Invitrogen™
life technologies

Instruction Manual

AFLP® Analysis System I
AFLP® Starter Primer Kit

Catalog nos. 10544-013 and 10483-014

Version B

July 30, 2003

50512

Table of Contents

This page is intentionally left blank.

Notices to Customer

1

1.1 Important Information

This product is authorized for laboratory research use only. The product has not been qualified or found safe and effective for any human or animal diagnostic or therapeutic application. Uses for other than the labeled intended use may be a violation of applicable law.

1.2 Precautions

Warning: This product contains hazardous reagents. It is the end-user's responsibility to consult the applicable MSDS(s) before using this product. Disposal of waste organics, acids, bases, and radioactive materials must comply with all appropriate federal, state, and local regulations.

1.3 Limited Label Licenses

Limited Label License No. 4: Products for PCR that include no rights to perform PCR

This product is optimized for use in the Polymerase Chain Reaction (PCR) covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche, Ltd. ("Roche"). No license under these patents to use the PCR process is conveyed expressly or by implication to the purchaser by the purchase of this product. A license to use the PCR process for certain research and development activities accompanies the purchase of certain reagents from licensed suppliers such as Invitrogen, when used in conjunction with an Authorized Thermal Cycler, or is available from Applied Biosystems. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

Limited Label License No. 17: AFLP® Products

The AFLP® technique is covered by patents or patented applications owned by Keygene n.v and this product is sold under license from Keygene n.v. This kit may be used for research purposes only. For use of this kit in plant breeding, contact the Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500. The use of this kit for any other purpose, including but not limited to the use for clinical, diagnostic, and/or therapeutic purposes; or for providing services to third parties, requires a license from Keygene n.v., P.O. Box 216, 6700 AE Wageningen, The Netherlands.

2 Overview

2.1 Principles of AFLP®

Amplified Restriction Fragment Polymorphism (AFLP) technology is a technique for fingerprinting genomic DNA (1,2). DNA fingerprinting is used to visualize DNA polymorphisms between samples. These fingerprints may be used as a tool for determining the identity of a specific DNA sample or to assess the relatedness between samples. Fingerprints are also used as the source for genetic markers to generate linkage maps or to identify molecular markers linked to phenotypic traits and/or genetic loci.

Many DNA fingerprinting techniques have been developed in the past few years and are generally based on one of two strategies:

- ***Classical, hybridization-based fingerprinting.*** Involves the cutting of genomic DNA with restriction endonucleases followed by electrophoretic separation of the DNA fragments. Restriction Fragment Length Polymorphisms (RFLPs) are detected by Southern hybridization with probes targeted to hypervariable regions of the DNA (3–6).
- ***PCR-based fingerprinting.*** Involves the *in vitro* amplification of particular DNA sequences using specific or arbitrary primers and a thermostable polymerase. Amplification products are separated by electrophoresis and detected by staining or use of labeled primers. Techniques in this category include Random Amplified Polymorphic DNA (RAPD) (7), DNA Amplification Fingerprinting (DAF) (8), and Arbitrarily Primed-PCR (AP-PCR) (9).

AFLP® technology is a DNA fingerprinting technique that combines both of these strategies. It is based on the selective amplification of a subset of genomic restriction fragments using PCR (figure 1). DNA is digested with restriction endonucleases, and double-stranded DNA adapters are ligated to the ends of the DNA fragments to generate template DNA for amplification. Thus, the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. Selective nucleotides extending into the restriction fragments are added to the 3´ ends of the PCR primers such that only a subset of the restriction fragments are recognized. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified. The subset of amplified fragments are then analyzed by denaturing polyacrylamide gel electrophoresis to generate the fingerprint.

When the DNA fingerprints of related samples are compared, common bands, as well as different bands will be observed. These differences, referred to as DNA polymorphisms, are observed in an otherwise identical fingerprint. Polymorphisms detected in DNA fingerprints obtained by restriction cleavage can result from alterations in the DNA sequence including mutations abolishing or creating a restriction site, and insertions, deletions, or inversions between two restriction sites. The DNA polymorphisms identified using AFLP® are typically inherited in Mendelian fashion and may therefore be used for typing, identification of molecular markers, and mapping of genetic loci.

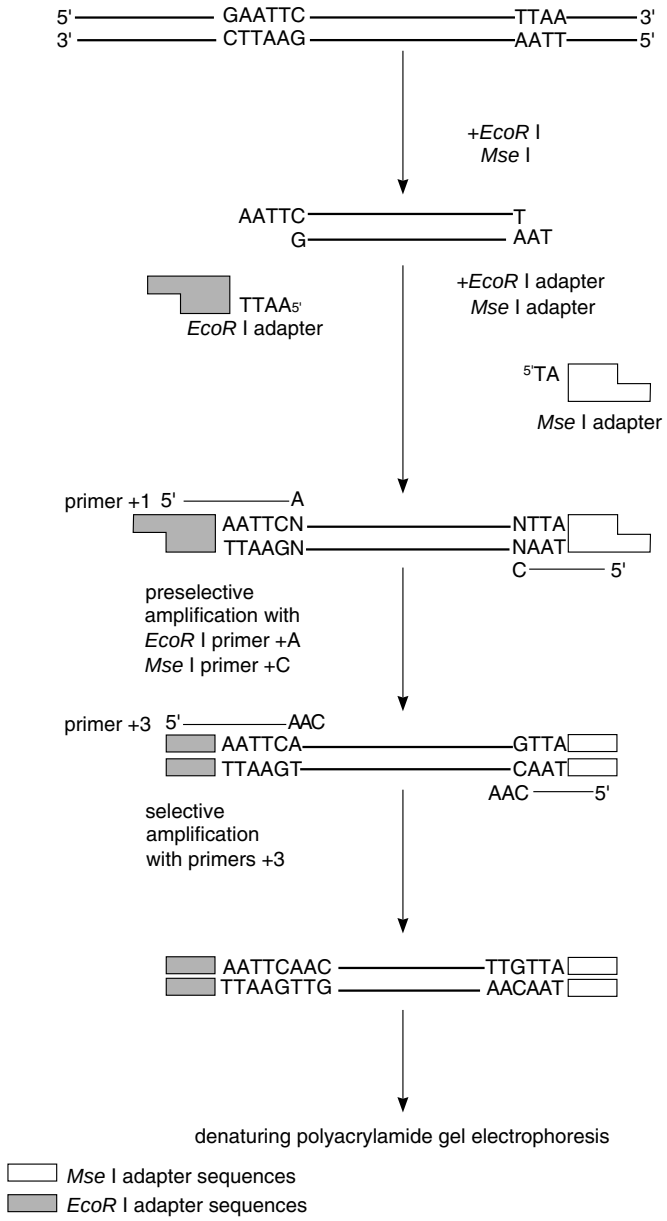

Figure 1. Example of the AFLP® procedure using one primer pair.

2.2 Summary of the AFLP® Analysis System I

The AFLP® Analysis System I has been designed for use with plants having genomes ranging in size from 5×10^8 to 6×10^9 bp, including tomato, corn, soybean, cucumber, lettuce, barley, cotton, oilseed rape, potato, sunflower, pepper, and brassica. The AFLP® technique involves three major steps:

1. Restriction endonuclease digestion of the DNA and ligation of adapters.
2. Amplification of the restriction fragments.
3. Gel analysis of the amplified fragments.

Typically, 50 to 100 restriction fragments are coamplified in each AFLP® reaction and detected by denaturing gel electrophoresis. This technique is, therefore, extremely powerful for the identification of DNA polymorphisms.

Overview

2.2.1 Restriction Endonuclease Digestion

To prepare an AFLP® template, genomic DNA is isolated and digested with two restriction endonucleases simultaneously. This step generates the required substrate for ligation and subsequent amplification.

The restriction fragments for amplification are generated by two restriction endonucleases: *Eco*R I and *Mse* I. *Eco*R I has a 6-bp recognition site, and *Mse* I has a 4-bp recognition site. When used together, these enzymes generate small DNA fragments that will amplify well and are in the optimal size range (<1 kb) for separation on denaturing polyacrylamide gels. Due to primer design and amplification strategy, these *Eco*R I–*Mse* I fragments are preferentially amplified (rather than *Eco*R I–*Eco*R I or *Mse* I–*Mse* I fragments).

The success of the AFLP® technique is dependent upon complete restriction digestion; therefore, much care should be taken to isolate high quality genomic DNA, intact without contaminating nucleases or inhibitors.

2.2.2 Ligation of Adapters

Following heat inactivation of the restriction endonucleases, the genomic DNA fragments are ligated to *Eco*R I and *Mse* I adapters to generate template DNA for amplification. These common adapter sequences flanking variable genomic DNA sequences serve as primer binding sites on these restriction fragments. Using this strategy, it is possible to amplify many DNA fragments without having prior sequence knowledge.

2.2.3 Amplification Reactions

PCR is performed in two consecutive reactions. In the first reaction, called preamplification, genomic DNAs are amplified with AFLP® primers each having one selective nucleotide. The PCR products of the preamplification reaction are diluted and used as a template for the selective amplification using two AFLP® primers, each containing three selective nucleotides. (The *Eco*R I selective primer is ^{32}P- or ^{33}P-labeled before amplification.) This two-step amplification strategy results in consistently cleaner and more reproducible fingerprints with the added benefit of generating enough template DNA for thousands of AFLP® reactions.

The most important factor in determining the number of restriction fragments amplified in a single AFLP® reaction is the number of selective nucleotides in the selective primers. The selective primers in the AFLP® Analysis System I contain three selective nucleotides. In practice, using the AFLP® Analysis System I with plants having genomes ranging in size from 5×10^8 to 6×10^9 bp, the number of fragments amplified per sample per primer pair averages 50, but may range from as low as 10 to ~100 depending on the sequence context of the selective nucleotides, and the complexity of the genome (1,2,10).

A second factor in determining the number of amplified fragments is the C and G composition of the selective nucleotides. In general, the more Cs and Gs used as selective nucleotides in the amplification primers, the fewer DNA fragments amplified. Also, the smaller the genome being analyzed, the fewer fragments amplified and the simpler the fingerprint.

2.2.4 Separation of Amplified Fragments on Denaturing Polyacrylamide Gels

Products from the selective amplification are separated on a 5% or 6% denaturing polyacrylamide (sequencing) gel. The resultant banding pattern (“fingerprint”) can be analyzed for polymorphisms either manually or using analytical software (1,2).

Methods

3

3.1 Components

The components of the AFLP® Analysis System I are as follows. Sufficient material is provided for 50 templates and up to 1,600 reactions for final AFLP® reaction. Store all components at –20°C. **Note:** *Taq* DNA polymerase is not included with this system.

Component	Volume
AFLP® Core Reagent Kit:	
*Eco*R I/*Mse* I [1.25 units/µl each in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 50% (v/v) glycerol, 0.1% Triton® X-100]	100 µl
5X reaction buffer [50 mM Tris-HCl (pH 7.5), 50 mM Mg-acetate, 250 mM K-acetate]	250 µl
Distilled water	1.25 ml
adapter/ligation solution [*Eco*R I/*Mse* I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate]	1.2 ml
T4 DNA ligase [1 unit/µl in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50% glycerol (v/v)]	50 µl
TE buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA]	4.5 ml
Arabidopsis DNA (100 ng/µl)	10 µl
tomato DNA (100 ng/µl)	10 µl
product profile	one
AFLP® Starter Primer Kit:	
pre-amp primer mix	2 ml
T4 kinase [10 units/µl in 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 1 mM 2-mercaptoethanol, 0.1 µM ATP, 50% (v/v) glycerol]	32 µl
5X kinase buffer [350 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 500 mM KCl, 5 mM 2-mercaptoethanol]	160 µl
*Eco*R I primers (27.8 ng/µl):	
primer E-AAC	46 µl
primer E-AAG	46 µl
primer E-ACA	46 µl
primer E-ACT	46 µl
primer E-ACC	46 µl
primer E-ACG	46 µl
primer E-AGC	46 µl
primer E-AGG	46 µl
Mse I primers (6.7 ng/µl, dNTPs):	
primer M-CAA	900 µl
primer M-CAC	900 µl
primer M-CAG	900 µl
primer M-CAT	900 µl
primer M-CTA	900 µl
primer M-CTC	900 µl
primer M-CTG	900 µl
primer M-CTT	900 µl
10X PCR buffer plus Mg [200 mM Tris-HCl (pH 8.4), 15 mM $MgCl_2$, 500 mM KCl]	3.5 ml
TE buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA]	8 ml
Distilled water	1.25 ml
preamplified tomato DNA (control for selective amplification)	10 µl
manual	one

Note: Restriction endonuclease primers are complementary to the adapter primers. Select one primer pair (e.g., one *Eco*R I primer and one *Mse* I primer) for each PCR. Sixty-four combinations of primer pairs are possible. Please refer to table 1 for primer pair selection guidelines.

Methods

3.2 Additional Materials Required

The following items are required for use with the AFLP® Analysis System I, but they are *not* included in the system:

- *Taq* DNA polymerase
- 1.5-ml microcentrifuge tubes
- 0.2- or 0.5-ml thin-walled microcentrifuge tubes (depending on thermal cycler)
- automatic pipettes capable of dispensing 1 to 20 µl and 20 to 200 µl
- autoclaved, aerosol-resistant tips for automatic pipettes
- microcentrifuge capable of generating a relative centrifugal force of 14,000 × *g*
- 37°C, 70°C, 90°C water baths or programmable thermal cycler
- denaturing polyacrylamide gel reagents and apparatus
- X-ray film
- film cassette
- [γ-^{33}P or γ-^{32}P]ATP (for radioactive detection)
- AFLP® Non-radioactive Probe (Cat No. 10822-013) (for chemiluminescent detection)
- silicone oil (if thermal cycler requires)

3.3 Advanced Preparation

The use of Invitrogen Plant DNAzol® (Cat. No. 10978-021) is recommended to obtain high-quality plant genomic DNA. For optimal results, high-quality, intact DNA must be used as starting material for AFLP. It is essential to prevent incomplete restriction digestion of genomic DNA, which may result in the incorrect identification of polymorphisms.

3.4 Restriction Digestion of Genomic DNA

WARNING: The AFLP® Core Reagent Kit contains two control DNAs: tomato and *Arabidopsis*. Use the tomato control DNA with AFLP® Analysis System I. The *Arabidopsis* DNA is provided as a control DNA for the AFLP® Analysis System II.

1. Add the following to a 1.5-ml microcentrifuge tube:

component	control	sample
5X reaction buffer	5 µl	5 µl
tomato control DNA (100 ng/µl)	2.5 µl	—
sample DNA (250 ng in ≤18 µl)	—	≤18 µl
*Eco*R I/*Mse* I	2 µl	2 µl
Distilled water	15.5 µl	to 25 µl
total volume	25 µl	25 µl

2. Mix gently and collect the reaction by brief centrifugation. Incubate the mixture 2 h at 37°C.
3. Incubate the mixture for 15 min at 70°C to inactivate the restriction endonucleases. Place tube on ice and collect contents by brief centrifugation.

3.5 Ligation of Adapters

1. Add the following to the digested DNA from step 3 of Section 3.4:

component	volume
adapter ligation solution	24 µl
T4 DNA ligase	1 µl

2. Mix gently at room temperature, centrifuge briefly to collect contents, and incubate at 20°C ± 2°C for 2 h.
3. Perform 1:10 dilution of the ligation mixture as follows:
   a. Take 10 µl of the reaction mixture and transfer to a 1.5-ml microcentrifuge tube.
   b. Add 90 µl TE buffer and mix well.
4. The unused portion of the reaction mixture may be stored at –20°C. STOP

Note: Possible stopping points are noted in the protocol with the STOP icon.

3.6 Preamplification Reactions

1. Add the following to a 0.2- or 0.5-ml thin-walled microcentrifuge tube:

component	volume
diluted template DNA (from step 3 of Section 3.5)	5 µl
pre-amp primer mix	40 µl
10X PCR buffer plus Mg	5 µl
Taq DNA polymerase (5 unit/µl)	1 µl
total volume	51 µl

Warning: Do not denature samples prior to PCR because denaturing reduces the annealing efficiency of the primers.

2. Mix gently and centrifuge briefly to collect reaction. Overlay with 2 to 3 drops of silicone oil, if required. Perform 20 cycles at:

   94°C for 30 s

   56°C for 60 s

   72°C for 60 s

   Soak temperature is 4°C.
3. Perform a 1:50 dilution as follows: transfer 3 µl to a 1.5-ml microcentrifuge tube containing 147 µl TE buffer. This is sufficient for 30 selective AFLP® amplifications. If necessary, new dilutions can be made of the preamplification reactions to give additional template for the selective AFLP® amplifications. Both unused diluted and undiluted reactions can be stored at –20°C. STOP

3.7 Primer Labeling

Note: This procedure generates enough labeled primer for 100 selective AFLP® amplifications.

Primer labeling is performed by phosphorylating the 5´ end of the *Eco*R I primers with [γ-^{32}P or γ-^{33}P]ATP and T4 kinase. ^{33}P-labeled primers are preferred because they give better resolution of the PCR products on the gels. Also, the reaction products are less prone to degradation due to autoradiolysis. Please refer to table 1 for information regarding choice of primers.

Note: For nonradioactive detection primers are not end-labeled with ^{32}P or ^{33}P. Dilute 18 µl of *Eco*R I primer with 32 µl of distilled water for use with the AFLP® Non-Radioactive Probe and follow product protocol (11).

1a. **For ^{32}P-label,** add the following components to a 1.5-ml microcentrifuge tube:

component	volume
*Eco*R I primer (select one)	18 µl
5X kinase buffer	10 µl
[γ-^{32}P]ATP (3,000 Ci/mmol)	20 µl
T4 kinase	2 µl
total volume	50 µl

1b. **For ^{33}P-label,** add the following components to a 1.5-ml microcentrifuge tube:

component	volume
*Eco*R I primer (select one)	18 µl
Distilled water	10 µl
5X kinase buffer	10 µl
[γ-^{33}P]ATP (2,000 Ci/mmol)	10 µl
T4 kinase	2 µl
total volume	50 µl

2. Mix gently and centrifuge briefly to collect contents of tube. Incubate reaction at 37°C for 1 h.

3. Heat inactivate the enzyme at 70°C for 10 min. Centrifuge briefly to collect reaction contents.

3.8 Selective AFLP® Amplification

1. For each primer pair, add the following components to a 1.5-ml microcentrifuge tube and label it **"Mix 1"**.

component	volume
labeled *Eco*R I primer (from step 3 of Section 3.7)	5 µl
Mse I primer (contains dNTPs)	45 µl
total volume (sufficient for 10 reactions)	50 µl

2. Add the following components to another 1.5-ml microcentrifuge tube and label it **"Mix 2"**.

component	volume
Distilled water	79 µl
10X PCR buffer plus Mg	20 µl
Taq DNA polymerase (5 units/µl)	1 µl
total volume (sufficient for 10 reactions)	100 µl

3. Each AFLP® amplification is assembled by combining the following in a 0.2- or 0.5-ml thin-walled microcentrifuge tube:

component	volume
diluted template DNA (from step 3 of Section 3.6; or control preamplified tomato DNA, undiluted)	5 µl
Mix 1 (primers/dNTPs)	5 µl
Mix 2 (*Taq* DNA polymerase/buffer)	10 µl
total volume	20 µl

Table 1. Guidelines for primer pair selection.

BARLEY:

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	√	√	√	√	√	√	√	√
E-AAG	√	√			√	√	√	√
E-ACA	√	√	√	√	√	√	√	√
E-ACC	√	√	√	√	√	√	√	√
E-ACG	√	√	√	√	√	√	√	√
E-ACT	√	√		√	√	√	√	√
E-AGC	√	√	√	√	√		√	√
E-AGG	√	√	√	√	√	√	√	√

LETTUCE:

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	√	√	√	√	√	√	√	√
E-AAG	√	√	√		√	√		√
E-ACA	√	√	√	√	√	√	√	√
E-ACC	√	√	√	√	√	√	√	√
E-ACG	√	√	√		√	√	√	
E-ACT	√		√	√	√	√	√	√
E-AGC	√	√	√	√	√	√	√	√
E-AGG	√	√	√		√	√	√	√

MAIZE:

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC		√	√	√	√	√	√	√
E-AAG	√	√	√	√		√	√	√
E-ACA		√	√	√	√	√	√	√
E-ACC	√	√	√	√	√	√	√	√
E-ACG	√	√	√	√	√		√	
E-ACT	√		√		√	√		
E-AGC	√	√	√	√	√	√	√	
E-AGG	√	√	√	√	√	√	√	√

OILSEED RAPE:

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	√		√	√	√			√
E-AAG	√	√		√		√		√
E-ACA								√
E-ACC	√	√	√			√		
E-ACG								
E-ACT				√		√		√
E-AGC								
E-AGG		√	√	√	√	√		

PEPPER:

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	√	√			√		√	
E-AAG	√	√	√		√	√	√	√
E-ACA	√	√	√		√	√	√	√
E-ACC	√	√	√	√	√	√	√	
E-ACG	√	√	√	√	√	√	√	√
E-ACT		√	√	√	√	√	√	√
E-AGC	√	√	√	√	√	√	√	√
E-AGG	√	√	√	√	√	√		√

POTATO:

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC			√					
E-AAG		√						
E-ACA		√	√				√	
E-ACC	√			√	√			
E-ACG	√			√				
E-ACT		√	√	√			√	√
E-AGC	√				√		√	√
E-AGG	√		√				√	√

√: Recommended primer pair
n.d. No data
Blank: Primer pair **not** recommended

Table 1. Guidelines for primer pair selection continued.

SUNFLOWER:

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	√		√	√	√	√	√	√
E-AAG		√	√	√		√	√	√
E-ACA		√	√			√	√	√
E-ACC	√	√	√	√	√	√	√	√
E-ACG	√		√	√		√	√	√
E-ACT	√	√	√	√	√	√	√	√
E-AGC	√	√	√	√	√		√	√
E-AGG	√	√	√	√	√		√	√

SUGAR BEET:

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	√	√	√	√	√	√	√	√
E-AAG	√	√	√	√	√	√	√	
E-ACA	√	√	√	√	√	√	√	√
E-ACC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
E-ACG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
E-ACT			√	√	√	√	√	√
E-AGC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
E-AGG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

TOMATO:

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	√	√	√	√	√	√	√	√
E-AAG	√	√	√		√	√		√
E-ACA	√	√	√	√	√	√	√	√
E-ACC	√	√	√	√	√	√	√	√
E-ACG	√	√	√	√	√	√	√	√
E-ACT	√	√	√	√	√	√	√	√
E-AGC	√	√	√	√	√	√	√	√
E-AGG	√	√	√	√	√	√	√	√

√: Recommended primer pair
n.d. No data
Blank: Primer pair **not** recommended

Warning: Do not denature samples prior to PCR because denaturing reduces the annealing efficiency of the primers.

4. Mix gently and centrifuge briefly to collect reaction. Overlay with 2 to 3 drops of silicone oil, if required.

If using the Perkin-Elmer 9600 thermal cycler (or similar authorized thermal cycler):

Note: PCR is started at a very high annealing temperature to obtain optimal primer selectivity. In the following steps the annealing temperature is lowered gradually to a temperature at which efficient primer binding occurs. This temperature is then maintained for the rest of the PCR cycles.

A. Perform one cycle at 94°C for 30 s; 65°C for 30 s; and 72°C for 60 s.

B. Lower the annealing temperature each cycle 0.7°C during 12 cycles. This gives a touch down phase of 13 cycles.

C. Perform 23 cycles at:

94°C for 30 s;

56°C for 30 s; and

72°C for 60 s.

Total Time: 2 h, 2 min. STOP

If using the Perkin-Elmer 480 thermal cycler (or similar authorized thermal cycler):

To program the Perkin-Elmer PE 480 for "touch down" phase, 11 linked files must be used:

File	°C	Time (s)	°C	Time	°C	Time	No. cycles	Link to	Type
1	94	60	65	60	72	90	1	2	Step
2	94	60	64	60	72	90	1	3	Step
3	94	60	63	60	72	90	1	4	Step
4	94	60	62	60	72	90	1	5	Step
5	94	60	61	60	72	90	1	6	Step
6	94	60	60	60	72	90	1	7	Step
7	94	60	59	60	72	90	1	8	Step
8	94	60	58	60	72	90	1	9	Step
9	94	60	57	60	72	90	1	10	Step
10	94	60	56	60	72	90	1	11	Step
11	94	30	56	30	72	60	23		Step

Total Time: 3 h, 18 min. STOP

3.9 Gel Analysis

Note: For non-radioactive detection, please refer to protocol supplied with the AFLP® Non-Radioactive Probe.

1. After PCR, add an equal volume (20 µl) of formamide dye (98% formamide, 10 mM EDTA, bromophenol blue, xylene cyanol) to each reaction. Heat the samples for 3 min at 90°C and immediately place on ice.
2. Pour a 5% or 6% polyacrylamide gel (20:1 acrylamide:bis; 7.5 M urea; 1X TBE buffer) with 0.4-mm spacers and sharkstooth combs.
3. Pre-electrophorese the gel at constant power (*e.g.,* ~55 W for a Model S2 Apparatus) for ~20 min.

Note: For a Model S2 gel, at 55 watts, electrophorese for 1 1/2 to 2 h.

4. Load 3 µl of each sample on the gel.
5. Electrophorese at constant power until xylene cyanol (slower dye) is two-thirds down the length of the gel.
6. Dry the gel.

Note: If the gel is ^{33}P-labeled, a longer exposure time may be required. No intensifying screen is required.

7. Expose the gel to x-ray film (*e.g.,* Kodak® SB, XAR or Biomax® MR film) at -80°C overnight.

4

Interpretation of Results

Individual band intensity, size distribution of amplified products, and overall pattern should be similar for AFLP® analysis with the same primer pairs and the same DNA template, and will vary between different genomic DNA samples and different primer pairs (figure 2). Fingerprints of related plants should display common bands, as well as some difference in banding pattern due to DNA polymorphisms.

The total number of bands as well as the number of polymorphisms, will depend on the crop, variety, complexity of the genome and primer pair used. The AFLP® Analysis System I is optimized for genomes ranging in size from 5×10^8 to 6×10^9 bp. Some primer pairs work better in specific crops for identifying polymorphisms and those suggestions are summarized in table 1. Some primer pair combinations may result in either too few or too many bands for a particular sample. In the case of too few bands, we suggest using a primer pair containing fewer Gs and Cs in the selective nucleotides. In the case of too many bands, select a primer pair containing more Gs and Cs in the selective nucleotides. For additional guidance in selecting primer pairs, see table 1.

Note: Use of this product with plants having genomes outside of this range may result in inadequate fingerprints exhibiting too few or too many amplified fragments.

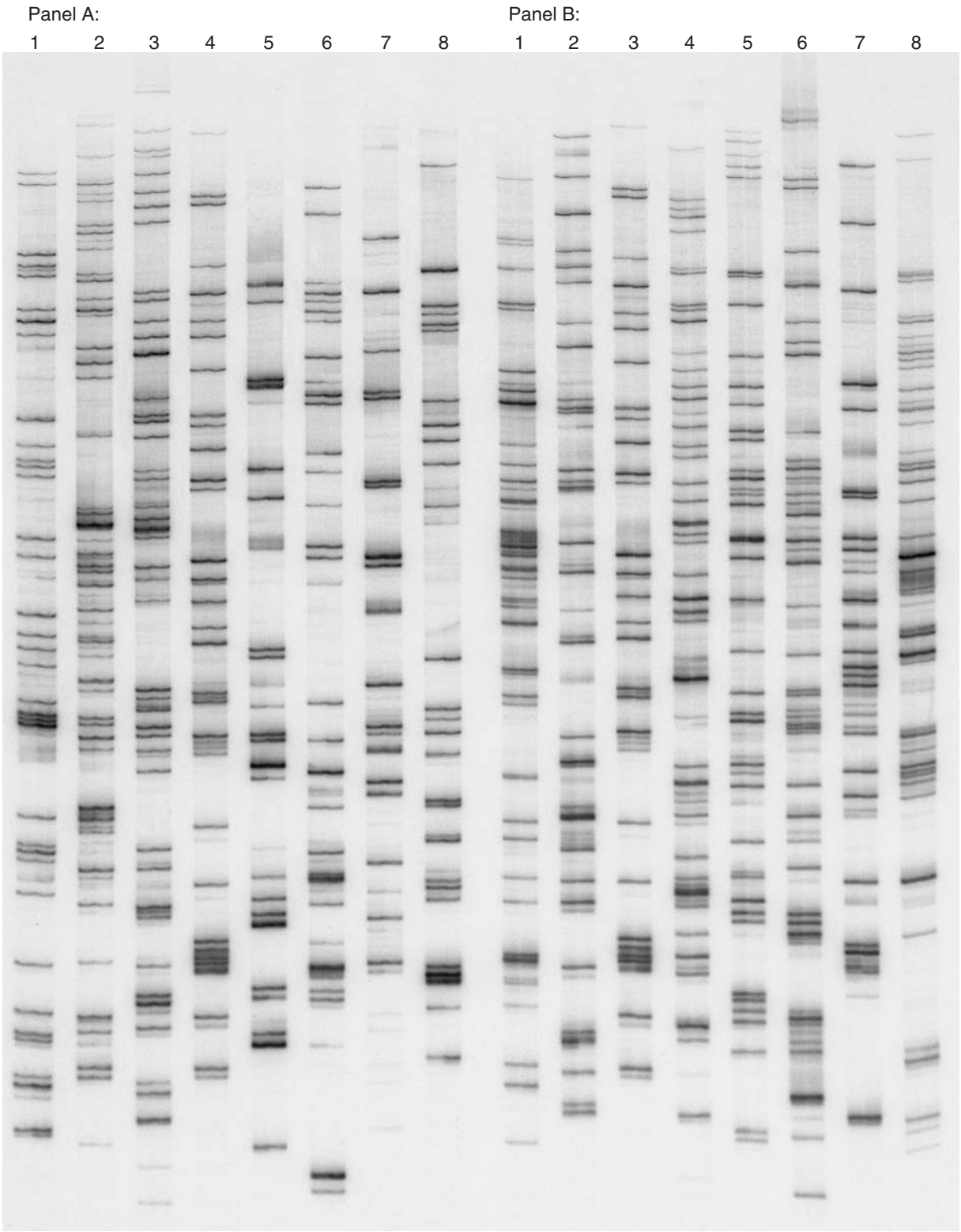

Figure 2. AFLP® fingerprints of control tomato DNA. *Panel A:* AFLP® fingerprints of control tomato DNA using *Mse* I primer M-CAG with the following *Eco*R I primers: E-AAC (lane 1), E-AAG (lane 2), E-ACA (lane 3), E-ACC (lane 4), E-ACG (lane 5), E-ACT (lane 6), E-AGC (lane 7), and E-AGG (lane 8). *Panel B:* AFLP® fingerprints of control tomato DNA using *Eco*R I primer E-ACC with the following *Mse* I primers:
M-CAA (lane 1), M-CAC (lane 2), M-CAG (lane3), M-CAT (lane 4), M-CTA (lane 5), M-CTC (lane 6), M-CTG (lane 7), and M-CTT (lane 8).

5 Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
AFLP® Reactions:		
No bands or weak bands on autoradiograph	Primer not labeled or poorly labeled	Be sure to use fresh, high specific activity γ-labeled ATP, not α-labeled dATP.
		Test the reaction with control primers.
	Not enough template DNA	Use ~100 to 500 ng of DNA per reaction.
		Test system with control DNA.
	DNA contaminated (*e.g.,* high salt, EDTA, Triton X-100, SDS, and protein)	Extract with phenol/chloroform followed by ethanol ammonium acetate precipitation (10). Wash with 70% ethanol.
	Incorrect thermal cycling	Verify the cycling program temperature, cycle number, and time.
		Verify that the thermal cycler is operating properly.
	Evaporation during thermal	Cover the reactions with silicone oil.
	cycling	Centrifuge briefly before incubation.
		Wipe tops of tubes with a wet paper towel.
		Check that caps fit properly for thermal cyclers that do not require oil.
	Low film sensitivity	Exposure time required of some films may be 2 to 5 times longer than Kodak XAR or Biomax MR film.
Too few/many or few heavy bands	Suboptimal primer pairs	Use suggested primer pair for your species (see table 1).
Missing lanes (nonspecific, variable)	Evaporation during cycle reaction	Cover the reactions completely with silicone oil. Centrifuge briefly before incubation.
		Wipe tops of tubes with wet paper towel to eliminate static electricity that may cause condensation above the oil.
		Check that caps fit properly for thermal cyclers that do not require oil.
	Pipetting error	Verify addition and mixing of all reaction components.
Many bands of high molecular weight	Partial digestion	Purify DNA using procedure in Section 3.3. Use autoclaved tubes, pipette tips, and solutions.
Bands only part way up gel	Low polymerase activity	Purify DNA template. Use fresh polymerase.
Sequencing Gels:		
Blurry bands	Urea diffusing into wells	After pre-electrophoresis, wash the urea from the wells before loading samples.
	Formamide in sample buffer degraded	Remake sample buffer with fresh formamide.
	Radiolabel properties lead to scattering of signal	Try ^{33}P-labeled γ-ATP instead of ^{32}P-labeled γ-ATP. Use a shorter exposure.
		Use a cassette that assures tight contact of gel to film.
		Do not use an intensifying screen.
	Urea diffused out of gel	Do not store precast gels under electrophoresis buffer overnight in the sequencing apparatus.
	Improper gel formation	Recast gel using fresh solutions, especially urea.

Problem	Possible Cause	Suggested Solution
Sequencing Gels:		
Smeared bands	Sample electrophoresing with the ion front	Pre-electrophorese gel until the current starts to drop (generally 20 min).
	Gel temperature too high	Do not electrophorese gel on constant current.
		Lower electrophoresis voltage.
		Glass surface temperature should be ~45°C to 50°C.
	Residual siliconizing agent on plate	Thoroughly rinse glass plates before gel preparation. Ethanol wipe before using.
		Soak glass plates in 2 N NaOH before using them.
		Thoroughly wash new glass plates with a strong lab soap before using.
	Formamide in sample buffer degraded.	Remake sample buffer with fresh formamide.
	Improper gel formation	Use fresh solutions to make gel, especially urea. Allow gel to polymerize fully before using.
	Differences in gel buffer and running buffer	Verify that the buffer in the gel and the running buffer are the same concentration.
Wavy bands	The gel surface did not polymerize evenly	Recast with extra acrylamide at the top of the gel. Clean the flat edge of the sharkstooth comb.
	Sharkstooth combs punctured the gel surface	Insert sharkstooth comb only to the point of dimpling the surface.
Strong bands in wells	Sample not denatured properly	Add 20 µl of loading dye to completed reactions. Heat for 5 min at 95°C *immediately* before loading gel.
Dark smear in length of lanes obscures bands	Detergent contamination	Rinse glass plates and glassware thoroughly with deionized water before gel preparation.
Glass plates crack during electrophoresis	Gel is too hot	Electrophorese with constant voltage or power, not constant current.
		Do not exceed settings that yield a gel surface temperature of 45°C to 50°C.
Smiling gels (corresponding bands in outer lanes electrophoresing slower than inner lanes)	Gel thickness is not uniform Gel temperature is not uniform	Allow the gel to polymerize at a 5° angle, instead of flat. Use an electrophoresis unit that has an aluminum plate to disperse the heat evenly.
Frowning gels (corresponding bands in inner lanes electrophoresing slower than outer lanes)	Improper clamping during gel casting Improper clamping of the gel into the electrophoresis chamber	Apply pressure points of the binder clips only over the spacer. Polymerize the gel at an angle of <10°. Do not over tighten the clamps on the apparatus. Make sure that the spacers are pushed against the polymerized acrylamide.
Lanes curve outward toward bottom of the gel	Improper gel casting	Do not use bottom clamps without a bottom spacer. Do not pull the plates apart while taping the gel.

5

Note: Extreme care should be exercised when working with the control DNA so the AFLP® Analysis System I reagents do not become contaminated. Use of aerosol barrier pipette tips significantly reduces the potential for the introduction of aerosols.

5.1 Testing the AFLP® Analysis System I Using the Control DNA

When using the AFLP® Analysis System I for the first time, perform an experiment using the control DNAs and any primer pair to become familiar with the AFLP® procedure and to verify proper functioning of all components and your reagents and equipment for PCR. Control tomato DNA (Moneymaker variety) is provided in the AFLP® Core Reagent Kit. Preamplified tomato DNA is provided in the AFLP® Starter Primer Kit. This control DNA is tomato DNA that has been cut with *Eco*R I and *Mse* I, ligated to adapters and pre-amplified, ready for selective amplification.

Typical results for the procedure using the control tomato DNA and the primer pairs indicated are shown in figure 2. Some minor differences in band intensity and position may be observed from experiment to experiment, but the overall pattern should match. If there is a marked difference between patterns pictured and results obtained using the same primer pairs, please refer to the troubleshooting table.

5.2 Common Problems

A major factor affecting the quality of an AFLP® fingerprint is the quality of the genomic DNA. If a DNA sample contains impurities, this may result in incomplete digestion by restriction endonucleases. Fingerprints generated from only partially digested DNA will display a different banding pattern than fingerprints of completely digested genomic DNA (figure 3). The shift to more numerous larger fragments is evident. If partial digestion is occurring, isolate the plant genomic DNA according to Section 3.3 and repeat the experiment.

Figure 3. Effect of partial digestion on AFLP® analysis. Genomic DNA isolated from *Arabidopsis* columbia was digested with different dilutions of *Eco*R I and *Mse* I and AFLP® analysis was performed. The concentrations of the mixtures of *Eco*R I and *Mse* I were 5X, 1X, 0.1X, 0.01X, and 0.001X in lanes 1 through 5, respectively.

References

6

1. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Freijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. (1995) *Nucleic Acids Res. 23,* 4407.
2. Zabeau, M. and Vos, P. (1993) *European Patent Application,* publication number EP 0534858.
3. Tanksley, S.D., Young, N.D., Paterson, A.H., and Bonierbale, M.W. (1989) *Bio/Technology 7,* 257.
4. Botstein, D., White, R.L., Skolnick, M.H., and Davies, R.W. (1980) *Am. J. Hum. Genet. 32,* 314.
5. Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kanlin, E., and White, R. (1987) *Science 235,* 1616.
6. Jeffreys, A.J., McLeod, A., Tamaki, K., Neil, D.L., and Monckton, D.G. (1991) *Nature 354,* 204.
7. Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. (1990) *Nucleic Acids Res. 18,* 6531.
8. Caetano-Anolles, G., Bassam, B.J., and Gressholf, P.M. (1991) *Bio/Technology 9,* 553.
9. Welsh, J. and McClelland, M. (1990) *Nucleic Acids Res. 18,* 7213.
10. Lin, J.J. and Kuo, J. (1995) *Focus® 17,* 66.
11. Lin, J.J., Kuo, J., and Ma, J. (1997) *Focus 19,* 36.

Additional References

Balvora, A., Hesselbach, J., Niewohner, J., Leister, D., Salamini, F., and Gebhardt, C. (1995) *Mol. Gen. Genet. 249*, 82.

Dean, C. and Schmidt, R. (1995) *Ann. Rev. Plant Physiol. Plant Mol. Biol. 46*, 395.

Eshed, Y. and Zamir, D. (1994) *Euphytica 79*,175.

Meksem, K., Leister, D., Peleman, J., Zabeau, M., Salamini, F., and Gebhardt, C. (1995) *Mol. Gen. Genet. 249*, 74.

Thomas, C.M., Vos, P., Zabeau, M., Jones, D.A., Norcott, K.A., Chadwick, B., and Jones, J.D.G. (1995) *The Plant Journal 8*, 785.

Valsangiacomo, C., Baggi, F., Gaia, V.E., Balmelli, T., Peduzzi, R., and Piffaretti, J.C. (1995) *J. Clinical Microbiology 33*, 1716.

7

Related Products

Product	Size	Cat. No.
AFLP® Products:		
AFLP® Analysis System II		10717-015
AFLP® Analysis System for Microorganisms		11352-010
AFLP® Core Reagent Kit	50 templates	10482-016
AFLP® Starter Primer Kit	≤ 1,600 reactions	10483-014
AFLP® Small Genome Primer Kit	≤ 1,600 reactions	10719-011
AFLP® Microorganism Primer Kit	≤1,200 reactions	11353-018
30-330 bp AFLP® DNA Ladder	300 applications	10832-012
Plant DNAzol™	100 ml	10978-021
AFLP® Pre-Amp Primer Mix I	1 ml	10792-018
AFLP® Pre-Amp Primer Mix II	1 ml	10781-011
AFLP® *Eco*R I Primers	16 available	see catalog
AFLP® *Mse* I Primers	8 available	see catalog
Enzymes:		
T4 DNA Ligase	100 units 500 units	15224-017 15224-025
T4 Polynucleotide Kinase	200 units 1,000 units	18004-010 18004-028
Taq DNA Polymerase, Native	100 units 500 units 1,500 units	18038-018 18038-042 18038-067
Taq DNA Polymerase, Recombinant	100 units 500 units 1,500 units	10342-053 10342-020 10342-046
Reagents:		
0.5 M EDTA, pH 8.0	4 × 100 ml	15575-020

United States Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, California 92008
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 603 7229
Email: tech_service@invitrogen.com

European Headquarters:
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel (Free Phone Orders): 0800 269 210
Tel (General Enquiries): 0800 5345 5345
Fax: +44 (0) 141 814 6287
Email: eurotech@invitrogen.com

International Offices:
Argentina 5411 4556 0844
Australia 1 800 331 627
Austria 0800 20 1087
Belgium 0800 14894
Brazil 0800 11 0575
Canada 800 263 6236
China 10 6849 2578
Denmark 80 30 17 40

France 0800 23 20 79
Germany 0800 083 0902
Hong Kong 2407 8450
India 11 577 3282
Italy 02 98 22 201
Japan 03 3663 7974
The Netherlands 0800 099 3310
New Zealand 0800 600 200
Norway 00800 5456 5456

Spain & Portugal 900 181 461
Sweden 020 26 34 52
Switzerland 0800 848 800
Taiwan 2 2651 6156
UK 0800 838 380
For other countries see our website

www.invitrogen.com