AFLP® Plant Mapping

Protocol

Applied Biosystems

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P/N 4303146F

Contents

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 plant mapping include:

- establishing linkage groups in crosses
- saturating regions of introgression with markers for gene landing efforts
- assessing the degree of relatedness or variability among cultivars

Examples of AFLP fingerprints are shown in Figure 1 on page 2 and Figure 2 on page 3. Literature references for the AFLP technique are found in Appendix D on page 48.

You can build a genetic map of markers showing Mendelian inheritance from AFLP data such as that shown in Figure 1. The four electropherogram panels in Figure 1 contain data from tomato DNA samples prepared using the AFLP technique. Samples were run on an ABI™ 373 DNA Sequencer and the resulting data analyzed using GeneScan® Analysis software.

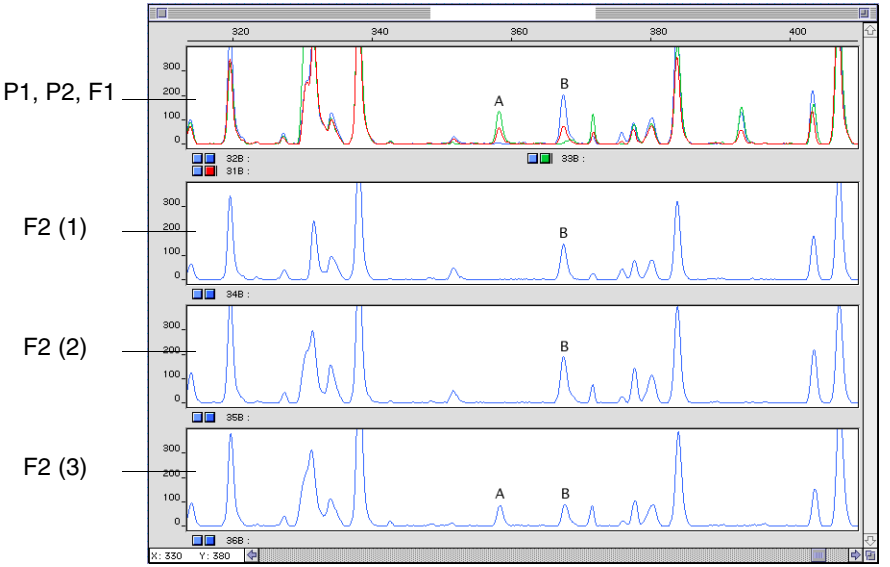
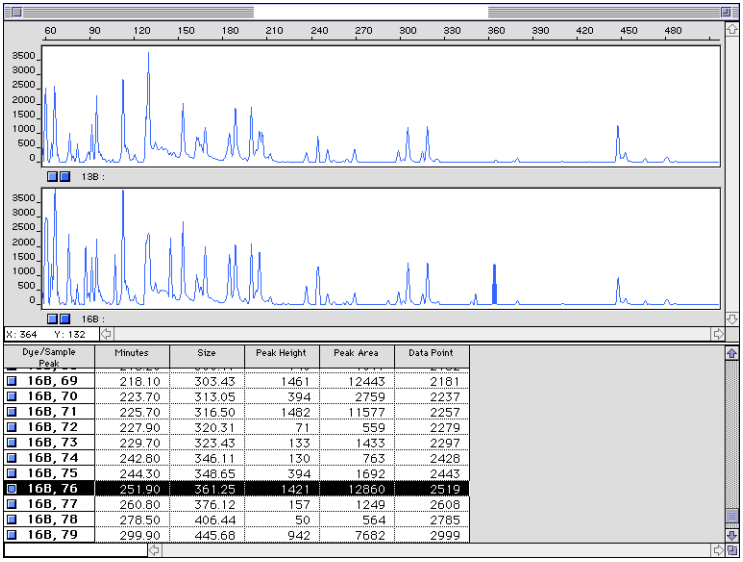


Figure 1 Tomato AFLP samples showing Mendelian segregation

The overlapping electropherograms in the top panel are AFLP results of sample DNA from three individuals: parent one (P1), parent two (P2), and F1 from a cross. A and B are the two significant peaks on this panel and appear only in P2 and F1.

The lower three electropherogram panels are AFLP results of sample DNA from three F2 generations. Peak A appears in F2 (3), but does not appear in either F2 (1), or F2 (2). Peak B is inherited in all three F2 individuals. The remaining non-polymorphic peaks appear in all three F2 electropherograms and show that the overall AFLP patterns are reproducible.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
16B, 69	218.10	303.43	1461	12443	2181
16B, 70	223.70	313.05	394	2759	2237
16B, 71	225.70	316.50	1482	11577	2257
16B, 72	227.90	320.31	71	559	2279
16B, 73	229.70	323.43	133	1433	2297
16B, 74	242.80	346.11	130	763	2428
16B, 75	244.30	348.65	394	1692	2443
16B, 76	251.90	361.25	1421	12860	2519
16B, 77	260.80	376.12	157	1249	2608
16B, 78	278.50	406.44	50	564	2785
16B, 79	299.90	445.68	942	7682	2999

Figure 2 Rice AFLP samples showing near-isogenic regions

The two electropherogram panels shown in Figure 2 contain data from rice DNA samples prepared using the AFLP technique. Samples were run on an ABI 373 DNA Sequencer and the resulting data analyzed using GeneScan Analysis software.

The rice DNA was isolated from near-isogenic lines (almost identical genetic material). It was selected for an introgressed region carrying a disease-resistance gene. By comparing peak patterns in the two electropherograms, you will find that the rice lines differ by only 1–2%. One of the peaks distinguishing the two lines has been highlighted in both the electropherogram display and the related tabular data beneath the electropherogram panels.

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). 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 take place in a single reaction. Ligation of the adaptor oligonucleotide to the restricted DNA does not regenerate the recognition site, so restriction does not recur after ligation (Figure 3).

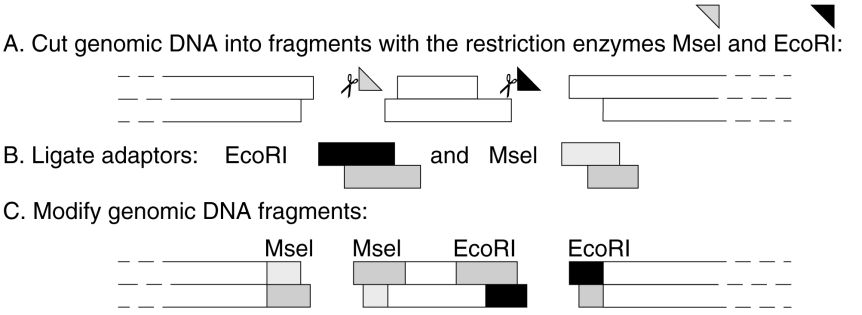


Figure 3 Template preparation and ligation of AFLP adaptors

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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.

The MseI complementary primer contains a 3´ C. The EcoRI complementary primer contains a 3´ A (Regular Plant Genome Kit modules) or no base addition (Small Plant Genome Kit modules).

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

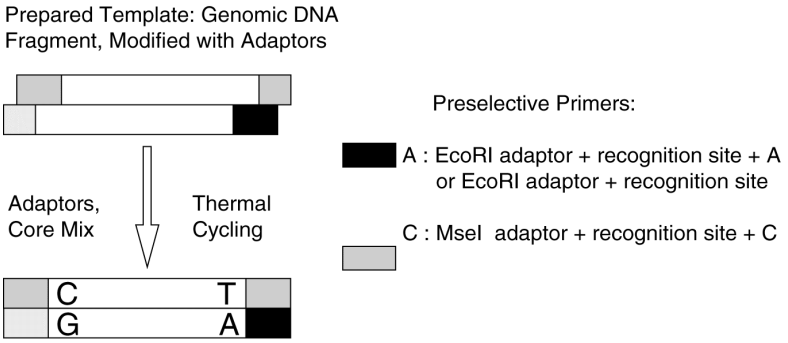

Figure 4 Preselective amplification of the prepared template

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Selective Amplification

Additional PCR amplifications are run to further reduce the complexity of the mixture so that it can be resolved on a polyacrylamide gel. These amplifications use primers chosen from the 24 available AFLP Selective Primers (eight MseI and sixteen EcoRI primers). After PCR amplification with these primers, a portion of each sample 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 5).

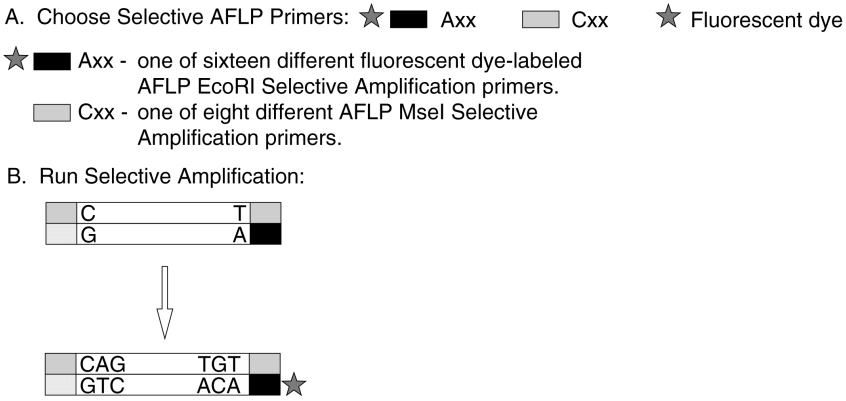

Figure 5 Selective amplification with fluorescent dye-labeled primers

Individual genomes yield distinctive restriction fragment profiles with each primer pair amplification. Those crop species genomes that have been analyzed successfully using MseI and EcoRI and the primers in this kit are shown in Table 7 on page 38.

continued on next page

Choosing Specific Primers for Amplification Screening

If you want to use a specific primer combination for the AFLP Selective Amplification reactions, you can order primer pairs in any combination of one EcoRI primer and one MseI primer. This gives you 128 possible primer pair combinations from which you can choose, for either regular or small plant genomes.

Order the AFLP Amplification Core Mix Module (P/N 402005) and the desired AFLP Selective Amplification Primers from Table 1.

Table 1. AFLP Selective Amplification Primers

EcoRI Primers, Regular Plant Genomes		
Primer	**Part Number (250 reactions)**	**Part Number (500 reactions)**
EcoRI-ACT FAM	402045	402037
EcoRI-ACA FAM	402038	402030
EcoRI-AAC NED	4303053	4303054
EcoRI-ACC NED	4303055	4303056
EcoRI-AGC NED	4303057	4303058
EcoRI-AAG JOE	402042	402034
EcoRI-AGG JOE	402043	402035
EcoRI-ACG JOE	402044	402036

EcoRI Primers, Small Plant Genomes	
Primer	**Part Number (250 reactions)**
EcoRI-TG FAM	402264
EcoRI-TC FAM	402265
EcoRI-AC FAM	402269
EcoRI-TT NED	4304352
EcoRI-AT NED	402955 (500 reactions)
EcoRI-TA JOE	402267
EcoRI-AG JOE	402268
EcoRI-AA JOE	402271

Table 1. AFLP Selective Amplification Primers *(continued)*

MseI Primers, Regular and Small Plant Genomes		
Primer	**Part Number (250 reactions)**	**Part Number (500 reactions)**
MseI-CAA	402021	402029
MseI-CAC	402020	402028
MseI-CAG	402019	402027
MseI-CAT	402018	402026
MseI-CTA	402017	402025
MseI-CTC	402016	402024
MseI-CTG	402015	402023
MseI-CTT	402014	402022

Testing New Genomes

If other genomes are to be tested, you need to be sure that they restrict appropriately with these enzymes. In general, the Regular Plant Genome Kit should produce quality genetic fingerprints with genomes of 5×10^8 to 6×10^9 base pairs, and the Small Plant Genome Kit with genomes of 5×10^7 to 5×10^8 base pairs.

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.

Fluorescent Dye-labeling and Marker Detection

Applied Biosystems has adapted the AFLP technique for use with our 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 in a fourth color in every lane or injection 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.

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Applied Biosystems web site address is:

http://www.appliedbiosystems.com/techsupport

We strongly encourage you to visit our web site for answers to frequently asked questions, and to learn more about our products. You can also order technical documents and/or an index of available documents and have them faxed or e-mailed to you through our site (see the "Documents on Demand" section below).

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Product	Hours
Chemiluminescence	9:00 a.m. to 5:00 p.m. Eastern Time
LC/MS	9:00 a.m. to 5:00 p.m. Pacific Time
All Other Products	5:30 a.m. to 5:00 p.m. Pacific Time

See the "Regional Offices Sales and Service" section below for how to contact local service representatives outside of the United States and Canada.

To Reach Us by Telephone or Fax in North America

Call Technical Support at 1-800-831-6844, and select the appropriate option (below) for support on the product of your choice at any time during the call. (To open a service call for other support needs, or in case of an emergency, press **1** after dialing 1-800-831-6844.)

For Support On This Product	Dial 1-800-831-6844, and...
ABI PRISM® 3700 DNA Analyzer	Press: **8** / FAX: 650-638-5981
ABI PRISM® 3100 Genetic Analyzer	Press: **26** / FAX: 650-638-5891
DNA Synthesis	Press: **21** / FAX: 650-638-5981

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<tr><th>For Support On This Product</th><th>Dial 1-800-831-6844, and...</th></tr>
<tr><td>Fluorescent DNA Sequencing</td><td><table><tr><th>Press</th><th>FAX</th></tr><tr><td>22</td><td>650-638-5891</td></tr></table></td></tr>
<tr><td>Fluorescent Fragment Analysis (includes GeneScan® applications)</td><td><table><tr><th>Press</th><th>FAX</th></tr><tr><td>23</td><td>650-638-5891</td></tr></table></td></tr>
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<tr><td>PCR and Sequence Detection</td><td><table><tr><th>Press</th><th>FAX</th></tr><tr><td>5, or call
1-800-762-4001, and press 1 for PCR, or 2 for Sequence Detection</td><td>240-453-4613</td></tr></table></td></tr>
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<tr><td>Protein Sequencing</td><td><table><tr><th>Press</th><th>FAX</th></tr><tr><td>32</td><td>650-638-5981</td></tr></table></td></tr>
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1-781-271-0045 (Tropix)</td><td>781-275-8581 (Tropix)
9:00 a.m. to 5:00 p.m. ET</td></tr></table></td></tr>
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by phone from outside the United States or Canada	a. Dial your international access code, then 1-858-712-0317, from a touch-tone phone. Have your complete fax number and country code ready (011 precedes the country code). b. Press **1** to order an index of available documents and have it faxed to you. Each document in the index has an ID number. (Use this as your order number in step “d” below.) c. Call 1-858-712-0317 from a touch-tone phone a second time. d. Press **2** to order up to five documents and have them faxed to you.

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Denmark (Naerum) Tel: 45 45 58 60 00 Fax: 45 45 58 60 01	Norway (Oslo) Tel: 47 23 12 06 05 Fax: 47 23 12 05 75

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Spain (Tres Cantos) Tel: 34 (0)91 806 1210 Fax: 34 (0)91 806 1206	South Africa (Johannesburg) Tel: 27 11 478 0411 Fax: 27 11 478 0349
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Korea (Seoul) Tel: 82 2 593 6470/6471 Fax: 82 2 593 6472	Thailand (Bangkok) Tel: 66 2 719 6405 Fax: 66 2 319 9788

What You Will Need to Perform AFLP

Overview

You will need the following:

- DNA—from 0.05–0.5 µg of good quality DNA, depending on the genome size. The plant mapping kits are optimized for small genomes of 50–500 Mb and medium (regular) genomes of 500–6000 Mb.
- AFLP Kit Modules and materials as specified on pages 7–8, 16–19, and in Appendix E, “Related Consumables and Accessories,” on page 50.

AFLP Kit Modules

The organization of the AFLP Plant Mapping Kit into individual modules allows for maximum flexibility. You can purchase individual modules separately depending on your research goals, as shown in Table 2.

Table 2. What to Order

Module	Regular Plant Genomes (500–6000 Mb)	Small Plant Genomes (50–500 Mb)
Ligation and Preselective Amplification	P/N 402004	P/N 402273
Amplification Core Mix	P/N 402005	P/N 402005
Selective Amplification Start-Up	P/N 4303050 or Individual primer pairs (one MseI and one EcoRI) that you select. See Table 1 on pages 7–8.	P/N 4303051 or Individual primer pairs (one MseI and one EcoRI) that you select. See Table 1 on pages 7–8.

The AFLP Ligation and Preselective Amplification Module contains sufficient reagents to prepare an initial mapping population of up to 100 individuals. For the testing of each additional 100 individuals in a population, you must use a new AFLP Ligation and Preselective Amplification Module.

The AFLP Amplification Core Mix Module supplies sufficient PCR mix to perform 500 individual AFLP reactions.

The AFLP Selective Amplification Start-Up Module supplies sufficient quantities of primers to test all 64 possible primer combinations on 30 individuals chosen from the 100 individuals prepared with the AFLP Ligation and Preselective Amplification Module.

For each primer combination you can compare:

- the total number of peaks amplified in the parents
- the number of polymorphic peaks between the parents
- the segregation ratios of polymorphic peaks in progeny of the cross

Once you establish the most useful primer combinations for your samples, you can purchase 250 or 500 reactions of primer along with the AFLP Amplification Core Mix Module. The Core Mix Module contains the necessary reagents for performing PCR.

The primer combination tables in Appendix A on page 38 show primer combinations best suited for analysis of ten different major crop species. You can order these primers separately (see pages 7–8).

AFLP Ligation and Preselective Amplification Module

Template preparation and preselective amplification require use of the AFLP Ligation and Preselective Amplification Module:

- Regular Plant Genomes (500–6000 Mb), P/N 402004
- Small Plant Genomes (50–500 Mb), P/N 402273

This module contains the following five tubes:

- Adaptor pairs that allow you to perform the ligation reactions during preparation of your genomic DNA template:
  - one tube of EcoRI adaptor pairs
  - one tube of MseI adaptor pairs
- Preselective primers, one tube
- Preselective Amplification mix (buffer, dNTPs, $MgCl_2$, and enzyme) necessary to perform the Preselective PCR amplification reactions, one tube
- AFLP Reference DNA you can use for a control, one tube

Sufficient reagents are supplied to perform up to 100 of each of these reactions. See “Preparing Enzyme Master Mix” on page 21 for the reagents needed for ligation and preselective amplification.

continued on next page

AFLP Amplification Core Mix Module

The AFLP Amplification Core Mix Module contains all of the components necessary to amplify modified target sequences. This module contains five tubes of Core Mix containing buffer, nucleotides, and AmpliTaq® DNA polymerase.

The Core Mix Module contains sufficient reagents for 500 amplification reactions of target genomic sequences. You determine how the selection occurs by choosing primer pairs from the AFLP Selective Amplification Start-Up Module or pairs of individually sold primers.

AFLP Selective Amplification Start-Up Module

To screen primer combinations, use the AFLP Selective Amplification Start-Up Module (Regular Plant Genomes, P/N 4303050; Small Plant Genomes, P/N 4303051) with the Core Mix Module.

Each AFLP Selective Amplification Start-Up Module contains 16 oligonucleotide primers (Table 1 on page 7). This provides you with 64 possible combinations of primer pairs that you can use in 30 reactions each for a maximum of 2000 Selective Amplification reactions.

- Eight of the primers are complementary to the MseI adaptor sequence and have three additional bases at the 3´ end.
- Eight of the primers are complementary to the EcoRI adaptor sequence. They have two (P/N 4303051) or three (P/N 4303050) additional bases at the 3´ end and have 5´ fluorescent dyes. The primers are labeled with FAM (blue), JOE (green), or NED (yellow).

Note Use a fourth color, red (ROX), for an internal size standard such as the GeneScan-500 ROX Size Standard, available from Applied Biosystems (P/N 401734).

Once you determine optimal primer combinations, you can purchase larger quantities (250 or 500 reaction equivalents) of specific primer combinations for testing of additional DNA samples.

Storage and Stability of Kit Components

Store all kit components at –15 to –25 °C in a non-frost-free freezer. If stored properly, the kit components will last 1 year from the time of receipt.

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Materials Required But Not Supplied

Reagents (see Appendix E on page 50 for more information)

- Nuclease-free distilled deionized water
- EcoRI restriction endonuclease, 500 Units (“high concentration” grade)
- MseI restriction endonuclease, 100 Units (“high concentration” grade)
- T4 DNA Ligase, 100 Units (“high concentration” grade)
- 10X T4 DNA ligase buffer containing ATP
- 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, for the ABI PRISM 310 Genetic Analyzer)
- Deionized formamide
- GeneScan-500 ROX Size Standard
- DNA size markers (*e.g.*, Boehringer Mannheim Set VI)
- Dye Primer Matrix Standard Kit
- NED Matrix Standard (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 (for the ABI PRISM 377)
- Applied Biosystems thermal cycler
- Sterile 0.5-mL microcentrifuge tubes
- Sterile 0.2-mL MicroAmp® Thin-Walled Reaction Tubes and caps (for the GeneAmp® PCR Instrument Systems 2400 and 9600)
- Sterile Thin-Walled MicroAmp 0.5-mL Reaction Tubes (for the DNA Thermal Cycler 480)

AFLP Plant Mapping Protocol

Before Starting an AFLP Experiment

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

Step	Action
1	Digest 1–3 µg of genomic 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.
4	View on a UV transilluminator. For an example of what a successful digest looks like, see Figure 6 on page 24 (left half).

Preparing Samples for PCR Amplification

IMPORTANT Before you prepare your samples, we strongly recommend that you run a control DNA reaction to verify that restriction, ligation, and amplification yield the expected products. A control DNA is supplied in the AFLP Ligation and Preselective Amplification Module (P/N 402004 for Regular Plant Genomes and 402273 for Small Plant Genomes) for this purpose.

To prepare samples for the AFLP Preselective Amplification and AFLP 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

Annealing Adaptor Pairs

You must anneal the adaptor pairs supplied with the AFLP Ligation and Preselective Amplification module before you can use them for the restriction-ligation reactions.

Step	Action
1	From the AFLP Ligation and Preselective Amplification Module, remove the tubes labeled MseI Adaptor Pair and EcoRI Adaptor Pair.
2	Heat tubes in a water bath at 95 °C for 5 minutes.
3	Allow tubes to cool to room temperature over a 10-minute period.
4	Spin in a microcentrifuge for 10 seconds at 1400 × *g* (maximum).

Preparing 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 ♦ 5 µL 1 mg/mL BSA (diluted from 10 mg/mL stock) ♦ 100 Units MseI ♦ 500 Units EcoRI ♦ 100 Weiss Units T4 DNA Ligase (or 6700 cohesive end ligation units) **IMPORTANT** Use high concentration preparations of the enzymes to avoid exceeding 5% glycerol in the reactions.
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 1–2 hours. Do not store the Enzyme 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.

Preparing 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: ♦ 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	Add DNA as follows:	
	Regular Plant Genomes	Add 0.5 µg genomic DNA in 5.5 µL sterile distilled water.
	Small Plant Genomes	Add 0.05 µg genomic DNA in 5.5 µL sterile distilled water.
	Control Reactions	Add 5.5 µL of control DNA (0.1 µg/µL) from the AFLP Ligation and Preselective Amplification Module.
3	Mix thoroughly, then place in a microcentrifuge for 10 seconds.	
4	Incubate at room temperature overnight, or for 2 hours at 37 °C. For incubation at 37 °C, use a thermal cycler with a heated cover, so that the evaporation does not lead to EcoRI* (star) activity. Be careful that the volume of enzyme added does not cause the amount of glycerol to be >5%, which also leads to EcoRI* activity.	

Diluting 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. **Note** Store the mixture at 2–6 °C for up to 1 month, or at –15 to –25 °C for longer than 1 month.

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 ramp times included in this protocol ensure identical products from any Applied Biosystems thermal cycler. Ramp time is crucial. See Appendix B on page 44 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 ♦ 1.0 µL AFLP preselective primer pairs ♦ 15.0 µL AFLP 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 following PCR method, entering all ramp times as 0.01 (1 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 after amplification.

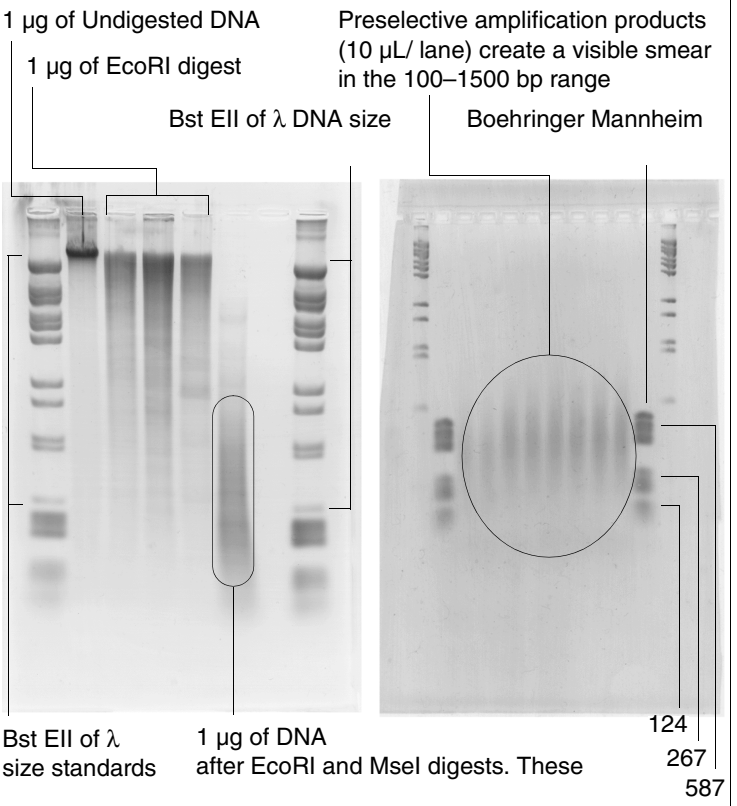
Table 3. Thermal cycler parameters for preselective amplification

HOLD	CYCLE			HOLD	HOLD
	Each of 20 Cycles				
72 °C 2 min.	94 °C 20 sec.	56 °C 30 sec.	72 °C 2 min.	60 °C 30 min.	4 °C (forever)

continued on next page

Verifying Successful Amplification

Run an agarose gel to see 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

Preparing 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 reaction product ♦ 190.0 µL $TE_{0.1}$ buffer
2	Mix thoroughly, then spin down in a microcentrifuge for 10 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 reaction tube (0.2-mL for the GeneAmp PCR System 9600 or 2400, 0.5-mL for the DNA Thermal Cycler 480): ♦ 3.0 µL diluted preselective amplification reaction product ♦ 1.0 µL MseI[Primer–Cxx] at 5 µM ♦ 1.0 µL EcoRI[Dye–primer–Axx] at 1 µM ♦ 15.0 µL AFLP Core 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 26. **Note** For the GeneAmp PCR System 9600 and DNA Thermal Cycler 480, enter all ramp times as 0.01 (1 second). For the GeneAmp PCR System 2400, enter all ramp times as 90%.
3	Store at 2–6 °C after amplification.

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.

Run Modules

The ABI 373 DNA Sequencer uses Filter Set A. The ABI PRISM 377 DNA Sequencer and ABI PRISM 310 Genetic Analyzer use Virtual Filter Set F.

For the ABI PRISM 377, Filter Set F module files can be obtained from the Applied Biosystems World Wide Web site as part of the ABI PRISM 377 Collection software version 2.1:

- www.appliedbiosystems.com/techsupport
  ABI_PRISM_377_v2.1.image.hqx

For the ABI PRISM 310, Filter Set F module files will be part of the next release of the ABI PRISM 310 Collection software (version 1.0.4).

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/25 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 1 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	**On the ABI 373 DNA Sequencer:**	**On the ABI PRISM 377 DNA Sequencer:**
	Add 2.5 µL of the loading buffer mix to a MicroAmp PCR tube for each sample.	Add 1.2 µL of the loading buffer mix to a MicroAmp PCR tube for each sample.
2	Add 0.8 µL of selective amplification product to the tube. **Note** To run multiple reactions in one lane, add 0.8 µL of each reaction.	Add 0.4 µL of selective amplification product to the tube. **Note** To run multiple reactions in one lane, add 0.4 µL of each reaction.
3	Heat tubes to 95 °C for 3 minutes.	Heat tubes to 95 °C for 3 minutes.
4	Quick-chill on ice.	Quick-chill on ice.
5	Load 2.5–4 µL of the sample onto a 6% denaturing polyacrylamide gel using 1X TBE running buffer.	Load the entire sample onto a 5% denaturing Long Ranger gel using 1X TBE running buffer.

IMPORTANT Use Filter Set A with the ABI 373 and Filter Set F with the ABI PRISM 377 DNA Sequencer when analyzing samples prepared with the AFLP Plant Mapping Kit modules (see "Run Modules" on page 27). 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 1 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 F run module and ABI PRISM 310 Genetic Analyzer Collection Software, version 1.0.2 or higher, with the AFLP Plant Mapping Kit modules (see "Run Modules" on page 27). 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[b]	15

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

b. Note the decrease in run time.

continued on next page

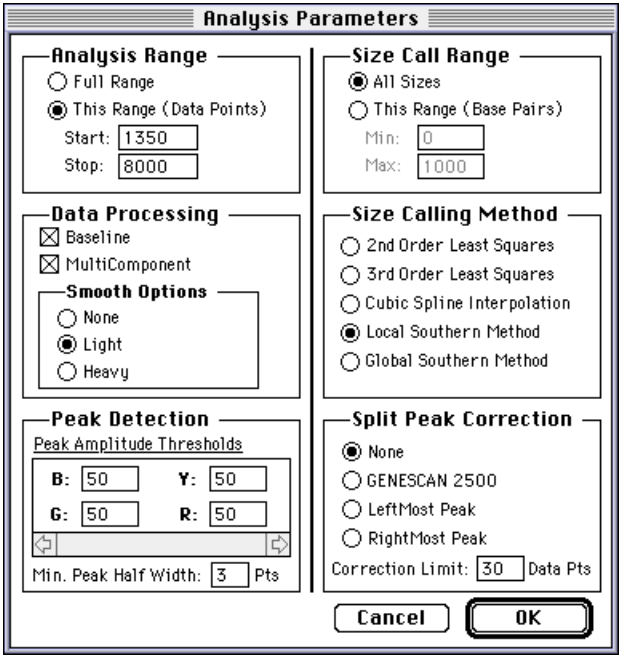
Using GeneScan to Analyze Results

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 for the ABI 373 and ABI PRISM 377 as shown below. On the ABI PRISM 310, use an analysis range of 2600–10000 data points and peak amplitude thresholds of 100.



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 7 on page 32).

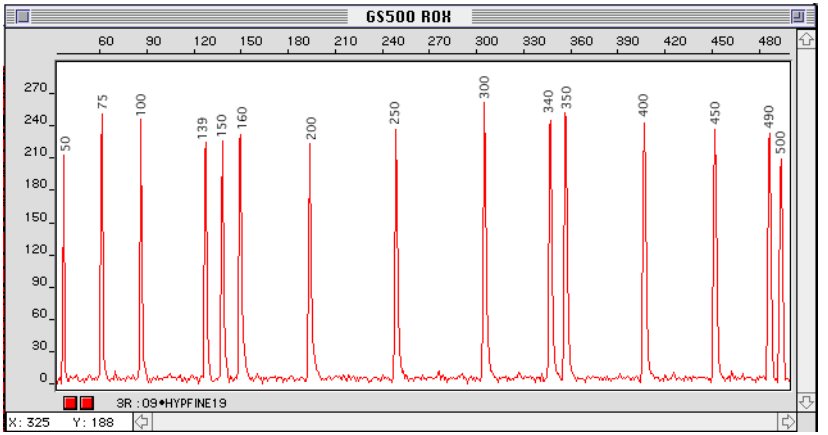


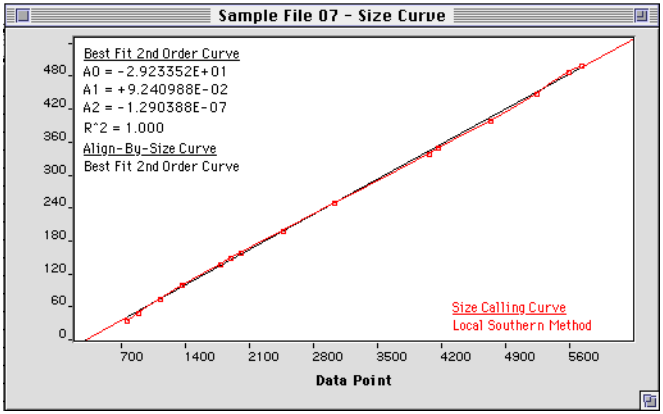
Figure 7 Electropherogram of GeneScan-500 run under denaturing conditions

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 window. To select several consecutive samples, shift-click the first and last sample in the group you wish to select.
2	Choose Size Curve from the Sample menu. The Standard Sizing Curve window appears.  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 8 shows the polymorphic peak patterns from a GeneScan analysis of two AFLP samples. Polymorphic peaks are labeled with size and origin.

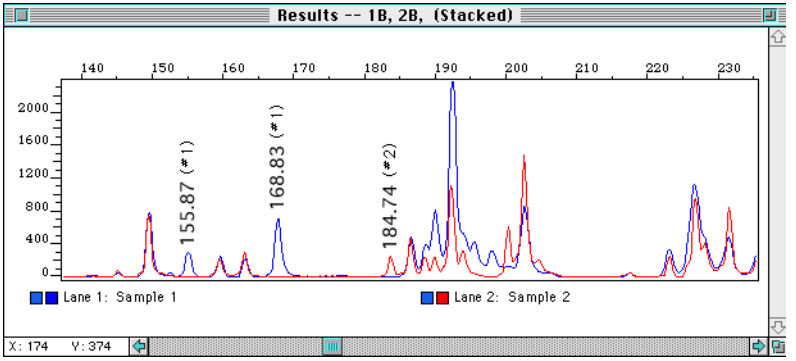


Figure 8 Overlapping electropherograms for two AFLP samples

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 9 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.

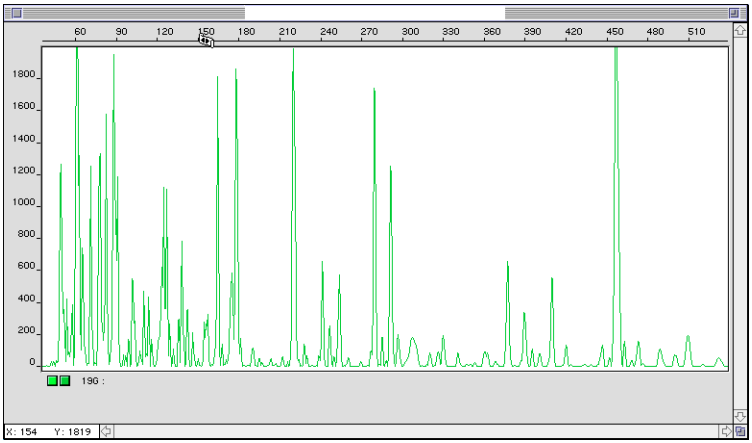


Figure 9 Electropherogram of AFLP sample run on an ABI 373 DNA Sequencer

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 10. 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.

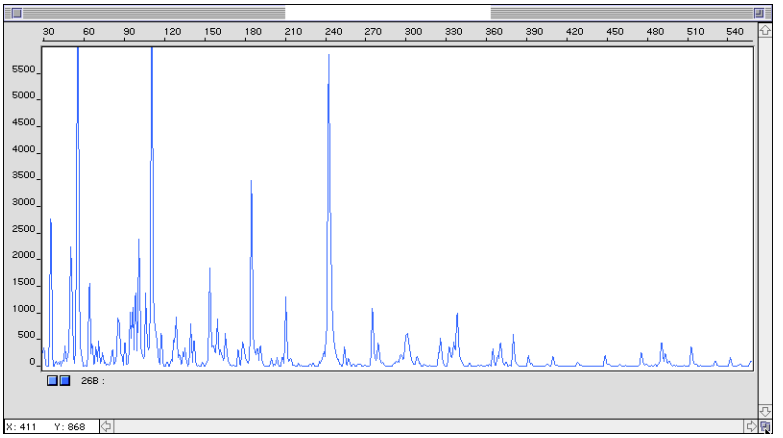
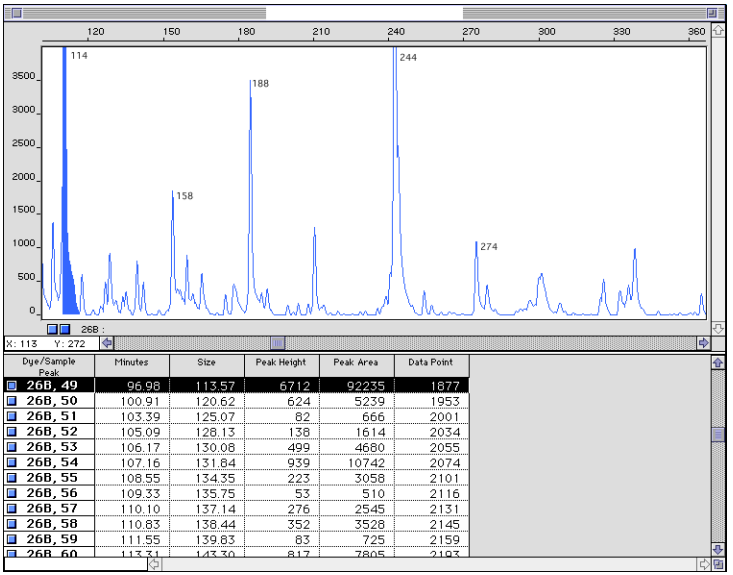


Figure 10 Electropherogram of AFLP sample run on an ABI PRISM 377 DNA Sequencer

Figure 11 on page 37 shows an expanded electropherogram of select peaks from the same AFLP samples shown in Figure 10. Tabular data in Figure 11 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 11 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 12. The MseI-CA and FAM-labeled EcoRI-A selective primers from the AFLP Microbial Fingerprinting Kit (P/N 402948) were used.

Note There are slight differences in fragment sizes on the ABI PRISM 310 compared to the ABI 373 and ABI PRISM 377.

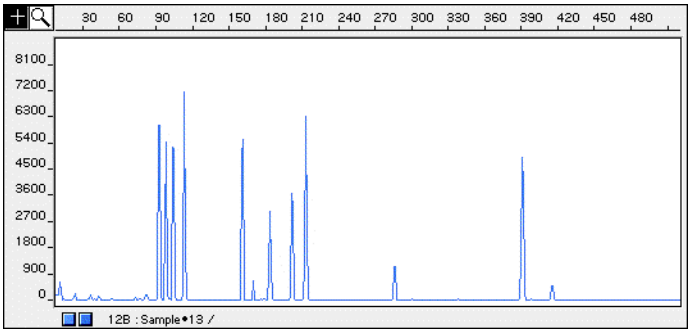


Figure 12 ABI PRISM 310 electropherogram of *E. coli* W3110 Reference DNA

Appendix A. Primer Combination Tables

Genomes Analyzed Using AFLP

Ten different crop species genomes were analyzed using the AFLP technique. For each crop species, primer combinations that produce the best DNA fingerprints were determined.

The names of each crop species tested and corresponding primer combination tables are given in Table 7. Those combinations of EcoRI and MseI Selective Amplification primers that are best suited for amplification screening of the designated crop genomes are shown in Table 8 through Table 17.

Table 7. Primer combination tables for crop species

Crop Species	Primer Combination Table
Regular Plant Genomes	
Sunflower	Table 8 on page 39
Pepper	Table 9 on page 39
Barley	Table 10 on page 40
Maize	Table 11 on page 40
Sugar beet	Table 12 on page 41
Tomato	Table 13 on page 41
Lettuce	Table 14 on page 42
Small Plant Genomes	
Arabidopsis	Table 15 on page 42
Cucumber	Table 16 on page 43
Rice	Table 17 on page 43

The following symbol indicates **unacceptable** primer combinations for amplification screening of designated species:

Table 8. Primer combinations for Sunflower species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC		⊘						
-AAG	⊘				⊘			
-ACA	⊘			⊘	⊘			
-ACC								
-ACG		⊘			⊘			
-ACT								
-AGC						⊘		
-AGG						⊘		

Table 9. Primer combinations for Pepper species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC			⊘	⊘		⊘		⊘
-AAG				⊘				
-ACA				⊘				
-ACC								⊘
-ACG								
-ACT	⊘							
-AGC								
-AGG							⊘	

Table 10. Primer combinations for Barley species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC								
-AAG			⊘	⊘				
-ACA								
-ACC								
-ACG								
-ACT			⊘					
-AGC						⊘		
-AGG								

Table 11. Primer combinations for Maize species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC	⊘							
-AAG					⊘			
-ACA	⊘							
-ACC								
-ACG						⊘		⊘
-ACT		⊘		⊘			⊘	⊘
-AGC								⊘
-AGG								

Table 12. Primer combinations for Sugar beet species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC								
-AAG								⊘
-ACA								
-ACC								
-ACG								
-ACT	⊘	⊘						
-AGC								
-AGG								

Table 13. Primer combinations for Tomato species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC								
-AAG				⊘			⊘	
-ACA								
-ACC								
-ACG								
-ACT								
-AGC								
-AGG								

Table 14. Primer combinations for Lettuce species

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AAC								
-AAG				⊘			⊘	
-ACA								
-ACC								
-ACG				⊘				⊘
-ACT								
-AGC								
-AGG				⊘				

Table 15. Primer combinations for Arabidopsis species (small genome)

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AA								
-AC								
-AG								
-AT	⊘							
-TA		⊘						
-TC								⊘
-TG								
-TT								

Table 16. Primer combinations for Cucumber species (small genome)

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AA							⊘	
-AC	⊘							
-AG								
-AT								
-TA	NOT DETERMINED							
-TC								
-TG								
-TT								

Table 17. Primer combinations for Rice species (small genome)

EcoRI Primers \ MseI Primers	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AA								
-AC								
-AG								
-AT								
-TA								
-TC								
-TG								
-TT								

Appendix B. Troubleshooting

Table 18. 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 18. 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 3 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 18. 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 C. Preparing Plant Genomic DNA

While the AFLP technique does not require as much genomic DNA as the RFLP technique, the quality of the DNA is very important. In particular, the DNA must first be restricted to completion with enzymes and then ligated to adaptors before the AFLP reactions are performed. This appendix supplies references for extraction and quantification methods for preparing genomic plant DNA.

DNA Extraction Techniques

Any particular plant species presents unique extraction problems, so it is up to researchers to optimize a DNA extraction technique for their system. Our scientists and those in many other labs have had excellent results using the various CTAB purification schemes (Doyle and Doyle, 1990).

For individual systems, journals such as *Biotechniques* contain numerous reports detailing modifications that improve the quality and or quantity of purified DNA in various species including cotton and pine (*e.g.*, Baker *et al.*, 1990).

Quantitating DNA

Refer to molecular biology manuals such as *Current Protocols in Molecular Biology for information* on:

- Quantitating the DNA, restriction digestion procedures
- Pouring and loading gels
- Running and interpretation of agarose gels

Another good source of general information is *Molecular Cloning: A Laboratory Manual.* See Appendix D on page 48 for specific references.

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Appendix E. Related Consumables and Accessories

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

Table 19. Related consumables and accessories

Name	Description	Vendor
AFLP Protocol Reagents and Equipment		
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
ABI PRISM 310 10X Genetic Analyzer Buffer with EDTA		Applied Biosystems P/N 402824
ABI PRISM 310 Genetic Analyzer Capillary	L_t = 47 cm, L_d = 36 cm, i.d. = 50 µm, labeled with a green mark	Applied Biosystems P/N 402839
10X TBE buffer stock		Gibco

Table 19. Related consumables and accessories *(continued)*

Name	Description	Vendor
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
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 Plant Mapping Kit	Applied Biosystems P/N 402996

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