

Model 4300

DNA Analyzer

IRDye[®] Fluorescent AFLP[®] Protocol for Large Plant Genome Analysis



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to this protocol will be posted at
<http://biosupport.licor.com>

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LI-COR[®]

Biosciences

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I. Required Reagents and Materials

AFLP® Components: Sufficient material is provided for 100 DNA templates and up to 1600 reactions for selective AFLP® reactions. Store all components at -20 °C.

AFLP® Template Preparation Kit Components

Volume

- Maize DNA (75 ng/μl) 10 μl
- *EcoRI/MseI* enzyme mix [1.25 units/μl each in 10 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 200 μg/ml BSA, 50% (v/v) glycerol, 0.15% 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
- T4 DNA ligase [5 units/μl in 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 50 mM KCl, 200 μg/ml BSA, 50% (v/v) glycerol] 50 μl
- Adapter mix [*EcoRI/MseI* adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate]. 1.2 ml
- TE buffer [10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA] 9.0 ml
- Water, deionized 1.0 ml
- AFLP® pre-amp primer mix 2.0 ml
- AFLP® protocol one

AFLP® Selective Amplification Kit Components

Volume

- Pre-amp Maize DNA 16 μl
- *MseI* primers (containing dNTPs)
 - Primer M-CAA 400 μl
 - Primer M-CAC 400 μl
 - Primer M-CAG 400 μl
 - Primer M-CAT 400 μl
 - Primer M-CTA 400 μl
 - Primer M-CTC 400 μl
 - Primer M-CTG 400 μl
 - Primer M-CTT 400 μl

- IRDye[®] infrared dye-labeled (either IRDye[®] 700 or IRDye[®] 800) *EcoRI* primers (1 μ M)
 - Primer E-AAC100 μ l
 - Primer E-AAG100 μ l
 - Primer E-ACA100 μ l
 - Primer E-ACT100 μ l
 - Primer E-ACC100 μ l
 - Primer E-ACG100 μ l
 - Primer E-AGC100 μ l
 - Primer E-AGG100 μ l
- 10X amplification buffer [100 mM Tris-HCl (pH 8.3), 15 mM MgCl₂, 500 mM KCl] 2.2 ml
- Blue stop solution 8.0 ml
- AFLP[®] protocol one

Additional Material Required

In addition to the kit components, the following items are required, but not included:

- LI-COR[®] DNA Analyzer.
- Programmable thermal cycler.
- Mineral oil or liquid wax (if thermal cycler is not equipped with a heated lid).
- Microcentrifuge capable of generating a relative centrifugal force of 14,000 x g.
- 1.5 ml microcentrifuge tubes or 0.2 or 0.5 ml thin-walled microcentrifuge tubes (depending on thermal cycler). For high throughput experiments, 96-well plates are recommended.
- Pipettes capable of dispensing 0.3 to 2 μ l, 1.0 to 20 μ l, and 20 to 200 μ l.
- Taq DNA polymerase.

II. Introduction

Amplified Fragment Length Polymorphism (AFLP[®]) is a DNA fingerprinting technique developed by Keygene N. V. (1, 2). Since 1995, AFLP[®] has been widely used for genetic diversity assessment (2, 3), linkage map construction (4, 5, 6), and gene profiling analyses (7, 8) in various genomes. A typical AFLP[®] analysis consists of five major steps (as illustrated in Figure 1).

The first step is a restriction digest in which genomic DNA is cut by two restriction enzymes (a rare cutter such as *EcoRI*, and a frequent cutter such as *MseI*) to generate small DNA fragments.

Step two is a ligation in which double-stranded DNA adapters are ligated to the ends of the restricted DNA fragments to generate templates for amplification.

Step three is a pre-amplification in which two primers, complementary to the adapter-ligated ends with one pre-selected nucleotide at the 3' end, are employed to amplify flanking regions containing the primer binding site and the restriction site.

Step four is a selective amplification in which selective primers, with an additional 1 to 3 nucleotides at the 3' end, are employed to amplify subsets of pre-amplified templates. In step five, selective amplification products are separated by denaturing polyacrylamide gel electrophoresis.

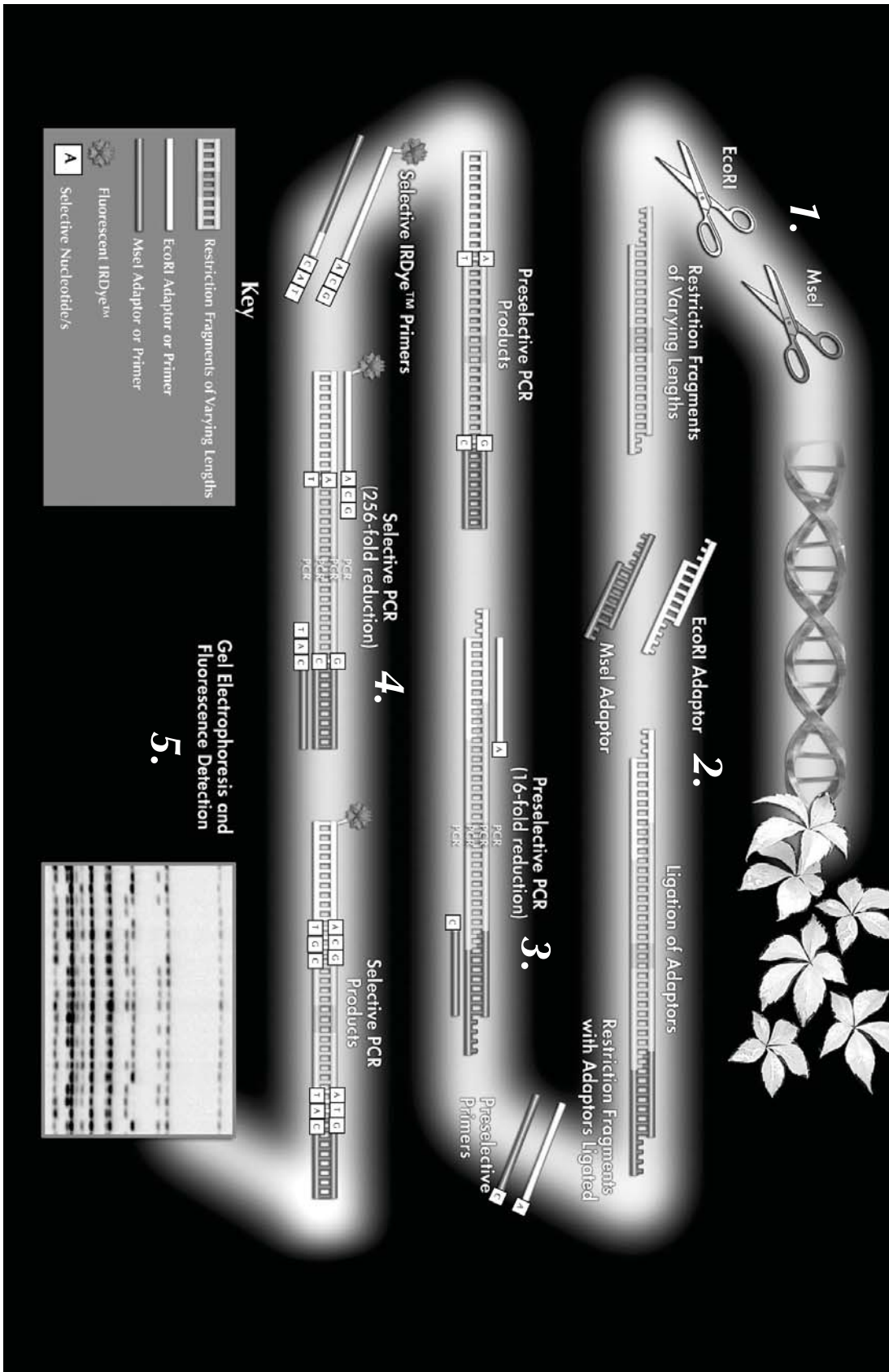


Figure 1. Schematic illustration of AFLP® analysis. A color version of this figure can be seen at http://www.licor.com/bio/applications/4300_applications/aflp.jsp.

The popularity of this fingerprinting and mapping technique has created the need for increased throughput via the automation of AFLP® analyses. Toward this end, LI-COR automated DNA analyzers can be used to efficiently generate true AFLP® images (3, 4, 5, 6, 9). When compared to conventional AFLP® detection methods, LI-COR® Biosciences' automated system provides at least four major advantages.

- IRDye® labels are much safer than alternative radioactive labels.
- Image data can be obtained from the automated system in several hours rather than the two to four days required for radioactive or silver staining procedures.
- The sensitivity of the automated system and availability of IRDye labeled AFLP® primers reduce overall cost and eliminate labeling steps.
- AFLP® images are scored quickly with software such as SAGA^{MX} (LI-COR). Software automation eliminates multiple data entry steps when scoring markers and preparing data for phylogeny programs such as PAUP, Treecon, or NTSYS and mapping software such as Mapmaker.

The protocols that follow are for plants having genomes ranging from 5×10^8 to 6×10^9 bp.

References

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9. Myburg, A.A., D.L. Remington, D.M. O'Malley, R.R. Sederoff, R.W. Whetten. 2001. High-throughput AFLP analysis using infrared dye-labeled primers and an automated DNA sequencer. *BioTechniques* 30:348-357.

III. AFLP® Template Preparation Kit

Preparing Genomic DNA Template

High quality genomic DNA is critical for obtaining reproducible AFLP® results. Contaminants in poor quality DNA may inhibit restriction, resulting in incomplete digestion that will produce variable AFLP® banding patterns following amplification. Although quantity is not as critical as quality, reliable quantification of DNA templates will ensure uniformity of AFLP® data among multiple individuals. Generally, 100 ng of genomic DNA per template is sufficient.

Note: Use of A260/A280 ratio to justify DNA quality may not be reliable and quantification based on A260 reading is often incorrect by several fold. Use of a fluorometer or gel with a set of standards is recommended.


Restriction Digestion of Genomic DNA

1.	Add the following to a 0.2 ml PCR tube (on ice):												
	<table> <tr> <td>5X reaction buffer</td> <td>2.5 µl</td> </tr> <tr> <td>Template DNA (100 ng in ≤9 µl)</td> <td>≤9.0 µl</td> </tr> <tr> <td><i>EcoRI/MseI</i> enzyme mix</td> <td>1.0 µl</td> </tr> <tr> <td>Deionized water</td> <td>to 12.5 µl</td> </tr> <tr> <td colspan="2"><hr/></td> </tr> <tr> <td>TOTAL VOLUME</td> <td>12.5 µl</td> </tr> </table>	5X reaction buffer	2.5 µl	Template DNA (100 ng in ≤9 µl)	≤9.0 µl	<i>EcoRI/MseI</i> enzyme mix	1.0 µl	Deionized water	to 12.5 µl	<hr/>		TOTAL VOLUME	12.5 µl
5X reaction buffer	2.5 µl												
Template DNA (100 ng in ≤9 µl)	≤9.0 µl												
<i>EcoRI/MseI</i> enzyme mix	1.0 µl												
Deionized water	to 12.5 µl												
<hr/>													
TOTAL VOLUME	12.5 µl												
2.	Mix gently, centrifuge briefly, and incubate the mixture at 37°C for 2 hours.												
3.	Incubate the mixture for 15 minutes at 70°C to inactivate the restriction enzymes and place tube on ice. Notes: <ul style="list-style-type: none"> A PCR thermal cycler is recommended for incubation in both steps 2 and 3. Program for one cycle of 37°C for 2 hours, one cycle of 70°C for 15 minutes, and 4°C soak. 												

Adapter Ligation

1.	Add the following to the previous tube (on ice):								
	<table> <tr> <td>Adapter mix</td> <td>12.0 µl</td> </tr> <tr> <td>T4 DNA ligase</td> <td>0.5 µl</td> </tr> <tr> <td colspan="2"><hr/></td> </tr> <tr> <td>COMBINED VOLUME</td> <td>25.0 µl</td> </tr> </table>	Adapter mix	12.0 µl	T4 DNA ligase	0.5 µl	<hr/>		COMBINED VOLUME	25.0 µl
Adapter mix	12.0 µl								
T4 DNA ligase	0.5 µl								
<hr/>									
COMBINED VOLUME	25.0 µl								
2.	Mix gently by pipetting up and down. Centrifuge briefly and incubate the mixture at 20°C for 2 hours.								
3.	After incubation, perform a 1:10 dilution of the ligation mixture by transferring 10 µl of the mixture to a new 0.5 ml microcentrifuge tube, adding 90 µl of TE buffer, and mixing well.								
4.	Store unused portion (15 µl) of the ligation mixture at -20°C for future experiments.								

Pre-amplification

1.	<p>On ice, add the following to a PCR tube (size depends on thermal cycler used):</p> <table border="0" style="width: 100%;"> <tr> <td>Diluted (1:10) ligation mixture (from steps above)</td> <td style="text-align: right;">2.5 μl</td> </tr> <tr> <td>AFLP® Pre-amp primer mix</td> <td style="text-align: right;">20.0 μl</td> </tr> <tr> <td>PCR reaction buffer (10X)</td> <td style="text-align: right;">2.5 μl</td> </tr> <tr> <td>Taq DNA polymerase (5 units/μl)</td> <td style="text-align: right;">0.5 μl</td> </tr> <tr> <td style="border-top: 1px solid black;">TOTAL VOLUME</td> <td style="text-align: right; border-top: 1px solid black;">25.5 μl</td> </tr> </table> <p>Notes:</p> <ul style="list-style-type: none"> Kit was optimized using Taq DNA polymerase (Cat. No. 1146173) and 10X PCR reaction buffer (Cat. No. 1271318) from Roche Molecular Biochemicals. Choice of DNA polymerase is left to the user; however, some modification or optimization may be required. Generally, 1 unit of Taq DNA polymerase per 10 μl reaction is recommended. For contents of suggested PCR buffer, see 10X amplification buffer listed earlier in this protocol under the AFLP Selective Amplification Kit Components (page 2). 	Diluted (1:10) ligation mixture (from steps above)	2.5 μ l	AFLP® Pre-amp primer mix	20.0 μ l	PCR reaction buffer (10X)	2.5 μ l	Taq DNA polymerase (5 units/ μ l)	0.5 μ l	TOTAL VOLUME	25.5 μ l					
Diluted (1:10) ligation mixture (from steps above)	2.5 μ l															
AFLP® Pre-amp primer mix	20.0 μ l															
PCR reaction buffer (10X)	2.5 μ l															
Taq DNA polymerase (5 units/ μ l)	0.5 μ l															
TOTAL VOLUME	25.5 μ l															
2.	<p>Mix gently by pipetting up and down. Centrifuge briefly and cap tightly. Add 2 drops of liquid wax or mineral oil if your thermal cycler is not equipped with a heated lid.</p>															
3.	<p>Place PCR tube in the thermal cycler and run the following program.</p> <p>Program:</p> <table border="1" style="display: inline-table; margin-right: 20px;"> <thead> <tr> <th>Step</th> <th>Temperature (°C)</th> <th>Time</th> </tr> </thead> <tbody> <tr> <td>1.</td> <td>94</td> <td>30 seconds</td> </tr> <tr> <td>2.</td> <td>56</td> <td>1 minute</td> </tr> <tr> <td>3.</td> <td>72</td> <td>1 minute</td> </tr> <tr> <td>4.</td> <td>4</td> <td>hold</td> </tr> </tbody> </table>  <p style="margin-left: 20px;">} 20 cycles total</p>	Step	Temperature (°C)	Time	1.	94	30 seconds	2.	56	1 minute	3.	72	1 minute	4.	4	hold
Step	Temperature (°C)	Time														
1.	94	30 seconds														
2.	56	1 minute														
3.	72	1 minute														
4.	4	hold														
5.	<p>Perform a 1:40 dilution by pipetting 5 μl of the pre-amplification DNA mixture into a 0.5 ml microcentrifuge tube and adding 195 μl of ddH₂O or low TE buffer (1.0 mM Tris-Cl, pH 8.0, 0.1 mM EDTA). The diluted pre-amp DNA solution is sufficient for 100 selective AFLP® amplifications.</p> <p>Notes:</p> <ul style="list-style-type: none"> Dilution factors (1:10, 1:20, 1:50, etc.) of the pre-amp DNA may vary, depending on species and templates. Low TE buffer is not included in this kit. 															
6.	<p>Store the unused portion (~20 μl) of the pre-amp template mixture at -20°C.</p>															

IV. AFLP® Selective Amplification Kit

Selective AFLP® Amplification

Selective amplifications are generally performed in 96-well microplates. Before beginning, determine how samples and primer combinations will be arranged in the plate. If an 8-channel syringe or pipetter will be used to load the gel, this partially determines how the plate will be set up (see Model 4300 Applications Manual or Model 4200 Genetic Analysis Manual for details).

The following **duplex** (one *MseI* with two IRDye-labeled *EcoRI* primers) PCR protocol is based on an 11 μ l total reaction volume per template-primer set. It is recommended that two labeled primers with similar T_m be used. A guide for selecting *EcoRI/MseI* pairs for several major crop species is provided in Table 1.

Taq DNA polymerase working mix (see recipe below)	6.0 μ l
Diluted pre-amp DNA	2.0 μ l
<i>MseI</i> primer containing dNTPs	2.0 μ l
IRDye 700 labeled <i>EcoRI</i> primer A	0.5 μ l
IRDye 800 labeled <i>EcoRI</i> primer B	0.5 μ l
<hr/> TOTAL VOLUME	<hr/> 11.0 μ l

Note: If a **monoplex** (one *MseI* with either one IRDye 700- or one IRDye 800-labeled *EcoRI* primer) PCR is to be conducted, eliminate primer A or primer B (total volume equals 10.5 μ l).

Reagent preparations

Because of the small amount of several reagents to be pipetted, it is strongly recommended to make a master reagent mixture involving multiple reagents whenever possible to reduce systematic and pipetting errors.

A) Taq DNA Polymerase Working Mix (recipe for 200 μ l, which is sufficient for 33 reactions):

Deionized water	158.0 μ l
10X Amplification buffer	40.0 μ l
Taq DNA polymerase (5 units/ μ l)*	2.0 μ l
<hr/> TOTAL VOLUME	<hr/> 200.0 μ l

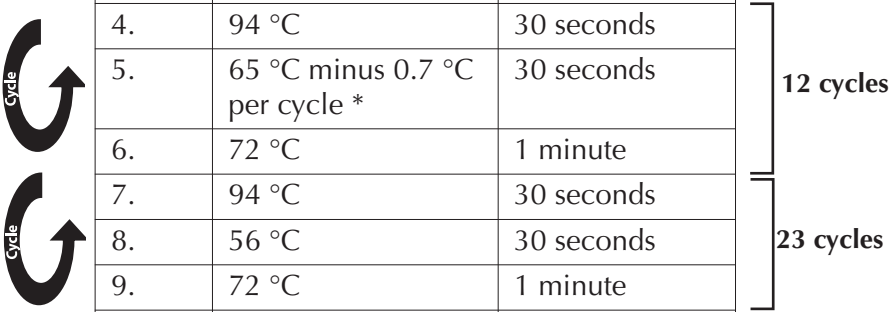
* Choice of DNA polymerase is left to the user; however, some modification to number of units needed may be required.

B) Taq DNA Polymerase and Primer Mix:

The amount needed depends on how many DNA templates are used per primer pair combination. For instance, to fingerprint 33 DNA templates using M-CAC coupled with E-AAC (IRDye 700 labeled) and E-AGG (IRDye 800 labeled) primer combinations, you will need to combine 198 μ l (= 6.0 μ l x 33) of Taq DNA polymerase working mix, 66 μ l (= 2.0 μ l x 33) of M-CAC primer solution, 16.5 μ l (= 0.5 μ l x 33) of IRDye 700 E-AAC, and 16.5 μ l (= 0.5 μ l x 33) of IRDye 800 E-AGG primers into a 1.5 ml tube. (A small amount of additional master mix is usually needed to compensate the loss due to pipetting errors.) Mix gently, centrifuge briefly, place on ice, and cover the ice bucket.

Note: IRDye® infrared dye-labeled primer is light-sensitive. To minimize exposure to light, wrap all tubes containing IRDyes (labeled primers and reaction mixes) with aluminum foil.

Thermal Cycling

1.	Pipette 9.0 μ l of <i>Taq Polymerase and Primer Mix</i> into each tube or well, then add 2.0 μ l of each diluted pre-amp template to bring the volume to 11 μ l per tube or well. Add a drop of liquid wax or mineral oil to each well if the thermal cycler has no heated lid.																																	
2.	Spin briefly. If 96-well plates have been used, centrifuge the microplate(s) at 3000 rpm for a few seconds to settle all the reagents to the bottom of the well. If such a centrifuge is not available, tap the plate(s) gently on a table or pipette all the reagents down to the bottom of the well.																																	
3.	<p>Perform PCR using a "touchdown" program:</p> <p>Program:</p> <table border="1"> <thead> <tr> <th>Step</th> <th>Temp</th> <th>Time</th> </tr> </thead> <tbody> <tr> <td>1.</td> <td>94 °C</td> <td>30 seconds</td> </tr> <tr> <td>2.</td> <td>65 °C</td> <td>30 seconds</td> </tr> <tr> <td>3.</td> <td>72 °C</td> <td>1 minute</td> </tr> <tr> <td>4.</td> <td>94 °C</td> <td>30 seconds</td> </tr> <tr> <td>5.</td> <td>65 °C minus 0.7 °C per cycle *</td> <td>30 seconds</td> </tr> <tr> <td>6.</td> <td>72 °C</td> <td>1 minute</td> </tr> <tr> <td>7.</td> <td>94 °C</td> <td>30 seconds</td> </tr> <tr> <td>8.</td> <td>56 °C</td> <td>30 seconds</td> </tr> <tr> <td>9.</td> <td>72 °C</td> <td>1 minute</td> </tr> <tr> <td>10.</td> <td>soak at 4 °C</td> <td></td> </tr> </tbody> </table> <p></p> <p>* Annealing temperatures are 65, 64.3, 63.6, 62.9, etc., for the 12 cycles.</p>	Step	Temp	Time	1.	94 °C	30 seconds	2.	65 °C	30 seconds	3.	72 °C	1 minute	4.	94 °C	30 seconds	5.	65 °C minus 0.7 °C per cycle *	30 seconds	6.	72 °C	1 minute	7.	94 °C	30 seconds	8.	56 °C	30 seconds	9.	72 °C	1 minute	10.	soak at 4 °C	
Step	Temp	Time																																
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9.	72 °C	1 minute																																
10.	soak at 4 °C																																	
4.	Add 5.0 μ l of Blue Stop Solution to each well, mix thoroughly, centrifuge briefly, and denature for 3 minutes at 94°C. After 3 minutes, place on ice and load immediately.																																	

V. Gel Electrophoresis

For AFLP[®] gel electrophoresis, LI-COR[®] 25-cm plates and KB^{Plus} (6.5%) gel are recommended. Gels with higher acrylamide concentration can be prepared using 40-50% polyacrylamide stock solutions from other manufacturers (see 4300 Applications Manual or 4200 Genetic Analysis Manual). The 0.25 mm thickness spacers and rectangular combs (either 48- or 64-tooth) are often the best choice. Some additional recommendations follow:

- Set voltage to 1500V, power to 40W, current to 40 mA, and temperature to 45°C. Set scan speed to 2 for the Model 4300 or 4 for the Model 4200.

- Pre-run the gel for 30 minutes. Flush the wells completely with a 20 cc syringe to remove urea precipitate or pieces of gel before loading.
- Load about 0.8 to 1.0 µl of each denatured sample using either the 8-channel Hamilton syringe or pipette. Load about 0.8 to 1.0 µl of the molecular size standard (50-700 bp) in the designated lanes. Two or three marker lanes are usually used for 48- or 64-well gels, respectively. The run will take about 3 hours to collect fragments up to 700 bp. The first bands will normally show up about 25 minutes after the run is started.
- After the first run is complete, gels can be reloaded once with a new set of samples, as long as the gel apparatus has not been moved. To reload a gel, create a new run, load samples and molecular weight markers, and start the second run (do not pre-run the gel).

VI. Image Collection and Analysis

AFLP® data (TIF images) from IRDye-labeled samples are automatically collected in real time during electrophoresis. Typical AFLP® fingerprints of control maize DNA, generated by several different *EcoR1/Mse1* primer combinations, are shown in Figure 2.

It is strongly recommended that you perform an AFLP® analysis using the control maize DNA samples and the protocols provided. Compare your images to Figure 2 prior to conducting other AFLP® analyses. Similar AFLP® banding patterns should be obtained. However, slight variations in the number of bands (especially faint bands) and the intensity of individual bands may be observed due to factors such as different Taq DNA polymerases, gel matrices, and running buffers.

Image data can be quickly viewed, printed, scored, analyzed, and converted into numerical data files using SAGA^{MX}, or other software. Articles cited earlier in this protocol provide additional guidance.

Figure 2. AFLP® fingerprints of control maize DNA.

Panel A: AFLP® fingerprints of control maize DNA using IRDye700 labeled *Eco*RI primer E-AGG with eight *Mse*I primers: M-CAA (1), M-CAC (2), M-CAG (3), M-CAT (4), M-CTA (5), M-CTC (6), M-CTG (7), and M-CTT (8).

Panel B: AFLP® fingerprints of control maize DNA using *Mse*I primer M-CAG with eight IRDye700 labeled *Eco*RI primers: E-AAC (a), E-AAG (b), E-ACA (c), E-ACT (d), E-ACC (e), E-ACG (f), E-AGC (g), and E-AGG (h).

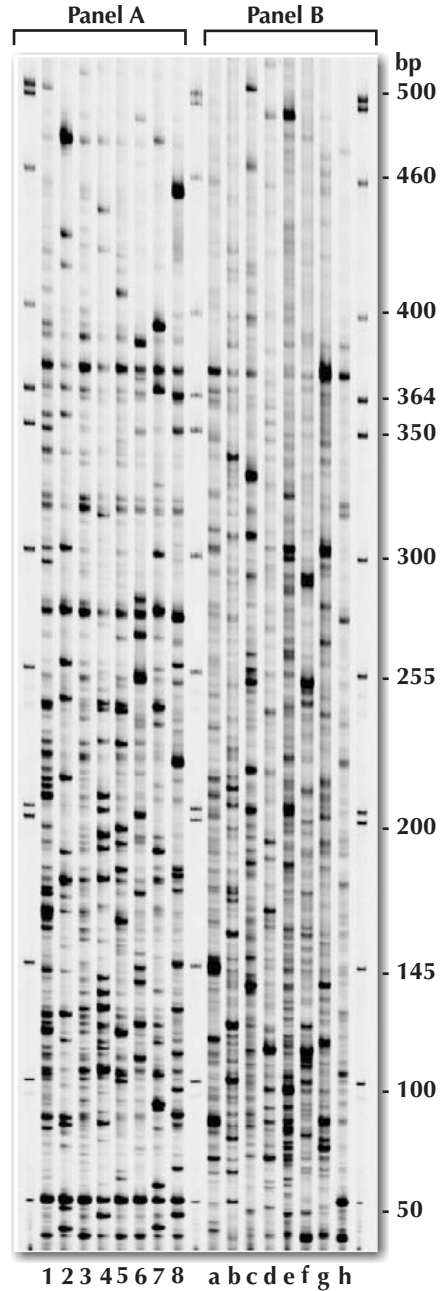


Table 1. Primer pair combinations recommended (■) for conducting selective amplifications.

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	■	■	■	■	■	■	■	■

Canola:

	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	■		■				■	■

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	■	■	■	■	■	■	■	■
E-ACG	■	■	■	■	■	■	■	■
E-ACT			■	■	■	■	■	■
E-AGC	■	■	■	■	■	■	■	■
E-AGG	■	■	■	■	■	■	■	■

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	■	■	■	■	■	■	■	

VII. AFLP® Troubleshooting Guide

Troubleshooting AFLP® Reactions

Problem	Possible Cause	Solution / Prevention
No bands or weak bands.	IRDye® infrared dye-labeled <i>EcoRI</i> primer(s) not added or degraded.	Be sure to add <i>EcoRI</i> primer(s) and avoid exposure of labeled primers to light.
	Not enough template DNA.	Be sure to have ≥75 ng of DNA per restriction digest reaction.
	DNA contaminated (e.g., high salt, EDTA, SDS, or protein).	Extract with phenol/chloroform followed by ethanol precipitation.
	Incorrect PCR conditions.	Verify the cycling program temperature, cycle number, and time; make sure that the thermal cycler is operating correctly.
	Evaporation during thermal cycling.	Cover the reactions with mineral oil or liquid wax; centrifuge briefly before incubation; check that caps fit correctly when using thermal cyclers with heated lids.
Too few high molecular weight bands.	Sub-optimal primer pair.	Use suggested primer pair for a given species (see Table 1).
	Sub-optimal quantities of primer and/or pre-amplified DNA.	Try a different quantity of IRDye <i>EcoRI</i> primer (0.3 to 0.8 µl per 11 µl selective PCR reaction) and a different dilution of pre-amplified DNA template (1:10 to 1:50).
Many high molecular weight bands.	Partial digestion.	Purify DNA.
Bands only partway up the gel.	Poor DNA and/or low polymerase activity.	Purify DNA templates and use fresh Taq DNA polymerase.
Missing lanes (nonspecific, variable).	Evaporation during thermal cycling.	Cover the reactions with oil or wax; centrifuge briefly before incubation; be sure that caps fit correctly when using thermal cyclers with heated lids.
	Pipetting error.	Verify addition and mixing of all reaction components.
Sub-optimal duplexed AFLP® results.	Competition effects of two labeled <i>EcoRI</i> primers over one <i>MseI</i> primer.	Choose two labeled <i>EcoRI</i> primers with similar predicted T _m . If the problem persists, try monoplex PCR first and pool samples later.

Troubleshooting Gel Images

Problem	Possible Cause	Solution / Prevention
Blurry bands.	Improper gel formation.	Recast gel using fresh solutions and allow gel to polymerize \geq 45 minutes.
Smeared bands.	Too much labeled primer(s).	Try less labeled <i>EcoRI</i> primer(s).
	Samples not denatured.	Add 5 μ l of stop/loading buffer and heat the sample at 95°C for 3 minutes immediately before loading gel.
	Incorrect electrophoresis conditions.	For running a 25 cm gel (LI-COR [®] 6.5% KB ^{Plus}), set temperature to 45°C, voltage to 1500- 2000 volts, current to 40 mA, power to 40 W, and scan speed to 2 (Model 4300).
	Differences between gel and running buffer.	Verify that the buffer in the gel and the running buffer are the same concentration (1X TBE).
Wavy bands.	The gel surface did not polymerize evenly.	Recast a gel and make sure that wells are free of excessive urea and unpolymerized gel solution.
	Wells are not formed properly.	Make sure to pull a rectangular-tooth comb straight out carefully.
	Binding silane not used in gel preparation.	Apply binding silane to front plate before casting gel.
	Air bubbles in gel.	Recast gel, tap gel apparatus to ensure smooth flow of gel.
Smiling gels.	Uneven gel thickness.	Do not over-tighten the rails or upper buffer tank to avoid uneven thickness of the gel.
Outer lane(s) missing.	Comb is not centered.	Be sure to insert the comb in the center of the gel.
	Improper running buffer.	Be sure to use freshly made buffer (1X TBE) to perform gel electrophoresis.

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3. Treatment of disease and disease predisposition in humans and animals;
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Biosciences

4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA

Technical Support: 800-645-4260

North America: 800-645-4267

International: 402-467-0700 • 402-467-0819

LI-COR GmbH (Germany, Austria, Switzerland, Czech Republic, Hungary, Slovakia): +49 (0) 6172 17 17 771

LI-COR UK Ltd.: +44 (0) 1223 422104

www.licor.com