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Protocols in Biotechnology

DNA Amplification Fingerprinting Using Arbitrary Oligonucleotide Primers

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ABSTRACT

DNA amplification fingerprinting (DAF) is a strategy for genetic typing and mapping that uses one or more very short (≥5 nt) arbitrary oligonucleotides to direct the enzymatic amplification of discrete portions of a DNA template resulting in a spectrum of products characteristic of the DNA starting material. Polymorphisms from simple banding patterns are useful as genetic markers while more complex and informative patterns are suitable for DNA fingerprinting. The use of polyacrylamide gel electrophoresis and silver staining can adequately resolve the spectrum of DAF products into detailed and reproducible patterns.

Index Entries: DNA amplification; fingerprinting; DAF; PCR; primer-template interactions; single oligonucleotide primers.

INTRODUCTION

Variant or polymorphic sites in the genome of eukaryotic and prokaryotic organisms can be studied by targeting specific DNA segments using molecular hybridization (*1*) or DNA amplification techniques, like the polymerase chain reaction (PCR) (2–4). These sites express changes in length or sequence of fragments resulting from the restriction or amplification of genomic DNA. Restriction fragment length polymorphisms (RFLPs) have been widely used as molecular markers in genetic linkage studies or in DNA

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fingerprinting applications. However, RFLP analysis requires prior knowledge of DNA sequence, cloned and characterized probes, or considerable experimental manipulation. Recently, DNA polymorphisms were revealed without these constraints. Arbitrary oligonucleotide primers were used to initiate amplification of discrete portions of a genome by targeting single (*5*) or multiple (*6–8*) amplification sites defined by primer-complementary sequences on each DNA strand. Some of these "amplicons" constitute amplification fragment length polymorphisms (AFLPs)—length polymorphisms that sometimes manifest as presence or absence of DNA fragments, and can be readily used in both genetic typing and mapping endeavors.

A model to explain how a single arbitrary primer amplifies DNA has been proposed (*9*). During the first few temperature cycles, the primer "screens" the possible DNA target sites and anneals to many of them. The thermostable DNA polymerase anchors to these sites and initiates extension of the annealed primers producing a defined population of "first-round" amplification products. In the PCR, these products are subsequently amplified with high efficiency. However, single primer amplification products have terminal sequences complementary to each other that allow production of hairpin loop structures. Because the primer must displace these hairpin loop complexes long enough for the enzyme to anchor and stabilize the duplex by strand extension, and because the extent of hairpin loop interference will be variable for each fragment, only some of the "first-round" products will be efficiently amplified.

Although RFLPs generally originate from sequence changes at restriction endonuclease sites, AFLPs can arise from a variety of mechanisms, including nucleotide substitutions that create or abolish primer sites, deletion, insertion, or inversion of a priming site or of segments between priming sites, and large insertions that separate priming sites rendering them unable to support detectable amplification. DNA amplification fingerprinting (DAF) is a multiple arbitrary amplicon profiling (MAAP) technique that uses one or more artibrary primers as short as five nucleotides (nt), but typically seven or eight nt in length, and either low- or high-stringency amplification conditions, to produce characteristic and relatively complex fingerprints (*6*). These fingerprints are adequately resolved by polyacrylamide gel electrophoresis and a highly sensitive DNA silver stain (*10*). Typical DAF profiles are shown in Fig. 1. Other MAAP techniques use longer arbitrary primers and produce far less complex profiles (*7,8*). DAF is able to detect genetic differences in a wide variety of organisms, including animals, plants, and bacteria. Although it is relatively simple to find differences between organisms at the species level, DAF can also differentiate those that are closely related, like bacterial isolates, plant cultivars, near isogenic lines, and human individuals (reviewed in refs. [*11,12*]). DAF can also be used to study complex mixtures of organisms, like those found in symbiotic or pathogenic relationships. Finally, DAF can generate molecular markers for genetic mapping and breeding applications, as well as for population and pedigree analysis.

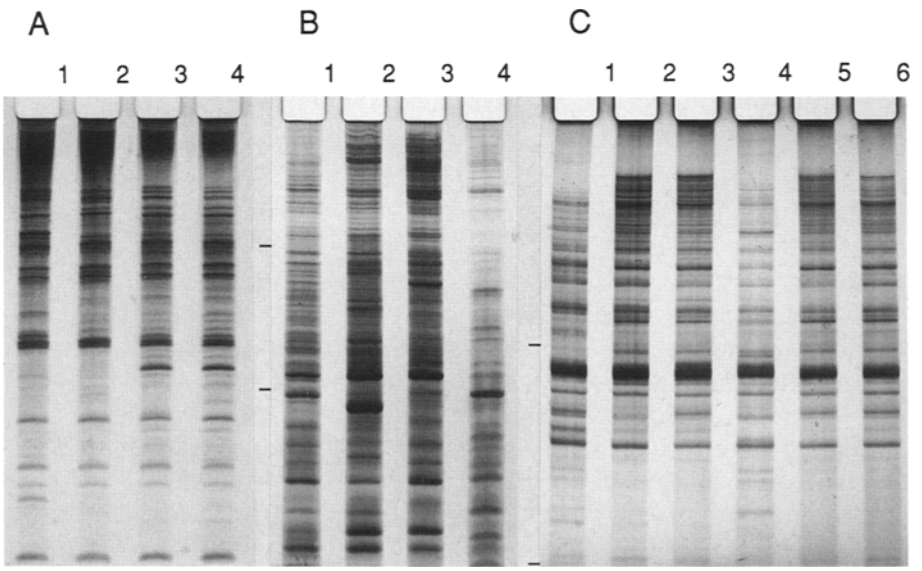

Fig. 1. DNA amplification fingerprints of bacterial isolates and plant cultivars. A. Genomic DNA from *Streptococcus uberis* isolates 1 and 2 from cow L829 (lanes 1 and 2), and isolates 15 and 16 from cow K1449 (lanes 3 and 4) amplified with primer 5′GTAACGCC3′. Results show that DAF offers high resolution at the subspecies level. B. Genomic DNA from the turfgrasses *Zoysia matrella* cv. Manilagrass (lane 1), *Zoysia japonica* cv. Korean common (lane 2), cv. El Toro (lane 3), and *Zoysia* sp. cv. Cashmere (lane 4) amplified with 5′GTGACGTAGG3′. DAF can readily separate the different cultivars. Cashmere expresses morphology of *Zoysia matrella* and *Zoysia tenuifolia*. C. Soybean cultivars *Glycine max* cv. AG10 (lane 1), cv. AG11 (lane 2), cv. AG12 (lane 3), *Glycine soja* PI468.397 (lane 4), *Glycine max* cv. Bragg (lane 5), and cv. nts382 (lane 6) amplified with the pentamer 5′AGCTG3′. Profiles show a high degree of monomorphism and therefore low molecular diversity. Soybean cultivar nts382 is an EMS-induced near-isogenic mutant of Bragg. Fragment sizes shown are in kilobase pairs.

MATERIALS

DNA Amplification

1. Reaction buffer: 100 m*M* Tris-HCl, 100 m*M* KCl, pH 8.3.
2. Deoxynucleoside triphosphate stock solution: 2 m*M* of each dNTP.
3. $MgCl_2$: 25 m*M* or 100 m*M* stock solution.
4. Thermostable DNA polymerase enzyme (preferably truncated such as AmpliTaq Stoffel fragment, from Perkin-Elmer/Cetus).
5. Oligonucleotide primer: 30 μM or 300 μM stock solution.
6. Template (usually diluted to give 1–10 ng/μL concentrations).

7. Chloroform or long disposable pipet tips.
8. Heavy mineral oil (depending on the thermocycler used).
9. Equipment: bench-top centrifuge and thermocycler.

DNA Separation

All reagents used in this step must be electrophoresis grade.

1. Urea.
2. Acrylamide/crosslinker stock solution: 38% Acrylamide and 2% piperazine diacrylamide. Acrylamide solutions are light-sensitive and should be stored in the dark at 4°C. Acrylamide is a potent neurotoxin; handle with care.
3. Tris-borate-EDTA (TBE) buffer stock (10 times stock: 1*M* Tris-HCl [121.1 g/L Trizma base], 0.83*M* boric acid [51.35 g/L], and 10 m*M* $Na_2EDTA \cdot H_2O$ [3.72 g/L], pH 8.3). Store at room temperature.
4. Ammonium persulfate. Prepare a fresh 10% solution each day.
5. *N,N,N′,N′*-tetramethylethylenediamine (TEMED).
6. Loading buffer: 5*M* urea, 0.02% xylene cyanole FF. Store at room temperature.
7. Equipment: electrophoresis apparatus, polyester gel-backing film (GelBond PAG from FMC Bioproducts, or similar), membrane syringe filter (optional), and small-bore flat pipet tips.

Silver Staining

All reagents used in this step must be of high purity analytical grade.

1. Fixer and stop solution: 7.5% (v/v) glacial acetic acid.
2. Silver impregnating solution: 1 g/L silver nitrate and 1.5 mL/L formaldehyde.
3. Developer solution: 30 g/L sodium carbonate, 3 mL/L formaldehyde, and 2 mg/L sodium thiosulfate.

METHODS

DAF involves three steps: the amplification of DNA, its subsequent separation usually by electrophoresis, and staining of individually resolved fragments (*see* Fig. 2 for a diagram and expected experimentation times).

DNA Amplification

1. Dilute DNA appropriately in sterile double-distilled water.
2. Add the standard amplification components in the following order (final concentrations are given in parentheses): 12.75 μL

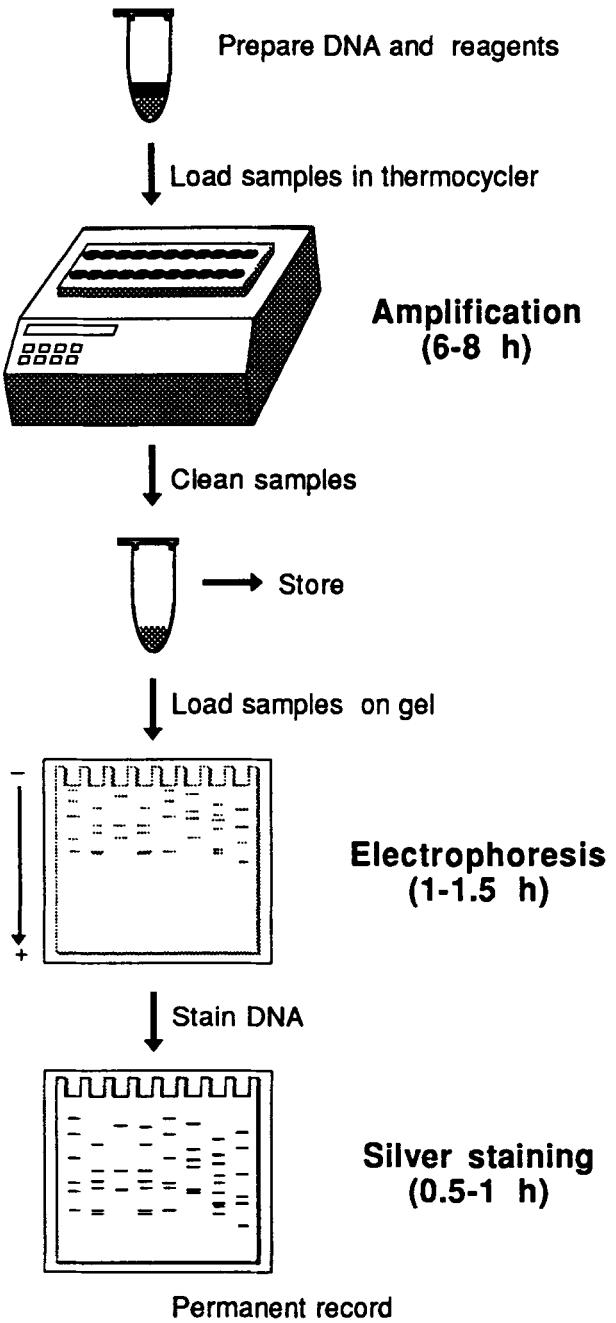

Fig. 2. DNA amplification fingerprinting flow diagram.

water, 2.5 μL deoxynucleoside triphosphates (200 μM of each dNTP), 1.5 μL $MgCl_2$ (1.5 or 6 mM), 2.5 μL reaction buffer (10 mM Tris-HCl and 10 mM KCl, pH 8.3), 0.75 μL enzyme (0.3 U/μL Stoffel fragment), 2.5 μL primer (3–30 μM), and 2.5 μL template (0.1–1 ng/μL). Shorter primers require higher concentration, and low-complexity genomes require higher $MgCl_2$ levels. Prepare a master mix (when possible) with reagents that are common. Distributing premixed reagents in this way avoids pipeting errors.

3. Mix (optional) and spin down (full power of bench-top centrifuge for 1–10 s).
4. Cover the amplification mix with one to two drops of mineral, oil and lock the tubes. When using an oven-thermocycler (Biosycler or similar), the oil layer may not be required.
5. Add one drop of heavy mineral oil into the block wells of the thermocycler, and place the tubes in position (when using some block-based thermocyclers) or directly in the oven (when using oven-based thermocyclers).
6. Run thermocycler (usually using two-step cycles of 20 s at 96°C and 20 s at 30°C in an Ericomp thermocycler or similar) for desired number of cycles (usually 35).
7. Retrieve amplification mixtures by either adding 100–200 μL of chloroform to each tube, pipeting out the aqueous droplet and placing it into a fresh tube, or directly from under the oil layer using a long pipet tip. Clean up the wells of the thermocycler with a Q-tip, if necessary. Dilute the samples, usually 5–10-fold to avoid gel overloading.

DNA Separation

DNA amplification products are usually electrophoresed in 0.45-mm-thick polyacrylamide slab gels (8 × 10 cm) backed on polyester film using a Mini-Protean II cell (Bio-Rad).

1. Assemble each electrophoretic rig in the following order: large glass plate on clamp assembly, polyester backing sheet (hydrophilic side up), spacers, and finally small glass plate. To avoid dust particles, assemble rig wet under running distilled water. All rig components must be flush against the bottom before tightening assembly screws. Finally, shake out excess water.
2. Dry gel rig in a dust-free area in the dark (backing sheets are light-sensitive) or in an oven at 25–30°C, overnight.
3. Assemble gel rigs in casting stand. Insert Teflon™ combs between glass plates leaving space to pour the gel mix.
4. Prepare to cast a 5% polyacrylamide—7M urea gel. Add the following reagents (enough for two miniature gels) into a 25-mL beaker: 4.2 urea, 1 mL of TBE buffer stock, and 1.2 mL

acrylamide/crosslinker stock. Dilute to 10 mL with double-distilled water and mix well.
5. Add 150 μL of 10% ammonium persulfate and 15 μL of TEMED to gel mix.
6. Deliver immediately the gel mix by injection through a 0.45-μm pore-size membrane syringe filter. This will remove dust particles. Adjust comb position. The gel mix begins to set in about 2 min and should fully polymerize in 30 min.
7. Attach gel rigs to electrode core, and place into buffer tank. Fill upper and lower buffer reservoirs with running buffer (TBE, one times stock).
8. Remove combs, rinse wells with running buffer using a 1-mL syringe, and prerun gels for 5 min to equilibrate.
9. Load gels with 3 μL each of the amplification (diluted to 30–40 ng DNA) and loading buffer using a small-bore flat pipet tip.
10. Electrophorese gels at 100 V for about 60–90 min or until dye front travels three-quarters of the length of the gel.
11. Disassemble gel rigs, and carefully remove backed-gels for silver staining.

DNA Silver Staining

After electrophoresis, the polyester-backed gels can be fixed and stained with silver using the procedure of Bassam et al. (*10*) (*see* related article by Bassam and Caetano-Anollés, this issue). Briefly, gels are fixed for 10 min, washed with distilled water (three times, 2 min each), impregnated with silver for 20 min, quickly rinsed with distilled water, and developed for about 4 min at 8–12°C until optimal image contrast. Image development is stopped in 7.5% acetic acid, and gels are washed with water. The staining protocol is tolerant of reaction times and detects about 1 pg DNA/mm^2 band cross-section. Silver stained gels can be preserved permanently by drying at room temperature.

NOTES

Primer design, amplification reaction conditions, and adequate separation and detection of DNA determine whether the spectrum of amplified products will be resolved into a characteristic and reproducible fingerprint pattern. Moreover, primer and amplification parameters tailor fingerprints in their complexity and polymorphic DNA content. Why are fingerprints produced by other MAAP procedures (*7,8*) less complex and sometimes less reproducible? Template sites become amplicons depending on many factors. For example, primer, magnesium, and deoxynucleoside triphosphate concentrations can all alter the amplification profile, perhaps by allowing more primer-template mismatch events (*see* discussion *below*).

Similarly, annealing temperature, especially during the first few cycles, can limit the number of amplicons by destabilizing primer-template and template-template interaction events. We will discuss many of these parameters, so that the protocol described in Methods can be modified to suit particular applications.

1. Primer design: DAF oligonucleotides are so short that they approach the functional limits for priming DNA amplification. We have examined characteristics inherent to these very short primers, and inferred primer–template interactions important for genome identification and the generation of molecular markers (*9*). We found that the first 8 nt from the 3′ terminus of the primer encompass one basic domain that largely conditions amplification. Although single base changes in sequence within this domain significantly alter the spectrum of amplified products, especially towards the 3′ terminus, regions beyond the basic domain alter the amplified spectrum only moderately. Since addition of nucleotides to this domain does not increase the information content of the patterns significantly, longer sequences, such as those used in other studies (*5,7,8*), are not necessary. Successful amplification requires a primer of at least 5 nt in length and annealing sites with perfect homology to the first 5 or 6 nt from the 3′ terminus. Decreasing primer length within the range of 5–8 nt simplifies the DNA profiles, whereas increasing length over the 8-nt domain does not decrease the number of amplification products obtained. Our results suggest that only a fraction of template annealing sites amplify efficiently, 5–8 nt primers amplify certain products preferentially because of competition for annealing sites between primer and terminal hairpin-loop structures of the template, and long primers allow for considerable primer-template mismatching at the 5′ terminus. We have not found any influence of primer GC content on amplification (although we routinely use primers that have 60–70% GC). However, some primers produce markedly fewer amplification products than others, and some produce very few or no products with genomic DNA from a wide range of organisms. The reasons for poor amplification are unclear, but may result from the existence of extremely rare oligonucleotide sequences as demonstrated in mammals and other organisms (*13,14*).
2. Annealing temperature: Temperature affects the formation, stability, and equilibrium between primer-template duplexes, hairpin loops, and other possible DNA molecular species formed during amplification. Although shorter primers require lower annealing temperatures for adequate amplification, a pentamer

can still produce products at about 55°C (*9*). Sequence-related primers of 7, 10, 12, and 15 nt in length failed to amplify DNA at annealing temperatures of 65, 70, 75, and 80°C, respectively. Temperatures over 80°C were unable to render profiles with any primer, probably because higher temperatures do not allow adequate primer annealing and extension. Although profiles tended to simplify with increasing annealing temperature, the boundary of the 8-nt primer domain is not altered.

3. DNA amplification reaction conditions: DAF is conceptually and mechanistically different from techniques like the PCR. Therefore, principles derived from other amplification techniques need not be applicable. A defined template and primer should be used in an iterative process to optimize the DAF amplification reaction carefully for a particular application. Moreover, fingerprints obtained with different thermostable DNA polymerases vary considerably and require different amplification parameters. Generally, truncated DNA polymerases, like Stoffel fragment, produce clearer fingerprints with a higher proportion of strong "primary" products and are less affected by experimental variables, such as primer or enzyme concentration (*15*).

   Several parameters are crucial for generating reproducible fingerprints. When compared with other protocols (*8*) and the PCR, over 10 times more primer (at least 3 μM) is required to reveal all amplification products. In the PCR, high primer concentration often results in increased primer mismatching and spurious annealing events. With DAF, higher concentrations favor the nonstringent reaction conditions typical of the amplification of arbitrary amplicons. We also found that template concentration is important, especially with genomes of low complexity, like bacteria or fungi (*15*). Concentrations of at least 1 $ng \cdot \mu L^{-1}$ of bacterial DNA are crucial for experimental consistency. Lower template levels result in the generation of inconsistent bands (usually corresponding to weakly amplified products), probably owing to stoichiometric misrepresentation of products amplified with low efficiency in later rounds of amplification. High template concentrations are not a requirement when using animal or plant genomes (*6*) probably because of their higher complexity.

   Several parameters have no major effect in the amplification reaction. DNA quality (purity and integrity) usually has little effect on fingerprints. Cycle parameters, like denaturing temperature, times of annealing, denaturation, and strand extension, as well as cycle number, had no important effect once within a safe range.

4. Fingerprint tailoring: Fingerprints can be tailored to be simple or complex simply by changing primer sequence, length, and number, as well as amplification parameters (*11*). Simple banding patterns appear desirable for genetic mapping, and more complex (i.e., informative) patterns appear better suited for DNA fingerprinting. Simple patterns are better obtained with shorter primer and higher annealing temperatures (*9,15*) than with lower primer or magnesium concentrations usually used in other MAAP analyses (*8*). Similarly, complex patterns can be better obtained with engineered DNA polymerases or by increasing magnesium concentration.

In cases where the template is composed of more than one genome, it is possible to differentiate each organism without resorting to physical separation of the contributing genomes. In the study of the *Azolla-Anabaena* symbiosis, we were able to generate characteristic DAF profiles for the fern or the cyanobacterial symbiont just by altering primer sequence (*16*).

In fingerprinting applications, an adequate number of monomorphic and polymorphic bands that can group and classify genomes individually must be obtained. In some cases, screening a set of primers and choosing those that provide clear fingerprints with useful ratio of monomorphic to polymorphic products is easily accomplished, as was the case with DNA from isolates of *Streptococcus uberis* (*17*) or cultivars of banana (*Musa* sp.) (*18*). In other cases, this proves to be very difficult. For example, isolates of *Discula destructiva*, the fungal pathogen that causes dogwood anthracnose, were very difficult to separate even after the use of a considerable number of arbitrary primers (*19*). In these cases, other tailoring strategies must be used in order to increase DNA polymorphism.

We found digestion of template DNA with restriction endonucleases prior to amplification enhances the detection of polymorphic DNA (*20*). We generally use one to three restriction endonucleases having 4-bp recognition sequences to digest stock DNA before dilution and amplification. Fungal and plant cultivars that were indistinguishable by DAF with several primers were easily separated in this way. Moreover, near-isogenic lines of soybean (*Glycine max*) generated by ethyl methane sulfonate (EMS) mutagenesis were differentiated and markers tightly linked to the mutated locus isolated without resorting to an extensive screening of arbitrary primers (*20*). This endonuclease-linked MAAP strategy can be used to efficiently identify sequence-tagged markers linked to genes of interest, for high-resolution linkage mapping of specific genomic regions,

and potentially for chromosome walking. This can be especially valuable in the analysis of induced mutants or near-isogenic lines obtained in plant breeding programs.

5. Resolution of DNA amplification products: Although it is obvious that many parameters of the amplification reaction can condition amplicon production as much as primer sequence, adequate resolution of amplification fragments will not only reveal the true number of amplified sites, but ensure that DNA patterns obtained are reproducible. We found that high-resolution separation and staining of DNA were crucial to detect products at the picogram level (*21*). If techniques with lower resolution are used, only the few predominant products are detected, which can result in the selection of amplification conditions tending to highlight few predominant products. We found that these conditions are usually borderline, i.e., subject to experimental variation (*15*); they could be responsible for inconsistencies that may discourage the novice user. Selection for predominant products can also tend to discard conditions that render reproducible patterns that are perhaps too complex to be detected with a lower resolution technique. For example, primer concentrations used in DAF tend to give smears when other MAAP analyses are used, or 7-mer and 8-mer primers that render complex profiles by DAF appear to produce no amplification products with these other techniques.
6. Automation: Robotic manipulation can accelerate reagent dispensal and thermocycling procedures. Automation techniques can also be used to separate and detect subnanogram quantities of DNA, so that fingerprints can be resolved quickly and accurately in real time. Of alternatives, the fluorophore tagging of DNA appears most promising. Fluorescently labeled groups affixed to the primer still allow efficient amplification, but simplify fingerprints. When amplification products are separated using a Gene Scanner ABI model 362, consistent profiles with polymorphic peaks are obtained and resolved at the base pair level (*12*). Other promising approaches, such as the use of an automated DNA sequencer or of capillary electrophoresis have yet to be explored.

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