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Jörg Klug
University of Marburg
Marburg, Germany

Silver Staining and Recovery of AFLP™ Amplification Products on Large Denaturing Polyacrylamide Gels

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The AFLP™ technique (Keygene N.V., Wageningen, The Netherlands) is a new method for DNA fingerprinting based on selective amplification of DNA restriction fragments (8,10). Briefly, the AFLP technique involves digestion of genomic DNA with two restriction enzymes followed by ligation of oligonucleotide adapters to the resulting restriction fragments. These DNA fragments are subsequently amplified by polymerase chain reaction (PCR) using primers that are homologous to the adapter and restriction site sequences. Primers carry also at their 3′ ends, extensions of one to several "selective" nucleotides. Only those restriction fragments starting with nucleotides complementary to the "selective" nucleotides of the primers are amplified. The restriction enzymes and the number and nature of the "selective" nucleotides can be varied, depending on the complexity of genomic DNA. According to the original procedure (8,10), one of the primers used for PCR amplification is radioactively labeled using phosphorus isotopes (^{32}P or ^{33}P), and the amplified fragments are separated on denaturing (sequencing) polyacrylamide gels and visualized by exposure to autoradiography films or by using phosphor-image analysis.

The AFLP technique is more powerful and reliable than other DNA fingerprinting techniques because it enables the generation of a large number of highly reproducible polymorphic markers (8,10). It has been successfully applied in several fingerprinting programs (3,7). However, the inclusion of radioactive isotopes to visualize AFLP products renders the technique relatively expensive, technically quite demanding and hazardous to health.

Silver staining of DNA in polyacrylamide gels has been used to visualize PCR-amplified fragments for a variety

of PCR-based techniques (2,4,6,9). In this report, we demonstrate that, with appropriate technical modifications, AFLP amplification products can be visualized by silver staining with sensitivity and resolution similar to those observed with radioactive labeling.

To illustrate the method, DNA extracted from the leaves of four different pea (*Pisum sativum* L.) cultivars was digested with *Mse*I ("frequent" cutter) and *Eco*RI ("rare" cutter) restriction enzymes (New England Biolabs, Bishop's Stortford, Herts, England, UK). The AFLP reactions were performed in a total volume of 20 µL as described by Vos et al. (8). Oligonucleotide adapters and PCR primers (hereafter designated according to their corresponding restriction sites) were kindly provided by Keygene N.V. Two PCR amplification steps were performed for AFLP. In the first step, primers were used with one selective nucleotide [*Eco*RI(+1) and *Mse*I(+1)]. The second PCR amplification step was done with 1/100 of the first PCR-amplified products, using primers with three selective nucleotides [*Eco*RI(+3) and *Mse*I(+3)]. Our initial experiments showed that, with three selective nucleotides for each of the two primers, 30–100 AFLP fragments were amplified.

For the radioactive AFLP procedure, the *Eco*RI(+3) primer was radioactively labeled with [γ-^{33}P]ATP (Du Pont de Nemours, Brussels, Belgium) and T4 DNA kinase (Pharmacia Biotech AB, Uppsala, Sweden). Aliquots of one-tenth of the amplified radioactive fragments (2 µL of PCR mixed with 2 µL of formamide-containing dye [Sigma Chemical, St. Louis, MO, USA]) were loaded onto sequencing gels of 6% polyacrylamide and 7 M urea (0.4-mm thickness, 40-cm height and 33-cm width). Electrophoresis was performed at a constant 60 W for 3–4 h. The gel was transferred onto Whatman MM paper (Maidstone, Kent, England, UK), dried and exposed to autoradiography film (Du Pont de Nemours) for 16 h.

AFLP reactions for silver staining were performed as described above except that the *Eco*RI(+3) primer was not radioactively labeled, and 30 ng of this primer were used per reaction, instead of 5 ng as for the radioactive AFLP reactions. Furthermore, one-fifth of the AFLP amplification products (4 µL of PCR mixed with 4 µL formamide-containing dye) was loaded onto the polyacrylamide gels. The polyacrylamide gels were poured as described above except that the short glass plate was treated with Silane® A-174 (Sigma Chemical) to bind the gel. The resulting glass-backed polyacrylamide gel is easy to handle during the silver staining procedure.

The silver staining of DNA was performed as described by Bassam et al. (1). At the end of the staining procedure, gels were thoroughly rinsed in distilled water (30 min), dried for 16 h and then photographed or directly scanned using a Studio/Scan apparatus (AGFA-Gevaert N.V., Morstel, Belgium) combined with the Adobe Photoshop™ 2.5.1 LE program (Adobe Systems, Mountain View, CA, USA) for Macintosh® computers (Apple Computer, Cupertino, CA, USA).

A representative comparison between the radioactive AFLP fingerprints visualized by autoradiography (A) and the nonradioactive AFLP fingerprints directly visualized on the polyacrylamide gel by silver staining (B) is presented in Figure 1. The sensitivity and resolution obtained by the two procedures are very similar.

In denaturing polyacrylamide gels, the two strands of dsDNA fragments are separated, and these may migrate as "doublet bands" because of the differences in their electrophoretic mobility. With the radioactive AFLP procedure, only one strand of the amplified fragments is visualized on the autoradiography films because only one of the two PCR primers is radioactively labeled (8,10). This is not the case for the silver-stained AFLP procedure where both strands of amplified fragments are visualized, and hence "doublets" or thick bands are frequently observed for each DNA fragment (Panel B). Nevertheless, no complexities in band patterns were observed in silver-stained gels compared to autoradiography (Panel B compared to Panel A).

A gradient in band intensity was observed in the silver-stained AFLP gels. Bands of higher molecular weight (at the top of the gel) were more intense than those of low molecular weight at the bottom of the gel. To increase the intensity of bands at the bottom of the silver-stained gels, we have adopted a modification, which simply consists of extending the time of development for the bottom half of the gel. When the bands at the top of the gel are sufficiently developed, the gel is inclined by hand in such a way that only the bottom half is still immersed in the developing solution. Stop solution (10% acetic acid) is then added directly onto the surface of the top half of the gel while

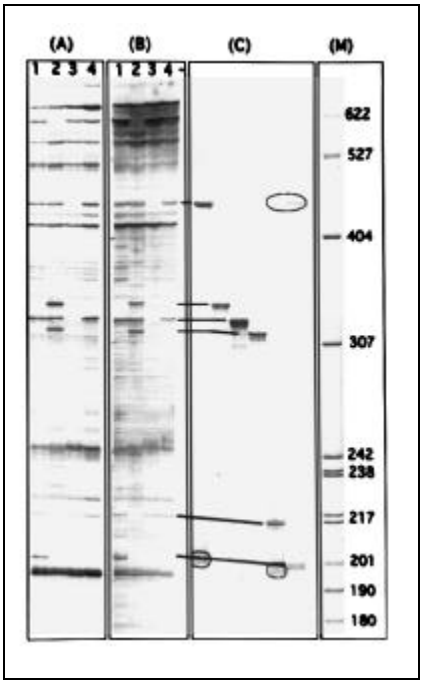

Figure 1. Comparison of radioactive and nonradioactive silver-stained AFLP. Reactions were performed as described by Vos et al. (8) and separated on 6% denaturing polyacrylamide gels. The primer combination used in the reactions presented are *Eco*RI (with the selective nucleotides AGG) and *Mse*I (with the selective nucleotides CGC). (Panel A) Radioactive AFLP performed on DNA, extracted from four pea (*Pisum sativum* L.) cultivars, (lanes 1–4) with the ^{33}P-labeled *Eco*RI(AGG) primer is visualized on autoradiography film. (Panel B) Nonradioactive AFLP of the same pea lines (lanes 1–4) and control (-) is directly visualized on the gel by silver staining. Plain lines starting from Panel B to Panel C indicate DNA fragments, which show polymorphism among the four pea cultivars and chosen for re-amplification. (Panel C) Re-amplification of individual AFLP fragments from the silver-stained gel. PCR conditions were as for the initial AFLP. Each lane represents the re-amplification of an individual AFLP fragment recovered from Panel B. Contaminant or "ghost" amplifications when observed are indicated (in circles). (Panel M) the pBR322-*Msp*I digest molecular size marker (New England Biolabs). Corresponding sizes (in nucleotides) are indicated.

the bottom half is left to continue developing for an additional 30–60 s.

We have also developed a simple method to recover and specifically re-amplify individual AFLP fragments from silver-stained gels. Bands of interest in the dried gels were rehydrated by placing 5 µL of sterile distilled water onto the band for 15–60 min. Another 2 µL of water were added and then transferred into a PCR tube containing the same sets of primers and reaction products as for the initial AFLP reaction. PCR amplification was then performed as for the initial AFLP procedure. Panel C shows successful re-amplification of several DNA fragments, which show polymorphism among the four pea cultivars.

Different protocols for the recovery and re-amplification of DNA fragments from silver-stained polyacrylamide gels have been described elsewhere (2,4,6, 9). These protocols usually require excision of the band and/or additional extractions of the DNA fragment. In our experiments, we observed difficulties in the re-amplification of DNA fragments when the bands were excised and used as DNA templates. On the contrary, using the protocol described above, successful and reproducible re-amplifications were obtained, even from silver-stained gels, which had been stored for more than 12 months. It is possible to recover DNA fragments from radioactive dried gels, but it is time-consuming and technically challenging.

In conclusion, we have shown that silver staining can be used to directly visualize AFLP amplification products, separated on large vertical polyacrylamide gels with a good degree of resolution and sensitivity. As discussed above, the silver-stained AFLP protocol results in significant simplifications over the standard radioactive AFLP procedure. An additional advantage of the silver-stained AFLP procedure is that dried gels provide a permanent record of the AFLP reaction from which fragments of interest can be easily recovered. This is of particular interest when these fragments are needed for further characterization, such as cloning, sequencing, preparation of probes or development of sequence-characterized amplified regions (SCARs) (5).

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B.A. Chalhoub, S. Thibault, V. Laucou, C. Rameau, H. Höfte and R. Cousin
Institut National de Recherche Agronomique (INRA)
Versailles, France