An Evaluation of Automated Fluorescent PCR-Based DNA Typing

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Abstract - Introduction

The recent revolution of new technologies in forensic DNA typing has presented several promising PCR-based identification systems. Compared to current RFLP VNTR systems, PCR offers the advantages of enhanced sensitivity, more discrete alleles and the efficacy of working with degraded DNA samples. In order to achieve the equivalent DNA discrimination experienced with RFLP VNTR analysis, the new PCR-based systems will require a greater number of STR systems to be run or additional procedures involving AmpFLPs systems or digital DNA typing (MVR). A concerted approach using several DNA typing technologies represents the future reality in the processing of numerous biological forensic samples. PCR-based DNA typing with automated real-time analysis of fluorescent amplification products is currently a major thrust of the RCMP DNA program.

AmpFLP Detection Procedures

Currently many laboratories have adopted the use of AmpFLP (amplified fragment length polymorphism) PCR identification for forensic applications (1,2), with specific interest in the loci D1S80, D17S5, Col 2A1, and Apo B. The use of fluorescent labelled primers allows the potential of simultaneous multilocus discrimination. In our own studies, we have found that the simultaneous analysis of the two AmpFLP systems, D1S80 and D17S5, offers excellent discrimination potential in a "diplex" reaction (Figure 1). Allele dropout encountered for the upper alleles in the D17S5 locus is generally a detection sensitivity issue that in the majority of cases is circumvented by using labelled fluoroprimers for both the forward and reverse reaction. In addition, although most alleles are easily designated by precise size identification using internal molecular weight size standards (ABI Genescan 1000 or 2500 size standards),variant and normal alleles are easily compared using fluorescent labelled allelic ladders (Figure 1). Several considerations, such as the great number of alleles found for each system, less problematic discrimination of variants due to gel resolution limitations, and the precedent for acceptance of these systems using silver-stained detection procedures, make AmpFLP systems excellent introductory automated fluorescent identification systems for PCR-based identification.

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STR DNA Analysis

The current scientific literature (3) suggests that the number of potential short tandem repeats (STRs) for polymorphic identification in the human genome could easily surpass 500,000. The more familiar STR systems (Table 1, ref 4,5), composed of trimeric and tetrameric repeats have fewer alleles but may provide high discrimination through the subsequent addition of cumulative STR systems. Ideally, routine forensic analysis of several STR systems could be accomplished by co-amplification in a multiplex procedure, or simply by analysing the products of single amplified reactions in a combined sample lane on a polyacrylamide gel. Both these approaches require complete knowledge of the optimized amplification conditions for each STR system and the resolution characteristics of non-overlapping alleles using silver-stained detection, or alternatively different fluorescent tagged STRs and differential colour detection.

The approach taken in our laboratory has involved the differential fluorescent labelling of each STR system and the utilization of optimized amplification conditions to eliminate potential PCR artifacts. As noted in Table 1, the empirical optimization of amplification conditions clearly delineates different optimal annealing temperatures, and therefore provides the initial criteria for combining several STR systems in a multiplex format. The multiplex analysis of single sample with a potentially limiting amount of target DNA could provide probative information on several STR systems in a timely manner. However, a multiplex reaction involving different primer sets, may not be as robust as a series of single STR systems amplified independently. Consequently, the limitations and advantages of multiplex reactions must be carefully examined and validated using forensically relevant samples with specific recommended reaction conditions and allele detection methodologies. For example, preliminary analysis of multiplex reactions has suggested that 5 ng of target DNA may provide an optimal multiplex template under the amplification conditions specified for many single amplification reactions. Clearly, conditions could be optimized for specific multiplex amplification reactions which would enable detection of multiple alleles with a smaller concentration of target DNA, but these conditions undoubtedly will differ from the "singleplex" reaction conditions. Numerous multiplex STR systems will undoubtedly be described in the future forensic literature, yet the ultimate challenge will be to develop a robust procedure with high discrimination that is easily detected in an automated fluorescent format. One such system under investigation in our laboratory (Table 2) combines the advantage of gender discrimination through the use of the amelogenin sex-typing locus (6) with discrimination at two other STR loci.

Automated Fluorescent Detection of MVRs (minisatellite variant repeats)

There has been considerable interest in the utilization of MVRs (7) for discrete identification. MVRs can potentially provide unlimited discrimination and appear to be immune to many potential problems encountered in other PCR-based systems (e.g. electrophoretic variation, the use of different size or allelic markers). However, deciphering complex mixtures or documenting the vast amount of discrimination data

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may prove to be more challenging than with conventional PCR-based systems. Ease of interpretation and recording the high number of potential alleles makes automated detection using fluorescent tagged alleles ideal for MVRs. Unlike silver-staining or intercalating detection methods that are based on the mass binding relationship of dyes or stains for detection sensitivity, fluorescent labelling is a simple molar incorporation phenomenon such that a DNA fragment with more fluorochromes attached will be detected with much higher sensitivity. After considerable empirical investigation, it was demonstrated that a fluorochrome dye labelled nucleotide provided the best method for tagging multiple MVR alleles. As noted (Figure 2), 20 ng of template DNA will provide sufficient material for the discrimination of greater than 30 MVR repeats. Optimization of the amplification reaction and methods for eliminating free fluorescent tagged nucleotides will have to be established before a practical and robust routine forensic analysis will become available. The use of internal size standards such as ABI fluorescent tagged Genescan 2500 in each MVR sample lane proved highly effective as an internal calibration standard for the potential adjustment of mobility shifts encountered when comparing several sample lanes. Ideally, the use of automated detection of fluorescent labelled MVR alleles should allow for the rapid generation of databases and the recording of the vast amount of discrimination data obtained from each sample as well as provide an excellent fluorescent quantitation method for unambiguous determination of homozygote repeat patterns.

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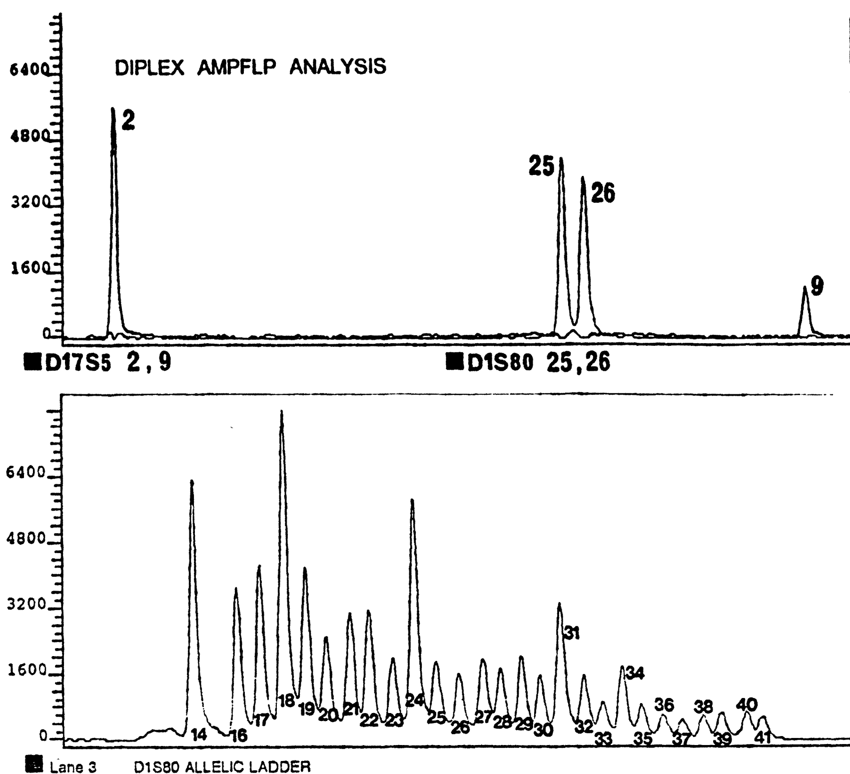
Figure 1. Ampflp Allele Measurements



D1S80 Allelic Ladder Size Comparison Using ABI Internal Size Standards:

Allele	Size bp	Allele	Size bp	Allele	Size bp	Allele	Size bp	Allele	Size
14	356.1	21	468.3	27	549.0	33	643.9	39	742.4
16	388.8	22	479.9	28	560.4	34	660.9	40	760.9
17	406.4	23	493.4	29	577.2	35	678.2	41	772.9
18	424.6	24	506.3	30	592.5	36	697.0		
19	441.6	25	520.0	31	608.8	37	711.2		
20	454.3	26	534.4	32	628.9	38	727.7		

DIPLEX AMPFLP ANALYSIS

Ampflp System	Most Common (2pq*)	Most Rare (2pq*)
D1S80	1/6	1/20,000
D17S5	1/12	1/10,000
Combined Diplex	1/72	200 million

*RCMP Canadian Caucasian Database

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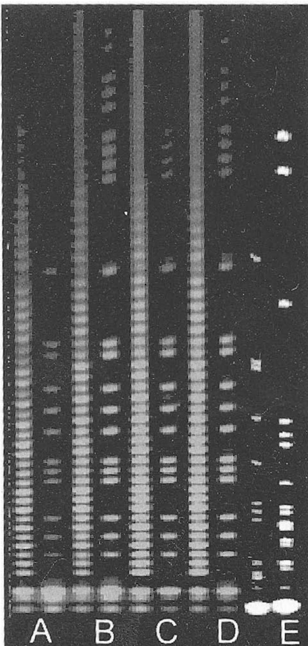
Table 1. STR Allele Number and Empirically Derived Annealing Temperatures

Locus	Primer/Length	%GC Content	Optimal Temperature °C	Product Size	Repeat Unit	Allele #
CD4	32/34	53/54	68	140-170	AAAG	7
vWF	24/28	38/36	64	102-154	TCTA	7
THO1	24/24	50/50	68	179-207	AATG	5
D21S11	18/22	50/36	64	172-264	TCTA/TCTG	12
FABP	25/24	44/50	64	199-223	AAT	6
ACTBP	20/20	50/50	64	231-339	AAAG	44
HPRT	24/24	46/42	60	257-297	AGAT	8
ARA	24/24	54/50	68	255-315	AGC	15

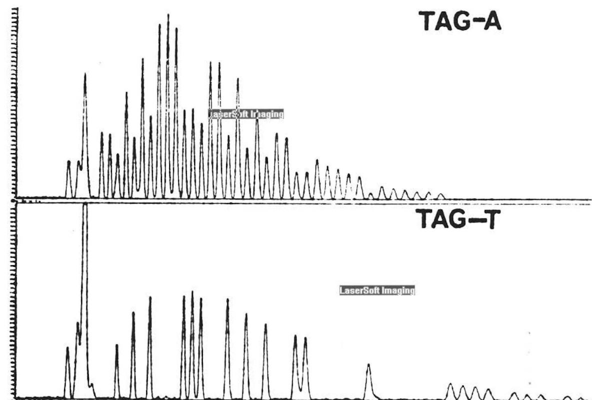
Table 2. Multiplex STR Triplex

STR System	Most Common (2pq*)	Most Rare (2pq*)
D21S11	1/8	1/15,900
FGA	1/17	1/30,700
Amelogenin	1/2	1/2
Combined Triplex	1/272	976 million

*RCMP Canadian Caucasian Database



Fluorescent MVR Analysis Using Tagged dUTP **Figure 2**



A 25 ng DNA
B 50
C 75
D 100
E standards