# AFLP technology for DNA fingerprinting

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**The AFLP technique is a powerful DNA fingerprinting technology applicable to any organism without the need for prior sequence knowledge. The protocol involves the selective PCR amplification of restriction fragments of a total digest of genomic DNA, typically obtained with a mix of two restriction enzymes. Two limited sets of AFLP primers are sufficient to generate a large number of different primer combinations (PCs), each of which will yield unique fingerprints. Visualization of AFLP fingerprints after gel electrophoresis of AFLP products is described using either a conventional autoradiography platform or an automated LI-COR system. The AFLP technology has been used predominantly for assessing the degree of variability among plant cultivars, establishing linkage groups in crosses and saturating genomic regions with markers for gene landing efforts. AFLP fragments may also be used as physical markers to determine the overlap and positions of genomic clones and to integrate genetic and physical maps. Crucial characteristics of the AFLP technology are its robustness, reliability and quantitative nature. This latter feature has been exploited for co-dominant scoring of AFLP markers in sample collections such as $F_2$ or back-cross populations using appropriate AFLP scoring software. This protocol can be completed in 2–3 d.**

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## INTRODUCTION

The AFLP (amplified fragment length polymorphism) method is a DNA fingerprinting technique based on selective PCR amplification of restriction fragments from a total digest of genomic DNA of any origin or complexity such as prokaryotes, plants, animals and human[1]. The AFLP technique was originally conceived for the construction of high-density linkage maps for application in positional cloning of genes and molecular breeding. Because the AFLP technology is essentially suited for fingerprinting and mapping of any genomic DNA, it is equally suited for applications in genetic analysis such as genetic relationship and diversity assessments (e.g., plants[2], bacteria[3]), establishment of 'essential derivation' among plant varieties[4,5] and association studies in natural[6] and breeding populations[7–9]. In addition, the AFLP technology is applicable for genome research, such as characterizing the level and target sites of cytosine methylation[10,11], fingerprinting and identifying overlapping clones in the construction of high-resolution BAC (bacterial artificial chromosome) physical maps[12], for integrating physical and genetic maps[13,14] and for high-throughput enrichment of radiation hybrid maps[15].

AFLP markers offer several advantages over other currently used DNA markers, such as simple sequence repeats and single nucleotide polymorphisms. Foremost among these is that the AFLP technology requires no prior sequence information and, hence, has a relatively low start-up cost. In addition, the AFLP technique is very amenable to automation and is highly multiplexed, which offers the potential to improve the efficiency and to increase the throughput of marker data production in organisms that lack the genomics platform necessary to allow the development of genotyping microarrays. The AFLP technique also has a number of limitations. In case of low (less than 90%) overall sequence homologies between samples, AFLP fingerprints will share very few common fragments. It has indeed been shown in bacteria that relationships at the subspecies level may not be detected by the AFLP technique[3]. The AFLP method cannot, therefore, be used for comparative genome analysis. Detection of markers in genomic DNA with very little sequence variation may be poor despite the large numbers of fragments that can be tested for polymorphisms. In these cases, marker systems that combine AFLP with microsatellites[16] or transposons[17] may be superior.

The first step of the AFLP procedure (see **Fig. 1**) involves the preparation of templates by restriction digestion of DNA, typically

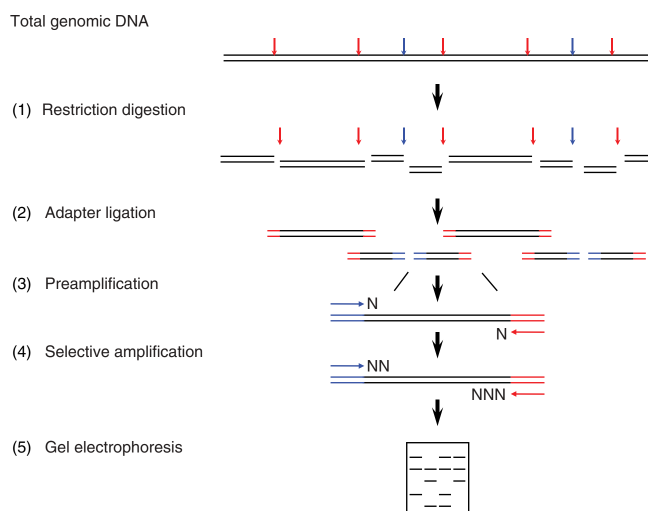


**Figure 1 |** Outline of the AFLP procedure. Template fragments are generated by: (1) digestion of genomic DNA with a combination of the two restriction enzymes *Eco*RI and *Mse*I (blue and red arrows represent *Eco*RI and *Mse*I restriction enzyme sites, respectively); (2) ligation of the double-stranded *Eco*RI- (blue) and *Mse*I- (red) specific adapters to the fragment ends; (3) a pre-amplification step using primers that match the adapter sequences and that carry each one selective nucleotide (represented by N) at their 3 end are used to PCR-amplify subsets of the *Eco*RI/*Mse*I templates; (4) a final selective PCR-amplification step in which additional selective nucleotides are added to the *Eco*RI and *Mse*I primers; and (5) the electrophoretic size fractionation and the display on denaturing polyacrylamide gels of the *Eco*RI/*Mse*I amplification products.

**Figure 2 |** Schematic for adapter and primer design for the two rare cutters *Eco*RI and *Pst*I and the frequent cutter *Mse*I. Adapters consist of a core sequence (black) and an enzyme-specific sequence (red). The enzyme-specific sequence allows the ligation of the adapters to the resulting restriction fragments (green) without restoring the original restriction sites. In this way, ligated adapters create a target site for the AFLP primers in the subsequent amplification reactions. For this purpose, primer design matches the core (black), the enzyme-specific (red) and the restriction-site remnant (green) sequence. Primers may have one or a number of additional bases at the 3-end extending into the restriction fragments, called the selective nucleotides (represented by N, in blue). AFLP primers are named '+0' when they have no selective bases (only the core, enzyme-specific and restriction-site remnant sequence), '+1' when they have a single selective base, '+2' when they have two selective bases, and so on. Adapters and primers for other restriction enzymes are similar to these but have enzyme-specific parts corresponding to the respective enzymes.

*Eco*RI-adapter

5′-CTCGTAGACTGCGTACC AATTCGAC-internal sequence-3′
3′-CATCTGACGCATGG-TTAAGCTG-internal sequence-5′

*Pst*I-adapter

5′-CTCGTAGACTGCGTACA-TGCAGGAC-internal sequence-3′
3′-CATCTGACGCATGT ACGTCCTC-internal sequence-5′

*Mse*I-adapter

5′-GACGATGAGTCCTGAG TAAGAC-internal sequence-3′
3′-TACTCAGGACTC-ATTCTG-internal sequence-5′

*Eco*RI-primer 5′-GACTGCGTACCAATTCNNN-3′

*Pst*I-primer 5′-GACTGCGTACATGCAGNNN-3′

*Mse*I-primer+0 5′-GATGAGTCCTGAGTAA-3′
*Mse*I-primer+1 5′-GATGAGTCCTGAGTAAG-3′
*Mse*I-primer+2 5′-GATGAGTCCTGAGTAAGA-3′

with two different restriction enzymes. The two restriction enzymes used are generally (but not necessarily) a rare cutter and a frequent cutter. The frequent cutter is used to generate fragments that are in the 50–500 bp length range resolvable by electrophoresis. The rare cutter is used to limit the number of fragments that can be amplified and, hence, to define the number of effective AFLP amplicons. Subsequently, double-stranded adapters are ligated to the ends of the restriction fragments. The second step of the AFLP procedure is the PCR amplification of subsets of restriction fragments using selective AFLP primers. The common parts of these primers correspond to the adapter and restriction enzyme recognition sequences, and they have a number of additional bases at the 3′-end extending into the restriction fragments, called the selective nucleotides (see **Fig. 2**). These selective nucleotides ensure that only a subset of restriction fragments is amplified to a detectable level, that is, those fragments where the nucleotides flanking the restriction site match the primer extensions. AFLP fingerprinting of low-complexity DNA (plasmids, cosmids and BACs) requires no selective nucleotides. For small genome-sized (5–100 Mb) organisms such as bacteria and fungi, up to two selective nucleotides for each of the AFLP primers are commonly required. AFLP fingerprinting of more complex, large genomes (greater than 100 Mb) is usually carried out with two or three selective bases in one or both primers and is generally performed in two consecutive steps: a pre-amplification step, reducing the complexity of the template mixture, and a final selective step. Detection of the AFLP fragments is made possible by radioactive or fluorescent labeling of one of the two AFLP primers used in the final selective amplification reaction. The final step of the AFLP technique is the electrophoretic size fractionation of the fingerprints. For this purpose the labeled reaction products are separated on denaturing polyacrylamide gels similar to those used for sequencing. In the case of conventional gel electrophoresis using radio-labeled primers, gels are either dried on paper or fixed on glass plates after electrophoresis, and AFLP images may be generated using either conventional autoradiography or phosphorimaging technology (**Fig. 3a**). In the case of gel electrophoresis using infrared dye (IRD) or fluorescently labeled primers, AFLP images may be generated using LI-COR (see **Fig. 4**) or Applied Biosystems (ABI) and MegaBACE automated DNA sequencers, respectively.

A crucial characteristic of the AFLP technology is the quantitative nature of the competitive PCR amplification of restriction fragments, which is based on the kinetics of the AFLP amplification reaction. Specifically, a single pair of PCR primers is used to amplify a subset of restriction fragments simultaneously, with each particular fragment from the subset competing for the same primers according to its initial relative abundance. These competitive amplification kinetics remain for as long as effective amplification can be sustained in the reaction mixture. Owing to this feature, the relative intensity of a band in an AFLP fingerprint pattern reflects the original abundance of that fragment in the AFLP template, such that band intensities can be compared among samples[18,19] as long as loading differences between lanes are accounted for. Because genomic polymorphisms manifest themselves predominantly as single-base mutations that affect either the restriction site or the selective bases immediately adjacent to them, such polymorphisms result in dominant PCR phenotypes; that is, the presence of a mutation causes the loss of a fragment from a fingerprint. In the case of a heterozygous mutation, however, the difference between $2n$ and $1n$ can be clearly distinguished from the band intensities, which reflect PCR product concentrations (100 and 50%, respectively). This quantitative nature of the AFLP technology has been exploited widely for routine co-dominant scoring of AFLP markers in segregating populations (see below for further discussion), establishing that the technology can clearly be used to discriminate homozygous from heterozygous loci in the majority of cases[20].

The AFLP protocol described below and illustrated in **Figure 1** details all steps in the AFLP procedure, except the isolation of total DNA, as follows: (i) the preparation of template fragments by digestion of the genomic DNA with a combination of the two restriction enzymes *Eco*RI and *Mse*I; (ii) the ligation of double-stranded adapters to the fragment ends; (iii) a first selective PCR amplification, also called pre-amplification, of *Eco*RI/*Mse*I fragments with a combination of an *Eco*RI and an *Mse*I primer with one selective base each; (iv) a final selective amplification step in which additional selective nucleotides are used in the *Eco*RI and *Mse*I primers; (v) the electrophoretic analysis of the 50–100 *Eco*RI/*Mse*I amplification products on standard denaturing polyacrylamide gels and detection of AFLP fragments using either conventional gel electrophoresis, radio-labeled primers and autoradiography, or LI-COR automated DNA sequencers and IRD detection technology. The two detection options are virtually identical through the preparation of template fragments, except in the final selective amplification step, where IRD-labeled primers are substituted for the radioactively labeled primers when using the LI-COR automated DNA sequencer.

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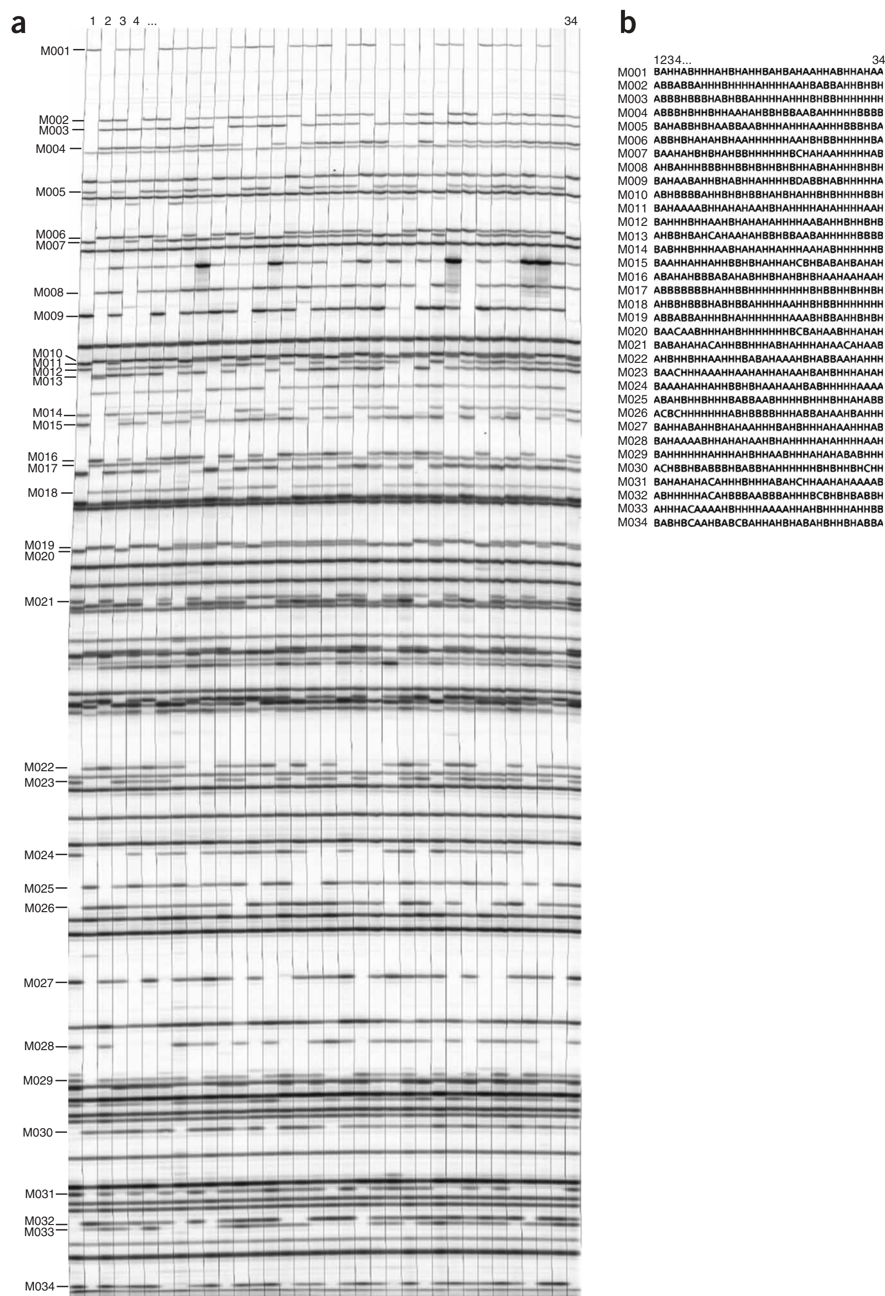


**Figure 3 |** AFLP analysis of 32 tomato $F_2$ segregants. (**a**) Gel image of the AFLP analysis of 32 tomato $F_2$ segregants (in lanes 3–34) and their parental lines (in lanes 1 and 2) using a *Pst*I + AT/*Mse*I + CTG primer combination. Selective amplification was performed using a [$\gamma$-$^{33}$P]ATP-labeled *Pst*I+AA primer and fingerprints were visualized using phosphorimaging technology. Thirty-four AFLP markers segregating in this $F_2$ population were co-dominantly scored using AFLP-QuantarPro software. The goal of co-dominant scoring of AFLP markers is unequivocally to distinguish homozygous from heterozygous genotypes based on quantitative measurements of the band intensities. This allows extraction of more genetic information from AFLP fingerprints than dominant (presence/absence) scoring. For homozygous individuals (2*n*), the band intensity is expected to be twice that for heterozygous individuals (1*n*). Thus, if band intensities can be measured quantitatively, the difference between 2*n* (100%) and 1*n* (50%) for an AFLP locus can be scored. In this example, 34 co-dominant AFLP markers are indicated, labeled M001-M0034 on the left-hand side. (**b**) AFLP dataset resulting from the AFLP analysis shown in (**a**). The dataset consists of 34 AFLP markers (labeled M001-M034 on the left hand side) which were co-dominantly scored using AFLP-QuantarPro software in the 32 $F_2$ individuals (labeled 3-34 across the top) and their parental lines (labeled 1-2 across the top). In the case of an $F_2$ population, individuals' marker genotypes may be assigned to one of the three genotype classes (A: homozygous absent; H: heterozygous; B: homozygous present). If heterozygous genotypes can not unequivocally be distinguished from homozygous B genotypes, a C genotypic score (i.e. not genotype A) will be assigned. If heterozygous genotypes can be distinguished from homozygous A genotypes, a D genotypic score (i.e. not genotype B) will be assigned. Such AFLP datasets typically serve as a starting point for further genetic analysis.

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## Experimental design

**Preparation and quality assessment of genomic DNA.** Complete restriction is crucial for good quality of AFLP fingerprints. DNA preparations, therefore, need to be of sufficient quality to allow complete digestion by the restriction enzymes. A minimum of 100 ng of eukaryotic DNA is recommended for template generation. DNA concentrations can be determined by measuring $OD_{260}$, but we recommend running a small aliquot of DNA on a 1% agarose gel, next to a series of phage-λ DNA dilutions ranging from 50 to 500 ng. The gel image will also allow inspection of the integrity of the original DNA. Substantial smearing below the main band of high molecular weight DNA may be detrimental for AFLP fingerprinting quality.

**Choice of restriction enzymes.** The protocol describes the generation of templates for AFLP reactions by the restriction of the DNA with the restriction enzyme combination (EC) *Eco*RI/*Mse*I, which is one of the most commonly used in plant species and micro-organisms (for the latter see, e.g., AFLP analysis system for micro-organisms; http://www.invitrogen.com). Obviously, altering the EC requires appropriate design of adapter and amplification primers and adaptation of the digestion conditions. The selection of the appropriate ECs is determined by the efficiency of polymorphism detection, the genome coverage and AFLP marker distribution. Genome coverage and AFLP marker distribution are mainly determined by the AT-content of the DNA. For AT-rich genomes, including most eukaryotic DNAs, AT-rich restriction enzymes such as *Mse*I (recognition sequence TTAA) and *Eco*RI (recognition sequence GAATTC) will generally produce good genome coverage and fragments that are in the optimal size range for both PCR amplification and separation on denaturing polyacrylamide gels. For CG-rich genomes, CG-rich restriction enzymes such as *Pst*I (recognition sequence CAGCTG) and *Taq*I (recognition site TCGA) are more appropriate restriction enzymes.

The efficiency of polymorphism detection by AFLP can be adjusted by targeting CpG dinucleotide motifs in mammals (e.g., by the use of *Taq*I) or CpNpG trinucleotide motifs (e.g., by the use of *Pst*I) in plants. Since such motifs are prone to an increased level of mutation, an increased level of polymorphism detection is observed. For example, in plants (e.g., maize and sorghum) sets of *Pst*I/*Mse*I primer combinations (PCs) have a significantly higher polymorphism information content than sets of *Eco*RI/*Mse*I PCs[11,21]. In farm animal species (chicken[22], cattle[23] and pig[24]), the EC of *Eco*RI/*Taq*I is most commonly used. However, other ECs have been used in farm animal species as well, such as *Eco*RI/*Hin*PI and *Eco*RI/*Msp*I in chicken[25] and *Eco*RI/*Hin*PI in turkey[26]. For parasites, such as *Oesophagostomum*, evaluation of different ECs demonstrated that the use of *Hin*dIII/*Bgl*II, a combination of two six cutters, was the most effective to investigate genetic diversity by the AFLP method[27].

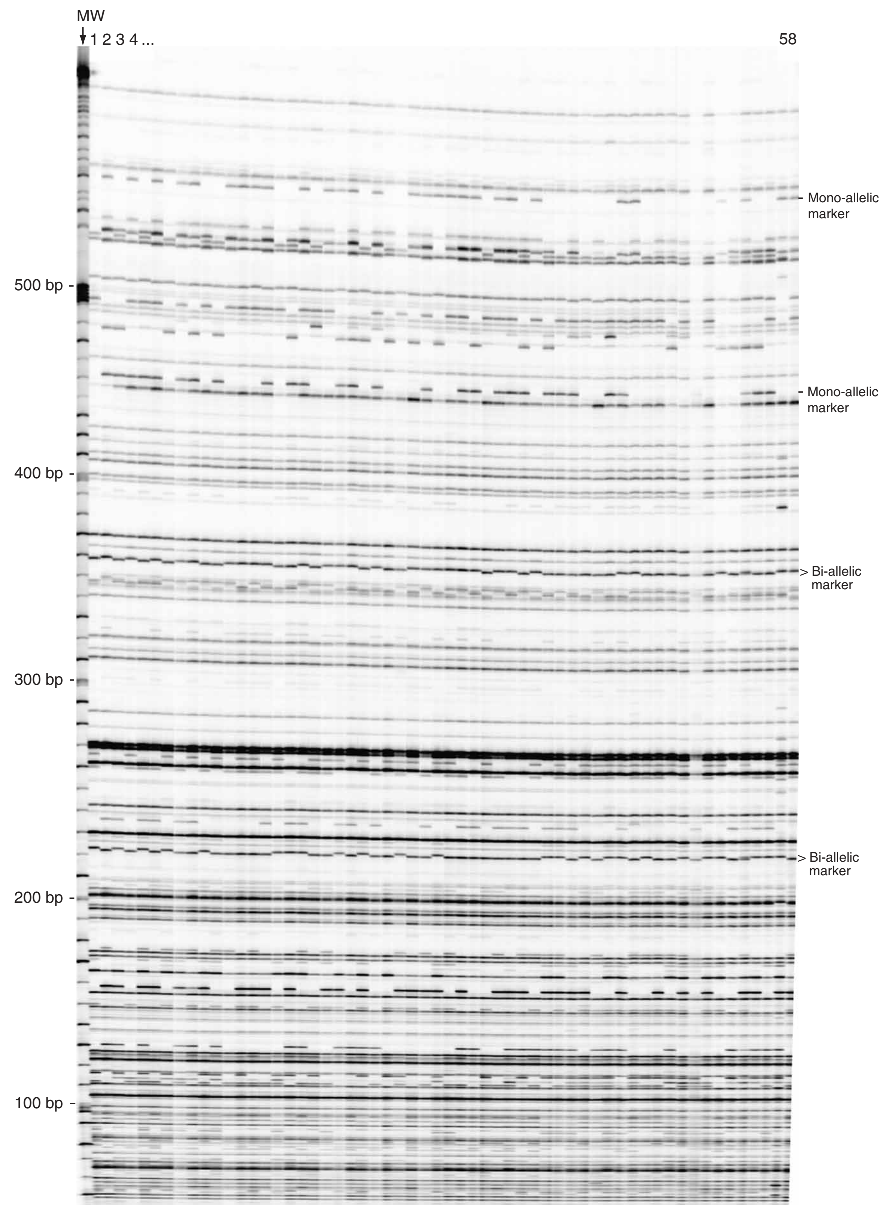


**Figure 4 |** Gel image of an AFLP analysis of 56 *Arabidopsis* Recombinant Inbred Line (RIL) segregants analyzed using an *Eco*RI + AA/*Mse*I + CAA primer combination. RILs are shown in lanes 3–58 and their parental lines in lanes 1 and 2. A 10-bp molecular weight (MW) marker was included on the left of the gel image. Selective amplification was performed using an infrared dye 700–labeled *Eco*RI + AA primer, and digital images of the fingerprints were obtained from the LI-COR automated sequencer. Because single-base mutations affect either the restriction site or the selection bases immediately adjacent to them, the presence of such a mutation causes the loss of an AFLP fragment from a fingerprint and results in a mono-allelic AFLP marker segregating present/absent in a RIL mapping population. An insertion/deletion in the sequence of one of the two AFLP fragment alleles causes a size difference between the two AFLP marker alleles, resulting in two AFLP markers showing a complementary present/absent segregation pattern. It is clear from this gel image that bi-allelic AFLP markers are identified with a much lower frequency than mono-allelic AFLP markers. Two examples of each type of AFLP marker are pointed out on the figure.

**Template preparation.** Adapters consist of a core sequence and a restriction enzyme–specific sequence (**Fig. 2**) and are prepared by adding equimolar amounts of both strands. Adapters are not phosphorylated, to prevent adapter–adapter ligation. The sequence allows the ligation of the adapters to he resulting restriction fragments without restoring the original restriction sites. Ligated adapters create a target site for the AFLP primers in the subsequent amplification reactions.

**Primer design and preparation.** For selective amplification of subsets of AFLP templates, primers are used that correspond to the core and the enzyme-specific sequence of the adapter and to the remnant sequence of the restriction site (**Fig. 2**). They have one or a number of (up to three) additional bases at the 3′-end extending into the restriction fragments, called the selective nucleotides (**Fig. 2**). AFLP primers are named '+0' when they have no selective bases (only the core and enzyme-specific sequence), '+1' when they have a single selective base, '+2' when they have two selective bases, and so on. Only one of the two selective AFLP primers used is labeled in the final selective amplification step because the mobilities of the two strands of a DNA fragment on sequencing gels are generally slightly different, resulting in a lower resolution of the PCR product on the gel. There is no preference which primer to label, but generally the primer corresponding to the rare cutter is chosen. Selective amplification of AFLP fragments obtained with other restriction ECs than detailed here can be performed using essentially the same protocol with appropriate primers and adapters.

**Choice of radioactive versus fluorescent detection systems.** LI-COR and ABI have adapted the AFLP technique for use with IRD or fluorescent dye detection technology, respectively. Detection of

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AFLP fragments using IRD or fluorescent dye detection technology offers several advantages over conventional detection using radio-labeled primers and autoradiography: the use of radioactivity is eliminated, the cost of dye-labeled primers is less than the cost of corresponding amounts of radionucleotides for radiolabeling primers and images are obtained in several hours rather than 1–3 d. In addition to the safety and convenience benefits of AFLP analysis on automated sequencers, the throughput of the AFLP analysis can be doubled by running multiplexed AFLP reactions on two-dye LI-COR[28] and tripled by detection using ABI or MegaBACE automated sequencers. Furthermore, owing to the larger separation power of capillary electrophoresis instruments, larger numbers of AFLP markers can be scored using these systems, specifically those in the 500–800-bp size range, which increases efficiency further.

This protocol does not describe the automated AFLP analysis using ABI or MegaBACE automated sequencers and fluorescent dye detection technology. For more information on AFLP microbial and plant fingerprinting using the ABI DNA automated sequencers, we refer the reader to http://docs.appliedbiosystems.com/pebiodocs/00402977.pdf and http://docs.appliedbiosystems.com/pebiodocs/04303146.pdf, respectively.

**Factors affecting co-dominant scoring of AFLP markers.** Various factors affect the proportion of AFLP markers that can be co-dominantly scored based on fragment intensity levels. One of the main factors in this respect is the genetic relatedness of individual samples included in the co-dominant scoring process. Specifically, it is commonly observed that high proportions of co-dominantly scored AFLP markers are usually reached in genetically less complex sample collections such as $F_2$ or back-cross populations, and lower percentages are observed when screening large germplasm collections. Second, the ploidy level of the organisms is important; that is, co-dominant scoring is easier in diploid organisms than in tetra- or hexaploid organisms. Third, DNA quality is important with respect to co-dominant scoring, as increased background signal levels (probably caused by incomplete restriction/ligation) adversely affect fragment quantification. Fourth, fixing gels on the glass plate results in a better resolution and, hence, in a more accurate estimation of the band intensities. Finally, appropriate AFLP scoring software such as AFLP-Quantar*Pro* (http://www.keygene-products.com) is essential for accurate fragment quantification and, hence, co-dominant scoring.

## MATERIALS

### REAGENTS

- Tris (Biosolve, cat. no. 20092391) **! CAUTION** Irritating to eyes and skin. Wear suitable protective clothing. Avoid contact with skin and eyes. Do not breath dust.
- Tris-buffers (see REAGENT SETUP)
- EDTA disodium salt:dihydrate ($M_r$ = 372.2 g mol$^{-1}$; Duchefa biochemie, cat. no. E0511.1000) **! CAUTION** Irritating to eyes. Avoid contact with the eyes. Wear suitable protective clothing.
- SYBR Safe DNA stain in DMSO solution (Invitrogen, cat. no. S33102) **! CAUTION** DMSO is irritating to eyes and skin. Avoid contact with the eyes. Wear suitable protective clothing.
- Ethidium bromide (EtBr; Merck, cat. no. 1.11608.0030) **! CAUTION** Harmful; possible risk of irreversible effects. Wear suitable protective clothing and gloves.
- *Eco*RI (New England Biolabs, cat. no. R0101S)
- *Mse*I (New England BioLabs, cat. no. R0525S)
- Acetic acid (HAc; Merck, cat. no. 1.00062.1000) **! CAUTION** Corrosive; flammable; causes severe burns. Do not breathe vapor. Wear suitable protective clothing, gloves and eye/face protection.
- Magnesium acetate (MgAc; Merck, cat. no. 1.05819.0250) (see REAGENT SETUP)
- Potassium acetate (KAc; Merck, cat. no. 1.04820.1000) (see REAGENT SETUP)
- Sodium acetate (NaAc; Merck, cat. no. 1.06268.250)
- DTT (Immunosource, cat. no. 502A) **! CAUTION** Harmful by inhalation, in contact with skin and if swallowed. Irritating to eyes, respiratory system and skin. Do not breathe dust. Wear suitable protective clothing.
- BSA (New England BioLabs, cat. no. B9001S)
- *Mse*I-F (Invitrogen) 5′-GACGATGAGTCCTGAG-3′
- *Mse*I-R (Invitrogen) 5′-TACTCAGGACTCAT-3′
- *Mse*I-adapter (see REAGENT SETUP)
- *Eco*RI-F (Invitrogen) 5′-CTCGTAGACTGCGTACC-3′
- *Eco*RI-R (Invitrogen) 5′-AATTGGTACGCAGTCTAC-3′
- *Eco*RI-adapter (see REAGENT SETUP)
- RL buffer (see REAGENT SETUP)
- $T_{10}E_{0.1}$ buffer (see REAGENT SETUP)
- T4 buffer (see REAGENT SETUP)
- IRD 700-labeled selective *Eco*RI primers for product detection using an automated LI-COR system (Biolegio)
- Selective *Mse*I primers (Invitrogen) 5′-GATGAGTCCTGAGTAAN$_{1–3}$-3′, where N represents the selective nucleotides
- Selective *Eco*RI primers (Invitrogen) 5′-GACTGCGTACCAATTCN$_{1–3}$-3′, where N represents the selective nucleotides
- T4 DNA-ligase (Invitrogen, cat. no. 15224-017)
- ATP 100 mM solution, 25 μmol (GE Healthcare, cat. no. 27-2056-01)
- Hydrochloric acid (HCl; Merck, cat. no. 1.00317.1000) **! CAUTION** Corrosive; causes burns. Irritating to respiratory system. Wear suitable protective clothing, gloves and eye/face protection.
- AmpliTaq DNA polymerase with PCR buffer 10× and $MgCl_2$ (25 mM) (ABI, cat. no. N8080153)
- dNTP set (dATP, dCTP, dGTP, dTTP), 100 mM solutions, 4 × 25 μmol (GE Healthcare, cat. no. 27-2035-01)
- [$\gamma$-$^{33}$P]ATP (GE Healthcare, cat. no. BF1000-8MCI) **! CAUTION** May cause cancer. May cause heritable genetic damage. Also harmful by contact with skin and if swallowed. Avoid exposure-obtain special instruction before use. Wear suitable protective clothing.
- T4 polynucleotide kinase (New England Biolabs, cat. no. M0201S)
- Spermidine-3HCl (Sigma-Aldrich, cat. no. S2501) **! CAUTION** Very toxic by inhalation. Irritating to the eyes, and to the skin. Wear suitable protective clothing, gloves and eye/face protection.
- Formamide (Sigma-Aldrich, cat. no. 47670) **! CAUTION** May cause cancer. May cause harm to the unborn child. May cause long-term adverse effects in the aquatic environment. Avoid exposure-obtain special instruction before use.
- Bromophenol blue (Merck, cat. no. 1.08122.0005) **! CAUTION** Irritating to eyes; avoid contact with skin. Wear suitable protective clothing.
- Xylene cyanol (Merck, cat. No. 1.10590.0005)
- Glycerol (Merck, cat. no. 1.04094.1000)
- 3-Methacryloxypropyltrimethoxysilane (Bind-Silane; Serva, cat. no. 28739.01) (see REAGENT SETUP) **! CAUTION** Irritating to eyes, respiratory system and skin. Wear suitable protective clothing.
- Acrylamide/bis-acrylamide (AabAA) 19:1 ready-made 40% mix solution (Biosolve, cat. no. 01352335) **! CAUTION** May cause cancer. May cause heritable genetic damage. Harmful by inhalation and in contact with skin. Also toxic if swallowed. Irritating to eyes and skin. Avoid exposure—obtain special instruction before use. Wear suitable protective clothing.
- Urea (USB, cat. no. 75826)
- Boric acid (Merck, cat. no. 1.00165.1000)
- Ammonium persulfate (APS; Sigma-Aldrich, cat. no. A9164) (see REAGENT SETUP) **! CAUTION** Oxidizing; harmful if swallowed. Toxic in contact with skin. Very toxic by inhalation. Irritating to eyes; may cause sensitization by inhalation and skin contact. Do not breathe the dust. Avoid contact with skin. Wear suitable gloves.

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- *N,N,N′,N′*-tetramethylethane-1,2-diamine (TEMED; Merck, cat. no. 1.10732.0100) ! CAUTION Highly flammable, corrosive, harmful by inhalation and if swallowed. Causes burns. Wear suitable protective clothing, gloves and eye/face protection. ▲ CRITICAL Store in the dark and keep bottle closed.
- AG501-X8 mixed-bed resin (BioRad, cat. no. 142-6424)
- Long Ranger stock solution 50% (Cambrex Bio Science Rockland, cat. no. 50611E) (see REAGENT SETUP) ! CAUTION May cause cancer. May cause heritable genetic damage. Harmful by inhalation and in contact with skin. Also toxic if swallowed. Irritating to eyes and skin. Avoid exposure—obtain special instruction before use. Wear suitable protective clothing.
- Ultrapure 10× TBE buffer (1.0 M Tris, 0.9 M boric acid, 0.01 M EDTA) (Invitrogen, cat. no. 15581-044)
- SmartLadder SF (Eurogentec, cat. no. MW-1800-04)
- Repel-Silane ES (GE Healthcare, cat. no. 17-1332-01), 2% ready-made solution of dimethyldichlorosilane dissolved in octamethylcyclo-octosilane ! CAUTION Possible risk of impaired fertility. May cause long-term adverse effects in the aquatic environment. Wear suitable protective clothing and gloves.
- Maxam 10× (see REAGENT SETUP)
- Agarose gel (see REAGENT SETUP)
- TAE running buffer (see REAGENT SETUP)

**EQUIPMENT**

- ICycler thermal cycler (BioRad)
- Thermomixer comfort (Eppendorf)
- Glass plates (BioRad)
- SequiGenGT 38 × 50 cm gel apparatus (BioRad)
- PowerPac 3000 (BioRad)
- Phosphorimager analysis system (Fuji Bas-2000 or GE-Healthcare 445 SI)
- Imaging plates (Fuji or GE-Healthcare)
- LI-COR long read-IR$^2$ 4200 (LI-COR Biosciences)
- Gel apparatus set (25 cm) (LI-COR Biosciences)
- Whatman pure cellulose blotting sheets (3 MM Chr) 35 × 43 $cm^2$ (Schleicher & Schuell BioScience, cat. no. 3030-347)
- Heto dry GD-I (Heto Lab Equipment Denmark, manufactured by Hoefer Scientific instruments)

**REAGENT SETUP**

**Tris-buffers [1 M Tris–HCl (pH 8.0), 1 M Tris–HCl (pH 7.5) and 1 M Tris–HAc (pH 7.5)]** Dissolve 12.1 g Tris in approximately 80 ml water. Add concentrated HCl or HAc (depending on the buffer) a little at a time to reach desired pH. Finally, add water to 100 ml and autoclave. ▲ CRITICAL Make sure buffer is at room temperature (20–22 °C) before making final pH adjustments, as the pH of Tris-buffers changes with increasing temperature. Store for up to 6 months at room temperature.

**1 M MgAc** Dissolve 2.145 g MgAc in water. Add water to 10 ml. Filter-sterilize. Store for up to 6 months at room temperature.

**4 M KAc** Dissolve 3.926 g KAc in water. Add water to 10 ml. ▲ CRITICAL Store for up to 6 months at −20 °C.

**1 M $MgCl_2$** Dissolve 2.033 g $MgCl_2$ in water. Add water to 10 ml. Filter-sterilize. Store for up to 6 months at room temperature.

**0.5 M EDTA (pH 8.0)** Dissolve approximately 9 g NaOH in 400 ml water. Add 93.05 g EDTA and stir over low heat on stir plate until dissolved. ▲ CRITICAL EDTA does not dissolve at pH less than 7.0. Add NaOH pellets to reach pH 8.0. Add water to 500 ml and autoclave. Store for up to 6 months at room temperature.

**dNTP** 5 mM of each dNTP (dATP, dGTP, dCTP, dTTP) ▲ CRITICAL Store in aliquots (up to 2 ml) at −20 °C for up to 6 months.

***Eco*RI-adapter (5 pmol $\mu l^{-1}$)** 5 µl *Eco*RI-F(100 µM), 5 µl *Eco*RI-R (100 µM), 90 µl $H_2O$. Store for up to 6 months at −20 °C.

***Mse*I-adapter (50 pmol $\mu l^{-1}$)** 25 µl *Mse*I-F (100 µM), 25 µl *Mse*I-R (100 µM). Store for up to 6 months at −20 °C.

**RL buffer 10×** Mix 1 ml 1 M Tris-HAc (pH 7.5) with 1 ml 1 M MgAc, 1.25 ml 4 M KAc, 0.077 g DTT, 50 ng $\mu l^{-1}$ BSA (optional). Add water to 10 ml. ▲ CRITICAL Store in aliquots (up to 2 ml) at −20 °C for up to 6 months.

**$T_{10}E_{0.1}$ buffer** Mix 1 ml 1 M Tris-HCl (pH 8.0) with 20 µl 0.5 M EDTA (pH 8.0). Add water to 100 ml. Store for up to 6 months at room temperature.

**T4 buffer 10×** Mix 2.5 ml 1 M Tris-HCl (pH 7.5) with 1 ml 1 M $MgCl_2$, 0.077 g DTT and 0.013 g spermidine-3HCl. Add water to 10 ml. ▲ CRITICAL Store in aliquots (up to 2 ml) at −20 °C for up to 6 months.

**APS 10%** Dissolve 1 g APS in water and adjust to a final volume of 10 ml. ▲ CRITICAL The APS solution must be freshly made.

**Bind-Silane solution** Add 30 µl HAc and 30 µl Bind-Silane to 10 ml ethanol. ▲ CRITICAL The Bind-Silane solution must be freshly made immediately before use.

**Maxam 10×** Dissolve 309 g boric acid and 605 g Tris in water and adjust to a final volume of 5 l. Store for up to 6 months at room temperature.

**Running buffer for LI-COR** Dilute Ultrapure 10× TBE buffer tenfold. Must be freshly made.

**10× TAE running buffer for agarose gels** Dissolve 48.4 g Tris in 250 ml water and add 11.4 ml HAc and 20 ml 0.5 M EDTA (pH 8.0). Adjust to a final volume of 1 l. Store for up to 6 months at room temperature.

**1% agarose gel** Add 1 g agarose to 100 ml 0.5× TAE running buffer. Heat in a microwave oven until completely melted. Most commonly, EtBr is added to the gel (final concentration 0.5 µg $ml^{-1}$) at this point to facilitate visualization of DNA after electrophoresis. After the solution is cooled to approximately 60 °C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

**4.5% Denaturing polyacrylamide gel solution 10×** Mix 450 g urea and 112.5 ml AAbAA 19:1 40% stock solution. Add water to a final volume of 700 ml. Stir the solution at 60 °C and filter. Add 100 ml Maxam 10× and 4 ml EDTA 0.5 M. Add water to a final volume of 1,000 ml. ▲ CRITICAL Store the gel solution at 4 °C in the dark for up to 30 d.

**6% Long Ranger gel solution (7 M urea/1.2× TBE)** Mix 3 ml Long Ranger stock solution (50%), 10.5 g urea, 3 ml 10× TBE buffer and 11 ml water. ▲ CRITICAL Do not prepare and store pre-mix solutions made from 50% Long Ranger gel solution.

**Formamide loading dye for radioactive gels** Mix 98 ml formamide, 2 ml 10 mM 0.5 M EDTA (pH 8.0), 0.06 g bromophenol blue and 0.06 g xylene cyanol. ▲ CRITICAL Store at 4 °C in the dark or at −20 °C for up to 6 months.

**Formamide loading dye for LI-COR gels** Mix 30 g AG50 1-X8 mixed-bed resin, 480 ml formamide, 20 ml 0.5 M EDTA (pH 7.5). Stir for 20 min. Add 40 mg bromophenol blue, mix and filter. ▲ CRITICAL Store at 4 °C in the dark or at −20 °C for up to 6 months.

**6× Loading dye for agarose gel** Dissolve 0.025 g xylene cyanol in 5 ml of water. Add 3 ml glycerol and adjust to a final volume of 10 ml with water. ▲ CRITICAL Store at 4 °C in the dark or at −20 °C for up to 6 months.

**Radiolabeled selective *Eco*RI primers** see **Box 1** and **Table 1**.

**EQUIPMENT SETUP**

Casting gels (see **Box 2**).

## PROCEDURE

### Checking DNA quality ● TIMING Approximately 1 h

**1|** Check the integrity of the DNA. Run a small aliquot of DNA on a 1% (wt/vol) agarose gel in 1× TAE running buffer at 100 V for 10–15 min. Visualize using either EtBr or SYBR Safe DNA stain. Intact total DNA will have sharp high molecular weight band. Degraded DNA will appear as a low molecular weight smear.

? TROUBLESHOOTING

### BOX 1 | RADIOLABELING PRIMERS FOR SELECTIVE AFLP AMPLIFICATION

To allow TDF detection using a conventional autoradiography platform (Step 9A), one of the selective primers for selective AFLP amplification (Step 8) is radiolabeled by phosphorylating the 5′-end of the primer with [$\gamma$-$^{33}$P]ATP and polynucleotide kinase, as follows:

1. Incubate the labeling mix detailed in **Table 1** for 45 min at 37 °C.
2. Stop the reaction by holding the temperature at 80 °C for 10 min.

The labeled primer can be stored for up to 1 month at −20 °C.

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**TABLE 1** | Primer radiolabeling mix.

| | Volume to add (µl) for number of samples (*X*) | |
|---|---|---|
| **Compound** | ***X*** **= 50** | ***X*** **= 100** |
| *Eco*RI+$N_{1-3}$[a] primer (50 ng µl$^{-1}$) | 5 | 10 |
| [$\gamma$-$^{33}$P]ATP (370 MBq µl$^{-1}$) | 5 | 10 |
| T4 polynucleotide kinase (10 U µl$^{-1}$) | 1 | 2 |
| 10× T4 buffer | 2.5 | 5 |
| Water | 11.5 | 23 |
| Final volume | 25 | 50 |

[a]*N* represents a number of selective nucleotides that may be added; see section "Experimental design" in the INTRODUCTION for details of primer design.

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**Template preparation ● TIMING Approximately 4 h**

**2|** Incubate 10 µl genomic DNA (approximately 0.1–0.5 µg) with 30 µl of the restriction digestion mix shown in **Table 2** for 1 h at 37 °C and mix gently:

**! CAUTION** Prolonged incubation with the restriction enzyme *Eco*RI (e.g., overnight) is not recommended because of its possible 'star' activity, giving reduced cleavage specificity and, ultimately, aberrant AFLP fingerprints.

**3|** Add 10 µl ligation mix as detailed in **Table 3** and continue the incubation for another 3 h at 37 °C:

*Note:* Do not inactive the restriction enzymes before the ligation.

**4|** After ligation, dilute the reaction mixture to 200 µl with $T_{10}E_{0.1}$ buffer. This will serve now as template for the pre-amplification reaction.

■ **PAUSE POINT** If necessary, the template can be stored for up to 1 year at −20 °C.

**Pre-amplification ● TIMING Approximately 3 h**

**5|** Add 45 µl of the pre-amplification mix detailed in **Table 4** (for an *Eco*RI/*Mse*I primer pair) to 5 µl of the AFLP template prepared in Step 4.

**6|** Use the following PCR program:

| Cycle number | Denature | Anneal | Extend |
|---|---|---|---|
| 1–25 | 94 °C, 30 s | 56 °C, 1 min | 72 °C, 1 min |

■ **PAUSE POINT** The reaction mixture can be stored for up to 1 year at −20 °C.

## BOX 2 | CASTING GELS ● TIMING APPROXIMATELY 1 H 30 MIN

The AFLP reaction products are analyzed on 4.5% denaturing polyacrylamide gels or 6% Long Ranger gels. If conventional gel electrophoresis is to be used to detect radiolabeled products, cast 4.5% denaturing polyacrylamide gel according to option A. If a LI-COR automated DNA sequencer is to be used with infrared dye (IRD) technology for detection, cast a 6% Long Ranger gel by following option B.

**(A) Casting 4.5% denaturing polyacrylamide gels to detect radiolabeled products**

(i) Cast the gel according to the manufacturer's instructions at least 2 h before use to ensure the proper polymerization of the gel. We prefer the SequiGenGT gel apparatus (38 × 50 × 0.04 cm$^3$), but there is no reason other sequencing gel systems should not work equally well. The back plate of the gels, the so-called integrated plate chamber, is treated with 2 ml of Repel-Silane. In case the gels need to be fixed, the front plate is treated with 10 ml of Bind-Silane solution. The Bind-Silane treatments cause the gels to stick to the front plate upon disassembly of the gel cassette after electrophoresis.

(ii) The SequiGenGT sequence gels require approximately 100 ml of 1× 4.5% denaturing polyacrylamide gel solution to which 500 µl of 10% ammonium persulfate (APS) and 100 µl of TEMED is added.

▲ **CRITICAL** Make sure to add the APS solution and TEMED immediately before pouring the gel, because these polymerize the gel.

**! CAUTION** Acrylamide and bis-acrylamide are highly neurotoxic. When handling these chemicals, wear gloves and use a pipetting aid.

**(B) Casting 6% Long Ranger gels for LI-COR analysis**

(i) Cast the 25-cm long sequencing gel (0.25-mm spacer thickness) according to the manufacturer's instructions at least 2 h before use. This ensures sufficient time for gel polymerization.

(ii) The Long Ranger gel requires 25 ml of 6% Long Ranger gel solution to which 166.5 µl of APS 10% and 16.5 µl TEMED is added.

▲ **CRITICAL** Make sure to add the APS solution and TEMED immediately before pouring the gel, because these polymerize the gel.

**! CAUTION** Acrylamide and bis-acrylamide are highly neurotoxic. When handling these chemicals, wear gloves and use a pipetting aid.

**TABLE 2 |** Restriction digestion mix for template preparation.

| Compound | Volume to add (μl) for number of samples (*X*) | | | |
|---|---|---|---|---|
| | *X* = 10 | *X* = 50 | *X* = 70 | *X* = 100 |
| *Eco*RI (20 U μl$^{-1}$) | 2.5 | 12.5 | 17.5 | 25 |
| *Mse*I (10 U μl$^{-1}$) | 5 | 25 | 35 | 50 |
| 10× RL buffer | 40 | 200 | 280 | 400 |
| Water | 252.5 | 1,262.5 | 1,767.5 | 2,525 |
| Final volume | 300 | 1,500 | 2,100 | 3,000 |

**7|** Run 5 μl of the pre-amplification reaction product on a 1% agarose gel in 1× TAE running buffer at 100 V for 10–15 min. Use SmartLadder SF as molecular weight marker ranging from 100 to 1,000 bp. Use EtBr or SYBR Safe DNA stain to visualize the pre-amplification products. Substantial smearing in the range of 50–500 bp indicates a successful pre-amplification PCR.

**? TROUBLESHOOTING**

**8|** Dilute the pre-amplification reaction product obtained in Step 6 20-fold with $T_{10}E_{0.1}$ buffer. These diluted reaction products serve as templates for the final selective amplification reactions using primers with two or three selective bases in one or both primers.

**Selective amplification ● TIMING Approximately 3 h**

**9|** Selective amplification can be accomplished using either radiolabeled primers (option A, to allow subsequent detection using the conventional autoradiography platform) or IRD-labeled primers (option B, to allow subsequent detection using an automated LI-COR platform).

**(A) Selective amplification using radiolabeled primers**

(i) Add 15 μl of the selective amplification mix shown in **Table 5** to 5 μl of diluted pre-amplification reaction mixture from Step 8.

(ii) Use the following PCR program:

| Cycle number | Denature | Anneal | Extend |
|---|---|---|---|
| 1–13 | 94 °C, 30 s | 65 °C, 30 s (reduced each cycle by 0.7 °C) | 72 °C, 1 min |
| 14–36 | 94 °C, 30 s | 56 °C, 30 s | 72 °C, 1 min |

**(B) Selective amplification using IRD-labeled primers**

(i) Add 15 μl of the selective amplification mix shown in **Table 6** to 5 μl of diluted pre-amplification reaction mixture from Step 8.

(ii) With IRD-labeled primers, the selective PCR profile is modified slightly to increase the relative intensity of larger fragments[28], and the following PCR program is used:

| Cycle number | Denature | Anneal | Extend |
|---|---|---|---|
| 1–13 | 94 °C, 10 s | 65 °C, 30 s (reduced each cycle by 0.7 °C) | 72 °C, 1 min |
| 14–36 | 94 °C, 10 s | 56 °C, 30 s | 72 °C, 1 min (extended 1 s per cycle) |
| 37 | | | 72 °C, 2 min |

**Electrophoresis and detection**

**10|** Amplification products can be detected using either a conventional autoradiography platform (option A) or an automated LI-COR platform (option B).

**TABLE 3 |** Ligation mix for template preparation.

| Compound | Volume to add (μl) for number of samples (*X*) | | | |
|---|---|---|---|---|
| | *X* = 10 | *X* = 50 | *X* = 70 | *X* = 100 |
| *Eco*RI adapter (5 pmol μl$^{-1}$) | 10 | 50 | 70 | 100 |
| *Mse*I adapter (50 pmol μl$^{-1}$) | 10 | 50 | 70 | 100 |
| 10× RL buffer | 10 | 50 | 70 | 100 |
| T4 DNA ligase (1 U μl$^{-1}$) | 10 | 50 | 70 | 100 |
| ATP (10 mM) | 10 | 50 | 70 | 100 |
| Water | 50 | 250 | 350 | 500 |
| Final volume | 100 | 500 | 700 | 1,000 |

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**TABLE 4 |** Pre–amplification mix for the pre–amplification step.

| Compound | Volume to add (μl) for number of samples (*X*) | | | |
|---|---|---|---|---|
| | $X = 10$ | $X = 50$ | $X = 70$ | $X = 100$ |
| *Eco*RI + N[a] primer (50 ng $\mu l^{-1}$) | 15 | 75 | 105 | 150 |
| *Mse*I + N[a] primer (50 ng $\mu l^{-1}$) | 15 | 75 | 105 | 150 |
| AmpliTaq (5 U $\mu l^{-1}$) | 2 | 10 | 14 | 20 |
| 10× PCR buffer | 50 | 250 | 350 | 500 |
| $MgCl_2$ (25 mM) | 50 | 250 | 350 | 500 |
| dNTP mix (5 mM) | 20 | 100 | 140 | 200 |
| Water | 298 | 1,490 | 2,086 | 2,980 |
| Final volume | 450 | 2,250 | 3,150 | 4,500 |

[a]N represents a number of selective nucleotides, either 1 or 2, that may be added; see section "Experimental design" in the INTRODUCTION for details of primer design.

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**(A) Product detection using conventional autoradiography platform ● TIMING Approximately 2 h 30 min (electrophoresis) + 12–72 h (detection)**

(i) Mix the selective amplification reaction products from Step 9A(ii) with an equal volume (20 μl) of formamide loading dye. Mix carefully and store overnight at −20 °C.

■ **PAUSE POINT** The mixture can be stored for up to 2 weeks at −20 °C.

(ii) Cast a 4.5% denaturing polyacrylamide gel (see **Box 2**).

**? TROUBLESHOOTING**

(iii) Fill the upper buffer tank with 1× Maxam buffer. For the lower buffer tank dissolve 8.8 g NaAc in 400 ml 1× Maxam buffer. This warrants no running off of smaller AFLP fragments.

(iv) Pre-run the gel for 15 min at 100 W to warm up the gel to approximately 50–55 °C. This temperature is maintained through electrophoresis.

(v) Denature the samples at 90 °C for 3 min and cool on ice.

(vi) Rinse the surface of the gel well with 1× TBE using a syringe and needle. Push sharkstooth combs carefully approximately 0.5 mm into the gel surface to create the gel slots.

(vii) Load 1.6–2.0 μl sample depending on the comb used (48, 64 or 96 wells). Load the molecular weight marker preferably in the first lane. If two or more PCs are run in parallel on one gel, load the molecular weight marker preferably in the lanes preceding the first sample lanes.

(viii) Perform electrophoresis at constant power, 100 W for approximately 150 min. A constant temperature of 50–55 °C throughout electrophoresis is favorable.

(ix) After electrophoresis, disassemble the gel cassette and either place the gel on blotting paper, covered with plastic (Saran), and dry for 1 h on a vacuum dryer at 75 °C or fix on the glass plate by soaking in 10% HAc for 30 min, rinsing with water for 10 min and drying at elevated temperatures in a fume hood.

**? TROUBLESHOOTING**

(x) Autoradiograph the gel by exposing to a standard X-ray film for 2–3 d. Exposure times are reduced to 12 h when using phosphorimaging technology.

(xi) Develop autoradiograph or visualize the fingerprint patterns using phosphorimager technology.

**? TROUBLESHOOTING**

**(B) Product detection using an automated LI-COR platform ● TIMING Approximately 2 h 30 min**

(i) Mix 6 μl of the selective amplification reaction products from Step 9B(ii) with 3 μl of formamide loading dye.

**TABLE 5 |** Selective amplification mix for the selective amplification step using radiolabeled primers.

| Compound | Volume to add (μl) for number of samples (*X*) | | | |
|---|---|---|---|---|
| | $X = 10$ | $X = 50$ | $X = 70$ | $X = 100$ |
| [γ-$^{33}$P]-labeled[a] *Eco*RI+*N*[b] primer (10 ng $\mu l^{-1}$) | 5 | 25 | 35 | 50 |
| *Mse*I + *N*[a]-primer (5 ng $\mu l^{-1}$) | 60 | 300 | 420 | 600 |
| AmpliTaq (5 U $\mu l^{-1}$) | 1.2 | 6 | 8.4 | 12 |
| 10× PCR buffer | 20 | 100 | 140 | 200 |
| $MgCl_2$ (25 mM) | 20 | 100 | 140 | 200 |
| dNTP mix (5 mM) | 8 | 40 | 56 | 80 |
| Water | 35.8 | 179 | 250.6 | 358 |
| Final volume | 150 | 750 | 1,050 | 1,500 |

[a]Procedure for radiolabeling primers can be found in **Box 1** and **Table 1**. [b]*N* represents a number of selective nucleotides, either 1,2 or 3 that may be added; see section "Experimental design" in the INTRODUCTION for details of primer design.

**TABLE 6 |** Selective amplification mix for the selective amplification step using infrared dye 700 (IRD 700)–labeled primers.

| Compound | Volume to add (μl) for number of samples (*X*) | | | |
|---|---|---|---|---|
| | $X = 10$ | $X = 50$ | $X = 70$ | $X = 100$ |
| IRD700-labeled *Eco*RI+*N*[a] primer (1 pmol $\mu l^{-1}$) | 8 | 40 | 56 | 80 |
| *Mse*I+*N*[a] primer (10 ng $\mu l^{-1}$) | 30 | 150 | 210 | 300 |
| AmpliTaq (5 U $\mu l^{-1}$) | 2 | 10 | 14 | 20 |
| 10× PCR buffer | 20 | 100 | 140 | 200 |
| $MgCl_2$ (25 mM) | 12 | 60 | 84 | 120 |
| dNTP mix (5 mM) | 8 | 40 | 56 | 80 |
| Water | 70 | 350 | 490 | 700 |
| Final volume | 150 | 750 | 1,050 | 1,500 |

[a]*N* represents a number of selective nucleotides, either 1, 2 or 3 that may be added; see section "Experimental design" in the INTRODUCTION for details of primer design.

(ii) Cast a 6% Long Ranger gel (see **Box 2**).
**? TROUBLESHOOTING**

(iii) Fill buffer tanks with running buffer 1× TBE.

(iv) Pre-run the gel for 15 min at 45 W, 1,500 V, 40 mA and 45 °C to warm up the gel.

(v) Denature the samples at 95 °C for 3 min and cool on ice.

(vi) Rinse the surface of the gel well with 1× TBE using a syringe and needle. Push sharkstooth combs carefully approximately 0.5 mm into the gel surface to create the gel slots.

(vii) Load 0.5–1.0 μl of each sample. Loaded volume depends on the comb used (48, 64 or 96 wells). Load the molecular weight marker preferably in the first lane. If two or more PCs are run in parallel on one gel, load the molecular weight marker preferably in the lanes preceding the first sample lanes.

(viii) Perform electrophoresis at 45 W, 1,500 V, 40 mA and 45 °C for 150 min (run time) and scan speed 'moderate'. Digital images are similar in appearance to the autoradiographs or phosphorimages produced with the conventional radiolabeling/standard sequencing gel protocol.
**? TROUBLESHOOTING**

## ● TIMING

Step 1, checking DNA quality: approximately 1 h
Steps 2–4, template preparation: approximately 4 h
Steps 5–8, pre-amplification: approximately 3 h
Step 9, selective amplification: approximately 3 h
Step 10A, product detection: approximately 2 h 30 min electrophoresis + 12–72 h detection; Step 10B: approximately 2 h 30 min

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 7**.

**TABLE 7 |** Troubleshooting table.

| Step | Problem | Possible cause | Solution |
|---|---|---|---|
| 1 | DNA is not of sufficient quality | DNA extraction was not performed properly | Retry DNA extraction to get higher-quality DNA |
| 7 | No pre-amplification product | No template | Check concentration of adapters and/or starting amount of DNA and generate new template |
| | | Amplification failed | Check amplification mix, concentration of primers and repeat the amplification |
| 10A(ii) and 10B(ii) | Air bubbles in the polymerized gel | Glass plates were not sufficiently clean(ed) | Clean glass plates thoroughly with soap. When re-using glass plates, gel remnants might be present. Remove gel remnants from glass plate when they are still moist |
| | Polyacrylamide gel does not polymerize well | Ammonium persulfate (APS) lost activity TEMED lost its catalytic activity | Always use freshly made APS solution Use TEMED within manufacturer's recommended expiration date. Store TEMED bottle closed and in dark |

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**TABLE 7** | Troubleshooting table (continued).

| Step | Problem | Possible cause | Solution |
|---|---|---|---|
| 10A(ix) | Gels stick to IPC upon disassembly of the gel cassette | | Avoid any contact between Bind-Silane and IPC. Treat the IPC thoroughly with Repel-Silane |
| 10B(viii) | Poor resolution of bands in LI-COR-generated fingerprint | Bromophenol blue quenches the infrared dye signal | Lower concentration of bromophenol blue in loading dye or order new bromophenol blue |
| 10A(xi) and 10B(viii) | No fingerprint | No template | Repeat dilution of pre-amplification reaction product obtained in Step 6 and repeat amplification |
| | | Labeling failed | Repeat labeling |
| | | Amplification failed | Check amplification mix, concentration of primers and repeat the amplification |

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## ANTICIPATED RESULTS

**Figures 3a** and **4** provide typical examples of AFLP gel images of segregating mapping populations and their parental lines (lanes 1 and 2) fingerprinted using this protocol and visualized using phosphorimaging technology (**Fig. 3a**) and the automated LI-COR platform (**Fig. 4**). Typically, 50–100 AFLP fragments are amplified and visualized in one single lane, and fragment size ranges from 50 to 500 bp, although a greater size range can generally be visualized and resolved with LI-COR automated sequencers (**Fig. 4**) than with the conventional sequencing gel electrophoresis (**Fig. 3a**). Digital images from the LI-COR sequencer are similar in appearance to the autoradiographs or phosphorimages with the conventional radiolabeling/standard sequencing gel electrophoresis. In contrast to conventional sequencing gels, all the fragments loaded on automated sequencers travel the same distance from the well before passing through the scan window. The bands representing the smallest fragments are therefore sharper and closer together than those representing the largest fragments on LI-COR gel images. The DNA fragments on conventional sequencing gels all spend the same amount of time in the gel but do not travel the same distance. The bands representing the largest fragments are therefore consequently sharper.

Only AFLP fragments for which the parental lines are polymorphic segregate in the mapping population. Because genomic polymorphisms manifest themselves predominantly as single-base mutations that affect either the restriction site or the selective nucleotides immediately adjacent to them, such polymorphisms result in a dominant PCR phenotype: the presence of a mutation causes the loss of an AFLP fragment from a fingerprint. Therefore, most AFLP markers are 'mono-allelic' markers because only one allele is actually visualized as a band in the fingerprint pattern. In contrast, an insertion/deletion polymorphism located in the internal sequence of an AFLP fragment results in a co-dominant PCR phenotype: the presence of an insertion/deletion causes a size difference between the two AFLP marker alleles, both of which are visualized as a band in the fingerprint pattern, showing a complementary segregation pattern. Such bi-allelic AFLP markers are identified at a much lower frequency than mono-allelic AFLP markers. Examples of segregating mono- and bi-allelic markers are shown in **Figure 4**.

**Figure 3a** represents a gel image of a tomato $F_2$ population, where the expected proportion of individuals heterozygous at a locus is 50%. The difference between samples homozygous (2*n*) or heterozygous (1*n*) for an AFLP marker can often be clearly distinguished from the band intensities (also by eye) and reflects PCR product concentrations (100 and 50%, respectively). This feature allows co-dominant scoring of AFLP markers based on relative fragment intensities with the aid of specific image analysis software AFLP-Quantar*Pro*.

**Figure 4** represents a gel image of an AFLP analysis of 56 *Arabidopsis* Recombinant Inbred Line (RIL) offspring and their parental lines (in lanes 1 and 2). Given that RILs are panels of genetically mosaic but homozygous strains generated by crossing parental strains and inbreeding the progeny, AFLP markers segregate as homozygous present or absent in the RIL progeny.
The scoring process of AFLP gels thus results in datasets consisting of dominantly scored markers, co-dominantly scored markers (**Fig. 3b**) or combinations thereof, depending on the population type involved and specifications of the scoring software used. These AFLP genotyping datasets typically serve as the starting point for further analysis in the context of the specific applications for which the AFLP fingerprints are generated (often involving dedicated software packages). For instance, in the case of genetic linkage mapping, AFLP marker datasets are used to group and order the AFLP markers in linkage groups and estimate genetic distances between them using genetic linkage mapping software. Such linkage maps are useful tools to identify genes affecting (complex) traits for breeding purposes. Another widely used application in plants is marker-assisted back-crossing.
In this case, AFLP marker genotypes are used to estimate the fraction of donor genome and the number of donor fragments that are introgressed into the recurrent parent line of each back-cross progeny, such that those progeny with the lowest number of

donor segments can be selected for the next generation of back-cross breeding. By repeating this process, the number of generations required for introgression of only a single donor fragment into elite cultivars can be reduced significantly. Yet another application of AFLP is the saturation of genomic regions with markers using pooled DNA samples obtained from (plant or animal) samples differing with respect to a phenotype of interest. By subjecting these pooled samples to AFLP fingerprinting, AFLP markers are discovered that are expected to be located near genetic loci that control the phenotype. This combination of AFLP with bulked segregant analysis[29] is a powerful approach for marker development, especially in species for which little sequence information is known. Finally, AFLP analysis is also widely used to estimate genetic relatedness of samples within a species across a wide range of taxa (plant, animal, micro-organism). Most of these applications are attractive because of the combination of the high multiplexing level of AFLP (and therefore low cost per data-point) and the ability to apply the technique without prior sequence knowledge according to a fixed protocol.

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**COMPETING INTERESTS STATEMENT** The authors declare competing financial interests (see the HTML version of this article for details).

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# Corrigendum: AFLP technology for DNA fingerprinting

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In the version of this article initially published, the three genotype classes in Figure 3b were incorrectly described in the legend. "A: homozygous as the first parent; H: heterozygous, B: homozygous as the second parent" should have read "A: homozygous absent; H: heterozygous; B: homozygous present".

This error has been corrected in the HTML and PDF versions of the article.

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