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Validation of a DNA Method for the Individualization of Plant Evidence

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Introduction

Many forensic cases involve plant evidence that may be useful to link a suspect, a victim, a weapon, a vehicle and crime scenes. Currently, most plant evidence is identified to the Genus or species level by class characteristics such as leaf morphology, color, size, geographic distribution and any special structures such as shape and presence of trichomes or inclusion bodies (1-4). These characteristics may be observed macroscopically or microscopically but rarely can confirm the source of the plant unless a good physical match is obtained. With the advent of new DNA technologies, however, plant material may be chemically extracted for DNA, and typed using a multilocus detection method called amplified fragment length polymorphism (AFLP).

The AFLP method was developed in 1995 by Vos et al. and has been used for numerous years in research laboratories and for patent applications (5). For the samples tested, DNA was extracted from single source plant material, digested into fragments with two different restriction enzymes, amplified by polymerase chain reaction (PCR) and finally detected using a DNA sequencer. Customized computer macros aid in determining a candidate match and for searching a reference population database by converting DNA fragment patterns into binary code (6).

As a model system, *Cannabis sativa* (marijuana) was selected due to its wide geographic distribution and close association with forensic casework. Sourcing of marijuana is not only useful for drug cases and for tracking distribution networks but also for providing additional evidence in many burglaries and homicides. Previous work (6) demonstrated

that consistency in DNA profiles was obtained from different somatic tissues of a *Cannabis* plant, that the presence of resin on leaf surfaces was not inhibitory to PCR, and that plants suspected of clonal propagation did indeed have identical DNA patterns and unrelated marijuana samples had different patterns. Additional studies presented here further define the appropriate use of the AFLP method for plant testing.

Materials and Methods

A. Reproducibility Studies:

All of the DNA extraction, PCR amplification and analytical steps for evaluation of AFLP data were executed as previously described (6). The following studies were performed:

Different analysts-AFLP profiles were generated for five different marijuana samples by two individuals with molecular biology experience. Each analyst processed identical DNA extracts in duplicate using the same reagents and equipment. In addition, a set of plant samples was co-extracted and processed to compare the effect of different DNA extractions on the AFLP pattern.

Different thermal cyclers-PCR amplifications were performed on two different models of thermal cyclers (9600 and 9700; PE Corporation) by two different analysts; one analyst performed this experiment extensively with multiple experiments.

Different laboratories-To establish the difficulty in potentially training an analyst without prior molecular biology experience, DNA extracts were sent to a student at Florida International University for duplicate processing along with a set being processed at the Connecticut State Forensic Science Laboratory. A set of work instructions and equipment specifications were included with the samples. Data was co-reviewed by each facility.

Effect of drying plant material-Since marijuana is frequently purchased as dried leaf and stem fragments, fresh and dried material was processed and compared for DNA patterns. Two live plants were removed from the ground during a seizure operation and inverted to air dry in a dark room at 25C and relative humidity at the Connecticut State Forensic Laboratory.

Effect of time-A substantial amount of time may intervene between when a fresh plant is harvested and when it is purchased and used by the consumer. Therefore, the dried plants were sampled for an extended period of time (0, 1, 2, 3, 5, 7 days; 2, 3, 4, 8, 12, 16, 20, 25 and 32 weeks) to determine the effect of drying and potential DNA degradation on the DNA patterns.

Effect of DNA degradation-DNA from a single fresh plant was digested into fragments with an enzyme, DNase I (Gibco BRL). Aliquots of the same DNA stock were removed and incubated with the enzyme for the following time points: 0, 0.5, 1, 1.5, 2, 4, 6, 9, 15, 23 and 35 minutes. The samples were then visualized for the level of degradation by

electrophoresis on a 1% agarose gel stained with ethidium bromide. In addition, a ten year old plant sample (*Jacaranda*) was processed multiple times and the DNA patterns compared.

Effect of DNA template concentration-Starting DNA concentration in the PCR reaction may have an effect on the resultant AFLP profile. To test this hypothesis, different amounts of DNA (450, 200, 100, 50, 20, 10, 5, 2, 0.5 and 0.2ng) were processed in duplicate and the DNA patterns compared. In addition, single seeds of marijuana and tomato (*Lycopersicon* spp.) were extracted for DNA to determine if sufficient quantity and quality DNA could be obtained from a single seed for AFLP testing.

B. Ability to discriminate between related plant samples and street seizure samples:

Half-sibling and seed stocks-Three marijuana seizure samples were obtained with different case numbers. Each had a female flower from a single plant that contained a few seeds. The seeds were dissected into two components: the embryo and the pericarp. DNA was also extracted from the parental somatic tissue. Each sample was processed separately and the patterns compared. Several bags of labeled seeds were seized from one case by the Royal Canadian Mounted Police (RCMP). Representative samples were extracted and processed for DNA patterns from several bags that were labeled with genetic breeding information. The number of fragments that differed between parent and seed and the different seed samples were compared.

Random street seizures-A population database of donated marijuana street seizure samples from multiple states was generated to gain a better understanding of how similar or different one might expect marijuana samples to be based on unknown genetic heritage. The donated samples were acquired from a variety of locations where plants were being grown outdoors in fields, indoors in soil or as hydroponic operations (6-8); also as drug samples packaged for individual use.

Results and Discussion

A. Reproducibility:

No differences in AFLP profiles were observed between the duplicate samples that were processed by two trained analysts at the Division of Scientific Services in Connecticut. In fact, multiple students on internship were also trained to perform this DNA testing method, most with complete success in obtaining reproducible results. The most frequent sources of error that were identified as affecting test result reproducibility both within a laboratory and between laboratories based on individual analysts were the following: pipetting skills, attention to protocol details, lack of mixing of samples prior to PCR amplification, incorrect dilutions of restriction enzymes to the recommended concentrations for AFLP and computer errors. Appropriate training and prior molecular biology experience were the two most significant factors in analysts achieving reproducible DNA patterns.

The use of different models of thermal cyclers for PCR amplification may affect AFLP patterns based on ramp speeds which can alter the manner in which PCR primers anneal

to the DNA template. Most significantly, one analyst observed single peak drop out between samples that should have been identical when using two different models of thermal cyclers. This same observation was made when a ten-year old plant sample (with marginal quality DNA) was processed by the AFLP method. Both types of observations can be best explained by a similar mechanism (Figure 1). In the case of the different ramp speeds on thermal cyclers, PCR primers may not anneal efficiently to the DNA fragment and peaks may drop out of a profile due to inefficient amplification. This same issue has been noted for RAPD markers (9) and can be corrected by using comparable equipment and testing known standards prior to performing tests on the evidence. The ten year old plant sample contained degraded DNA based on a visual exam on an agarose gel. This test case and the enzymatic digestion studies both indicate that if the starting DNA for AFLP is badly damaged, some fragments will not amplify by PCR. To avoid this situation, only DNA that is of sufficient quality (>1Kb) should be used for AFLP analysis (Figure 2).

No differences in AFLP patterns were observed when comparing fresh and dried marijuana samples even over an extended time period of thirty-two weeks. In addition, a wide range of DNA template concentrations resulted in comparable AFLP profiles as long as the relative peak heights were within approximately two-fold of each other (Figure 3). In other words, duplicate samples that were on a similar scale for relative fluorescence have identical DNA patterns. Samples that were more than two-fold different in relative fluorescence units may exhibit minor peak drop-out. The peaks that dropped out of the profile could be detected again once the samples were processed and

detected within the same scale. These results agree with that of Vos et al. (1995). High quality and sufficient quantity of DNA was obtained from both single marijuana and single tomato seeds indicating this method may be very useful for sourcing trace seed evidence from clothing or stomach contents (3).

B. Population studies:

The seeds collected from the marijuana flowers represent half-siblings since they all share at least the female parent. It was unknown whether they were pollinated from one or more male plants. When their DNA patterns were compared for multiple PCR primer sets, they averaged between two and ten peak differences between parent and seed. When compared to each other, the seeds averaged between one and seven peak differences for three different PCR primer sets. For the seeds collected from the RCMP, it was expected that they may be any one of the following based on the label: siblings, half-siblings, or individuals of the same variety; however, the variety was not specifically designated. In all cases, a single PCR primer set was sufficient to distinguish between the seed samples.

A survey of existing street seizures of marijuana with unknown cultivar designations revealed some interesting results. In one case from Iowa, an entire field of marijuana samples (approximately 500 plants) had identical DNA profiles indicating they were propagated clonally and then planted outdoors. In another case in Connecticut, multiple cultivars were being expanded clonally in an indoor hydroponic grower operation. For most of the random street seizures from across the United States, the DNA profiles were

unique indicating either that the marijuana samples were generated by seed or the sampling was random enough that clonal marijuana samples were not encountered repeatedly except when in local geographic regions. For example, two samples from the Northeast that were collected from different states had identical DNA profiles suggesting they originated from a common clonal population.

The validation of the AFLP method for plant evidence has defined the conditions for when plant evidence can be appropriately processed for DNA comparisons between evidentiary samples and known reference samples. The quantity and quality of the DNA needs to be sufficient for an accurate comparison; otherwise a false exclusion may result due to peak drop out during the PCR amplification steps. The DNA template concentration has relatively little effect on the DNA profile as the same profiles were observed for 200, 20 and 2 nanograms of DNA in the AFLP reaction. The generation of a marijuana DNA database of approximately three hundred unrelated cases contained both unique and clonally propagated samples and gave a relative estimate of the extent of genetic diversity one might expect from marijuana seizure samples.

The AFLP method is a useful technique for any single source plant sample since it can be applied to any organism without prior knowledge of the target segments of DNA. The DNA fragment patterns generated by AFLP are considered multilocus in nature and genetic linkages between fragments are unknown. For this reason, when reference population databases are generated for comparative purposes, it is suggested that a modified counting method be used to determine random match probability estimates.

Although this may decrease the power of discrimination of the test method, a conservative statistical approach and the use of a generally applicable method for all forms of plant evidence should increase its usage in forensic casework.

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Figure Legends:

Figure 1. Two different mechanisms can result in a DNA fragment dropping out of an AFLP profile. First, differential annealing of PCR primers to the DNA template due to thermal cycler ramp speed differences can result in loss or reduction of a fragment. Secondly, a break in a DNA fragment can result in loss of amplification and a peak being absent from an AFLP profile.

Figure 2. The AFLP profiles generated from four treatments with DNaseI to determine the effect of degradation of template DNA on AFLP profiles. A) 2 minutes with DNA fragments >1Kb, B) 9 minutes with DNA fragments 500-50bp, C) 15 minutes with DNA fragments 300-50bp, D) 23 minutes with DNA fragments 200-50bp. The effect of DNaseI-induced degradation indicates larger fragments do not amplify well as DNA becomes increasingly more fragmented and reduced in size. DNA fragments greater than 1Kb yield a highly reproducible AFLP profile.

Figure 3. Varying the starting DNA template concentration shows no difference in the AFLP profiles (A-C; 200, 20 and 2 ng, respectively) unless the starting concentration is low (D, 0.2ng). In panel D, some minor peak drop out can be observed.

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Relevant Scientific Publications:

H. Miller Coyle, C.-L. Lee, T. Palmbach and H.C. Lee. 2005. Collection and preservation of plant evidence. Manuscript in preparation.

C.-L. Lee, H. Miller Coyle, E. Carita, C. Ladd, N. Yang, T.M. Palmbach, I. C. Hsu and H.C. Lee. 2004. DNA analysis of digested tomato seeds in stomach contents. American Journal of Forensic Medicine and Pathology (in press).

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Scientific Presentations:

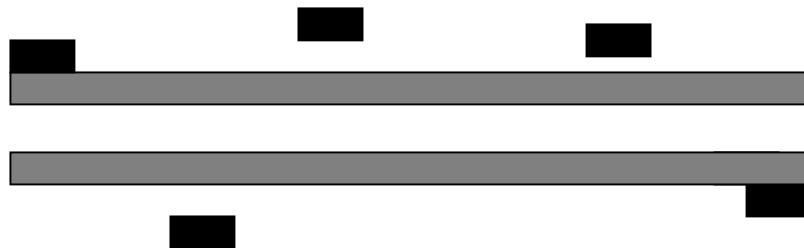
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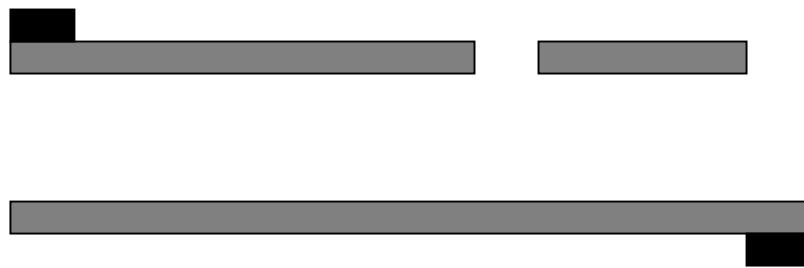
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Transfer of Technology Workshops:

- (2) Laboratory based training sessions for workshop attendees sponsored by the Henry C. Lee Institute for Forensic Sciences

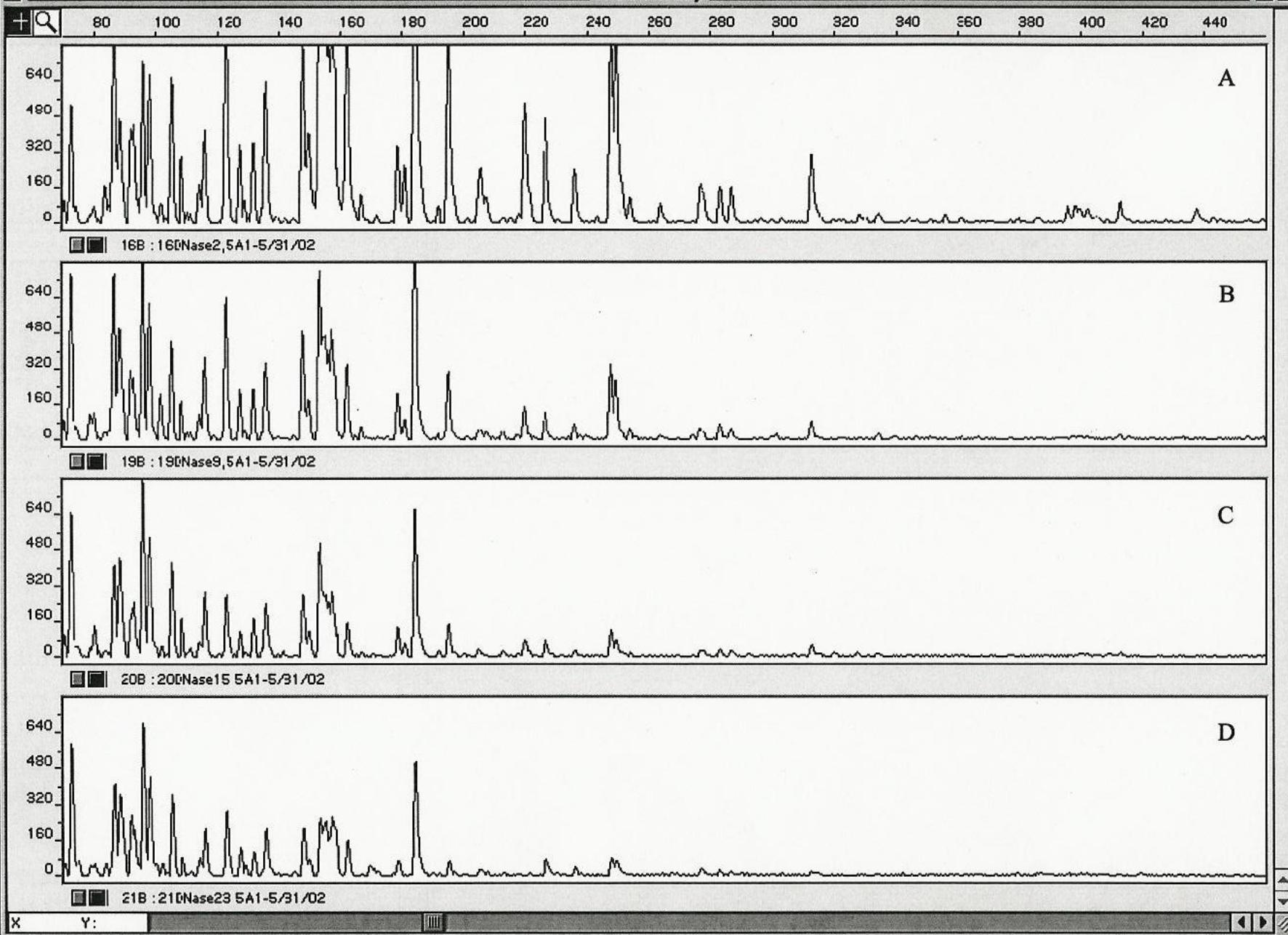


A) Differential amplification may result from PCR primers (black) annealing at different times and temperatures due to ramp speeds

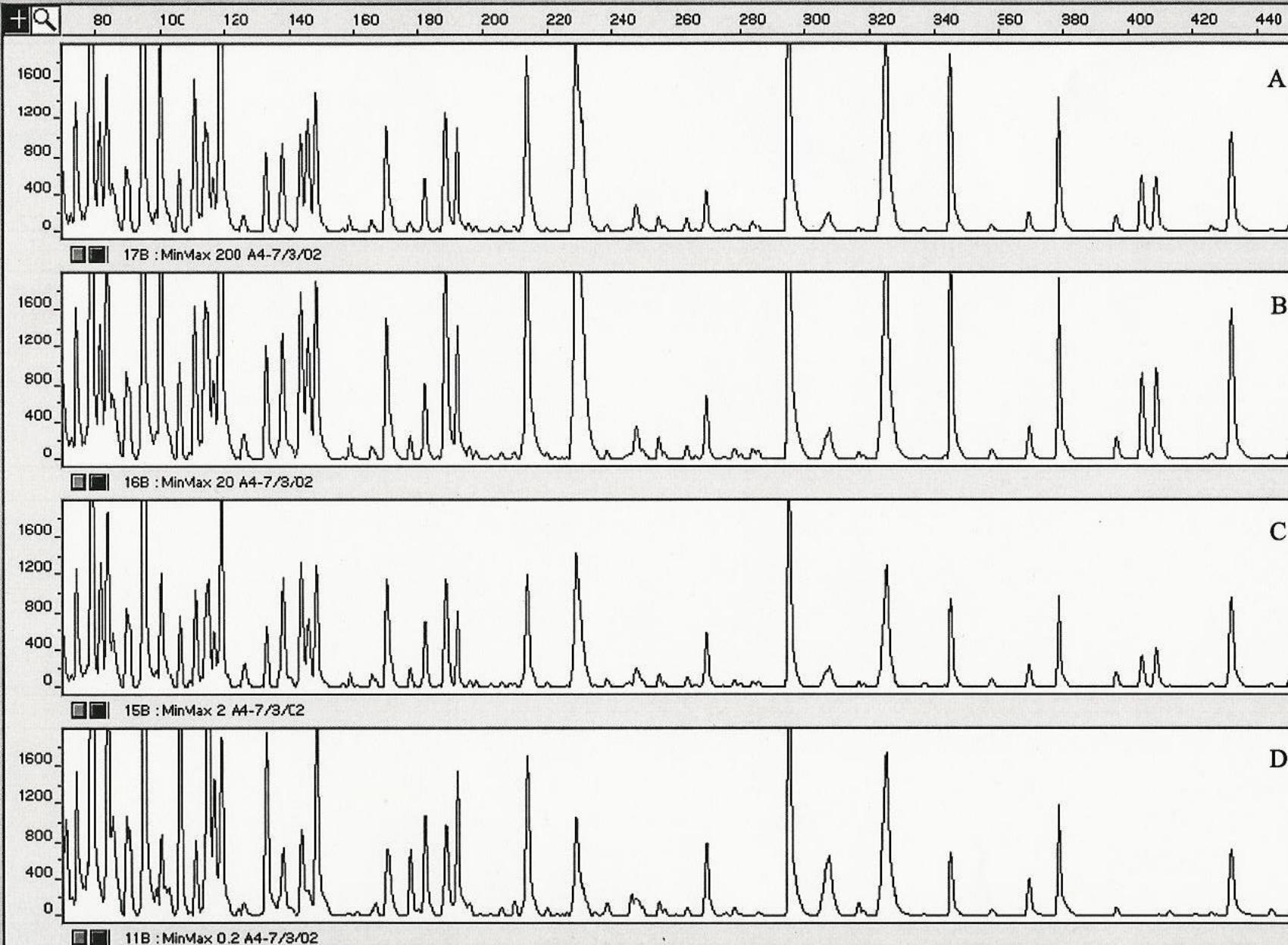


B) DNA strand breakage results in a loss of peak amplification

DNase Study



Sensitivity Study Display-1



X Y:

◀ ▶