (Johannesburg)	**27 11 478 0411**	**27 11 478 0349**
Middle Eastern Countries and North Africa (Monza, Italia)	**39 (0)39 8389 481**	**39 (0)39 8389 493**

Region	Telephone Dial...	Fax Dial...
Eastern Asia, China, Oceania		
Australia (Scoresby, Victoria)	**61 3 9730 8600**	**61 3 9730 8799**
China (Beijing)	**86 10 64106608**	**86 10 64106617**
Hong Kong	**852 2756 6928**	**852 2756 6968**
Korea (Seoul)	**82 2 593 6470/6471**	**82 2 593 6472**
Malaysia (Petaling Jaya)	**60 3 758 8268**	**60 3 754 9043**
Singapore	**65 896 2168**	**65 896 2147**
Taiwan (Taipei Hsien)	**886 2 2358 2838**	**886 2 2358 2839**
Thailand (Bangkok)	**66 2 719 6405**	**66 2 319 9788**
Europe		
Austria (Wien)	**43 (0)1 867 35 75 0**	**43 (0)1 867 35 75 11**
Belgium	**32 (0)2 712 5555**	**32 (0)2 712 5516**
Czech Republic and Slovakia (Praha)	**420 2 61 222 164**	**420 2 61 222 168**
Denmark (Naerum)	**45 45 58 60 00**	**45 45 58 60 01**
Finland (Espoo)	**358 (0)9 251 24 250**	**358 (0)9 251 24 243**
France (Paris)	**33 (0)1 69 59 85 85**	**33 (0)1 69 59 85 00**
Germany (Weiterstadt)	**49 (0) 6150 101 0**	**49 (0) 6150 101 101**
Hungary (Budapest)	**36 (0)1 270 8398**	**36 (0)1 270 8288**
Italy (Milano)	**39 (0)39 83891**	**39 (0)39 838 9492**
Norway (Oslo)	**47 23 12 06 05**	**47 23 12 05 75**
Poland, Lithuania, Latvia, and Estonia (Warszawa)	**48 (22) 866 40 10**	**48 (22) 866 40 20**
Portugal (Lisboa)	**351 (0)22 605 33 14**	**351 (0)22 605 33 15**
Russia (Moskva)	**7 095 935 8888**	**7 095 564 8787**
South East Europe (Zagreb, Croatia)	**385 1 34 91 927**	**385 1 34 91 840**
Spain (Tres Cantos)	**34 (0)91 806 1210**	**34 (0)91 806 1206**
Sweden (Stockholm)	**46 (0)8 619 4400**	**46 (0)8 619 4401**
Switzerland (Rotkreuz)	**41 (0)41 799 7777**	**41 (0)41 790 0676**
The Netherlands (Nieuwerkerk a/d IJssel)	**31 (0)180 331400**	**31 (0)180 331409**

Region	Telephone Dial...	Fax Dial...
United Kingdom (Warrington, Cheshire)	**44 (0)1925 825650**	**44 (0)1925 282502**
All other countries not listed (Warrington, UK)	**44 (0)1925 282481**	**44 (0)1925 282509**
Japan		
Japan (Hacchobori, Chuo-Ku, Tokyo)	**81 3 5566 6230**	**81 3 5566 6507**
Latin America		
Del.A. Obregon, Mexico	**305-670-4350**	**305-670-4349**

To Reach Technical Support Through the Internet

We strongly encourage you to visit our Web site for answers to frequently asked questions and for more information about our products. You can also order technical documents or an index of available documents and have them faxed or e-mailed to you through our site. The Applied Biosystems Web site address is

http://www.appliedbiosystems.com/techsupp

To submit technical questions from North America or Europe:

Step	Action
1	Access the Applied Biosystems Technical Support Web site.
2	Under the **Troubleshooting** heading, click **Support Request Forms**, then select the relevant support region for the product area of interest.
3	Enter the requested information and your question in the displayed form, then click **Ask Us RIGHT NOW** (blue button with yellow text).
4	Enter the required information in the next form (if you have not already done so), then click **Ask Us RIGHT NOW**. You will receive an e-mail reply to your question from one of our technical experts within 24 to 48 hours.

To Obtain Documents on Demand

Free, 24-hour access to Applied Biosystems technical documents, including MSDSs, is available by fax or e-mail or by download from our Web site.

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