Automated Analysis of Fluorescent Amplified Fragment Length Polymorphisms for DNA Typing

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SUMMARY

We have utilized fluorescently labeled PCR primers to amplify 20 ng of genomic DNA at three different VNTR loci. By using an instrument capable of discriminating the fluorescence emitted from four different dyes, it was possible to analyze the fluorescent PCR products from the three VNTR loci along with an in-lane fluorescently labeled size standard. The instrument software established a calibration curve for each lane of the gel based on the mobility of the standard in the lane, and then automatically calculated the length of the nucleic acid molecules in base pairs from the calibration curve. 24 individuals could be analyzed per gel for 3 VNTR loci within 6 hours on 2% agarose gels capable of resolving 15 base pair repeats with a high degree of precision and accuracy. In a validation study, approximately 100 samples were examined at the D1S80 locus, and the data compared perfectly with published results obtained by electrophoresis on polyacrylamide gels and detection by silver staining.

INTRODUCTION

Analysis of electrophoretic gels in the molecular biology laboratory has often been limited to visual inspection of the bands for differences in shape, intensity, and position. The desire for numerical values has led to the use of scanning densitometers and software to integrate the area associated with a band. However, these instruments are not always useful for establishing a match in the length of two DNA fragments. Some of the problems in determining a match in fragment size have been eliminated by switching from RFLP analysis to the PCR, but there still exists the possibility of lane-to-lane differences in DNA mobility. In order to alleviate this problem, we have used a size standard, which can be co-electrophoresed in the same lane as the sample. By this technique, the size of a sample is determined by the way it migrates in relation to the known sizes of the fragments of the standard that are subject to the same effects in the electrophoretic lane as the sample. In this communication, we describe the features of an instrument for automatic DNA fragment analysis that is both convenient to use and capable of high throughput. The performance of the instrument is discussed in terms of its analytical qualities.

AUTOMATION OF DNA FRAGMENT ANALYSIS

The use of fluorescence for DNA sequence analysis is well established, and the principles of this method can be found in numerous reviews. In this work, we have utilized the Applied Biosystems 362 GENE SCANNER, which incorporates fluorescent DNA sequencing technology in a horizontal gel configuration for agarose gels. The fluorescent DNA fragments are excited by a laser beam and the emitted fluorescence is collected as the fragments move past a window that allows the light to pass. The DNA fragments are thus detected in real time as the bands move through the gel during electrophoresis. The optics are mounted on a stage

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that moves back and forth in a direction perpendicular to the direction of electrophoresis, permitting the analysis of 24 lanes per gel. A filter wheel is placed in the optical pathway and made to rotate in synchrony with the scanning stage to permit the discrimination of the fluorescence emission bands of multiple dyes. The filtered emitted light is collected by a photomultiplyer and converted to digital data. Analysis software can correct for the spectral overlap of the blue, green, yellow, and red fluorescence emitted by the dyes and yields a collection file, from which one obtains a presentation of the data as a familiar gel image. Thus, bands of different color, representing different alleles, appear as individual entities. The sizes of the sample bands are then automatically determined from the calibration curve obtained from the co-electrophoresed standard size ladder. We used a second order least squares curve fit to the size ladder to obtain the calibration curve. The analyzed data are stored in a tabular form suitable for export to database management software.

METHODS OF VNTR ANALYSIS

We have analyzed 94 blind, pristine DNA samples from unrelated Caucasians for the VNTR loci D1S80 (Kasai et al., 1990), D17S5 (Horn et al., 1989), and COL 2A1 (Wu and Bell, 1990) using published sequences for the primers, generally using 20 ng DNA in the PCR. In addition, we examined Chelex (BioRad) extractions of bloodstains on cotton fabric for the same loci using 10 µl of the supernatant in the PCR. We used two methods for obtaining the allele size data. In one, we loaded per lane a pool of 1 µl of the amplification products of three individual PCRs obtained from the same DNA sample using primers fluorescing in blue for D17S5, in green for D1S80, and in yellow for COL 2A1. In the second method, we analyzed three different DNA samples per lane for the same VNTR locus, using a different color for each sample. We added 3 µl of a 2X load buffer containing a red fluorescing size standard, which had a fragment of 946 bp as the longest band. 5 µl of the mixture were loaded in the well of a 2.1% SeaPlaque (FMC) agarose gel at a distance of 4 cm from the detection window. The gel was subjected to electrophoresis at 4 volt/cm for 6 h. In some cases, we re-utilized gels from previous runs and compared the data with that obtained with freshly prepared gels. Used gels generally run faster than fresh ones, so that the electrophoresis and analysis could be reduced by 1 h. Samples found homozygous for the D17S5 locus were re-run on a 1.6% SeaPlaque gel for 5 h with a size standard having bands with lengths of at least 1740 bp.

RESULTS OF THE VALIDATION STUDY

To test the instrument performance, a validation study was carried out using samples that had been analyzed for their D1S80 locus allele frequency on polyacrylamide gels (Budowle et al., 1990) The samples had been binned at the FBI Academy, but the numbers associated with the samples for the Applied Biosystems 362 GENE SCANNER validation study gave no indication of the allele size. The analysis with the GENE SCANNER of these blind samples gave an identical allele frequency as that reported previously, thus demonstrating good precision for the data set. In multiple runs with the same sample, the precision was greater than 99.5%. The repeat length of 16 bp was readily apparent from the difference in size between adjacent bands. Allele 18 was called by the software as 428 bp in length, which must be composed of 16X18 = 288 bp from the repeated segment and 145 bp from the flanking regions established by the primer sites (Kasai et al., 1990). The difference between the size calculated from the electrophorectic analysis, 428 bp, and the expected size of 433 bp is only 5 bp, yielding an accuracy of 98.8%. This degree of accuracy was typical for all allele sizes found in the sample set. A higher degree of accuracy would be expected with a D1S80 allelic size ladder, since DNA fragments composed of specific sequence repeats should migrate differently than restriction fragments composed of a random base composition.

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Since the DNA fragments run off the gel during electrophoresis, the gel may be re-used for further separations. However, we noted that the DNA fragments migrate faster on the re-used gels, and it was necessary to determine whether an alteration of the gel matrix affects the size calling precision. Upon analysis of 20 randomly selected samples, we obtained the same allele frequency as we had determined from the electrophoresis through fresh gels. The observation that a difference in the gel matrix does not affect the allele assignment is probably derived from the fact that the size ladder also migrates faster in the used gels.

Sizes determined for the alleles were the same whether three differently colored samples of the same VNTR were loaded in a lane or whether one sample of three differently colored VNTRs of the same sample were loaded in a lane. In other words, it is possible to process 72 samples for the same VNTR when three colors are used for the primers during the PCR. This proves that the software can distinguish three fragments of different color co-migrating on the gel.

COMPARISON OF THE ALLELE FREQUENCIES OF THREE VNTR LOCI

In the 94 samples, we found alleles ranging in size from 428 to 776 bp for D1S80, from 168 to 1040 bp for D17S5, and from 584 to 779 bp for COL 2A1. The repeat lengths between adjacent bands were 16 and 70 bp for the D1S80 and D17S5 loci, respectively. For these two loci, it was easy to assign the DNA fragments to bins upon consideration of two factors: (1) the median value of a set of DNA fragments and (2) the observed repeat lengths. The DNA fragments for the COL 2A1 VNTR could be assigned to bins from the data base, but because there are two repeat lengths for this VNTR (31 bp and 34 bp), it was difficult to establish the bin boundaries. A wide range of lengths are possible for each COL 2A1 fragment, depending on the way the fragment is constructed from the two repeat segments. With a second order least squares curve fitting procedure for the data analysis, we generally found a repeat length of 33 base pairs for COL 2A1. The use of polyacrylamide gels might be preferable for this VNTR.

From the foregoing discussion, it is clear that the use of the PCR and the fluorescence analysis method should make it easy to obtain population data bases accurately and with a high degree of precision. However, the use of the fluorescence technique is not limited to pristine DNA samples. Bloodstains on cotton cloth (3 mm square), extracted with 5% Chelex (Walsh et al., 1991) yielded results of the same quality as those obtained with pristine DNAs. This result suggests that fluorescence methods can be useful in casework analysis. We plan to test cigarette butts, hairs, and mixed body fluids for DNA typing in the future.

REFERENCES

Budowle B, Chakraborty R, Giusti A, Eisenberg AJ, and Allen RC (1990) Analysis of the VNTR Locus D1S80 by the PCR Followed by High-Resolution PAGE. Am J Hum Genet 48: 137-144

Horn GT, Richards B and Klinger KW (1989) Amplification of a Highly Polymorphic VNTR Segment by the Polymerase Chain Reaction. NAR 17: 2140

Kasai K, Nakamura Y, and White R (1990) Amplification of a Variable Number of Tandem Repeats (VNTR) Locus (pMCT118) by the Polymerase Chain Reaction (PCR) and Its Application to Forensic Science. J Foren Sci 35: 1196-1200

Walsh PS, Metzger DA and Higuchi R (1991) Chelex 100 as a Medium for Simple Extraction of DNA for PCR-Based Typing from Forensic Material. BioTechniques 10: 506-513.

Wu S, Seino S and Bell GI (1990) Human Collagen, Type II, alpha 1, (COL2A1) Gene: VNTR Polymorphism Detected by Gene Amplification. NAR 18: 3102

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