An Investigation Into The Use of Single Nucleotide Polymorphisms for Forensic Identification Purposes

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Thesis submitted to the University of Wales in candidature for the degree of Doctor of Philosophy

March 2006

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DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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"CRIME IS COMMON. LOGIC IS RARE. THEREFORE IT IS UPON THE LOGIC RATHER THAN UPON THE CRIME YOU SHOULD DWELL"

Arthur Conan Doyle (1859 - 1930) Scottish author, physician

ACKNOWLEDGEMENTS

To Peter Gill. You are an inspiration, and if I can get away with being like you when I grow up, I will be a happy woman. Thank you for always having the time to help me, for never shouting at me and for always knowing where I could find the answer if it didn't come directly from you.

Thanks to Mike Bruford, for all his academic support.

Many thanks to Jon Wetton for agreeing (in a moment of insanity) to read through this thesis and for sharing all his intelligent thoughts.

And to my Mum and Dad. This is for you, for all the years of worry and for many more years of happiness. Thank you for believing in me and making me believe in myself.

SUMMARY

Major limitations of the short tandem repeat (STR) loci that form the basis of criminal DNA databases are the 'partial' profiles that result from degradation of the longer repeat sequences. In contrast, Single Nucleotide Polymorphisms (SNPs) can be encompassed in smaller amplicons increasing the chance of amplification in degraded and limited samples.

To aid SNP analysis a range of studies were performed including creation of the ASGOTH (Automated SNP Genotype Handler) software for rapid and accurate sample genotyping on microarrays. A multiplex assay for simultaneous detection of 20 SNPs plus a sex-determining locus by single-tube PCR amplification and electrophoretic detection was also developed. All loci conformed to Hardy-Weinberg equilibrium and showed independent inheritance. Computer simulations characterised the effects of inbreeding and supported the use of current STR F_{ST} correction factors. Both paternity testing and kinship analysis were compared to STR DNA profiling results.

Interpretation criteria were formulated for correct genotyping of the 21-SNP multiplex to control for stochastic variation at low DNA inputs. Each locus was individually characterised for allele dropout, homozygous thresholds and heterozygous balance.

The performance of the 21-SNP multiplex on degraded samples was compared with the AMP*Fl*STR® SGMplus™ (SGM+) STR method currently used for the UK National DNA Database®and other DNA profiling techniques used across Europe. Applying the 21-SNP multiplex to casework samples previously profiled using low copy number (LCN) SGM+ amplification indicated that partial SNP profiles could be generated in samples that had given partial LCN SGM+ profiles, but samples failing to amplify using LCN PCR parameters would also fail with SNPs.

This study demonstrated the use of SNPs for human forensic identification purposes as an adjunct to current STR methods and has formed the basis of further work on degraded DNA and the design of the next generation of DNA profiling systems.

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ABBREVIATIONS

µg	Microgram
µL	Microlitre
µM	Micromolar
6-FAM	6-carboxyfluorescein
A, dATP	Adenine, deoxyadenosine triphosphate
AB	Applied Biosystems
ANOVA	Analysis of variance
ARMS	Amplification refractory mutation system
ASGOTH	Automated SNP Genotype Handler
bp	Base pairs
BSA	Bovine serum albumin
C, dCTP	Cytosine, deoxycytidine triphosphate
CE	Capillary electrophoresis
°C	Degrees Celsius
DNA	Deoxyribonucleic Acid
E	Evidence
EDNAP	European DNA profiling Group
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
ENFSI	European Network of Forensic Science Institutes
FSS	The Forensic Science Service™
$\mathbf{F_{ST}}$	Fixation index (θ)
G, dGTP	Guanine, deoxyguanosine triphosphate
GDA	Genetic Data Analysis
HET or het	Heterozygote
HMW	High molecular weight
HOM or hom	Homozygote
H_d	Hypothesis for the defence
H_p	Hypothesis for the prosecution
HWE	Hardy-Weinberg equilibrium
JOE	2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein
LCN	Low copy number
LMW	Low molecular weight
LR	Likelihood ratio
Mg	Magnesium
$\mathbf{MgCl_2}$	Magnesium chloride
min	Minute
mL	Millilitre
mM	Millimolar
MtDNA	Mitochondrial DNA

NDNAB	National DNA Database®
ng	Nanogram
NIST	National Institute of Standards and Technology (US)
nM	Nanomolar
nt	Nucleotide
OLA	Oligonucleotide ligation assay
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
$\mathbf{P_E}$	Probability of exclusion
pg	Picogram
PI	Paternity index
Pm	Match probability
Pr	Probability
R&D	Research and Development
RFLP	Restriction Fragment Length Polymorphism
rfu	Relative fluorescent unit
ROX	6-carboxy-X-rhodamine
SD	Standard deviation
SDW	Sterile Distilled Water
sec	Second
SGM	Second Generation Matrix
SGM+	AMP*FI*STR® SGM Plus™ system
SLP	Single locus probe
STR	Short Tandem Repeat
SNP	Single Nucleotide Polymorphism
SWGDAM	Scientific Working Group on DNA Analysis Methods
T, dTTP	Thymine, deoxythymidine triphosphate
Tm	Melting temperature
Tris	Tris(hydroxymethyl)methylamine
U	Units
URP	Universal reporter primer
UV	Ultraviolet
VBA	Visual Basic for Applications
VNTR	Variable number tandem repeat

1 Introduction

1.1 Human DNA Polymorphisms

1.1.1 Mutations in the human genome

DNA polymorphisms are present throughout the human genome and have been well studied and documented over the last forty years. In the 1990s the Human Genome Project set out to determine the entire human DNA sequence, and revealed the presence, and location, of millions of these DNA sequence variants throughout the genome (Chakravarti 1999; Kruglyak and Nickerson 2001; Kwok 2001; Venter *et. al.* 2001). Variations consist of insertions and deletions of a few to many nucleotides, variation in the repeat number of a motif (mini- and micro-satellites) or single nucleotide polymorphisms (SNPs) and, on a larger scale, chromosomal mutations such as inversions and translocations.

The process that produces heritable variations in DNA is driven by mutation. A mutation appearing in the germline can be transmitted to subsequent generations, whereas mutations in somatic cells are not inherited. If a mutation event occurs in an important region of the genome (especially in a coding region) then it is possible that a genetic disease may be the result. The process of mutation can be linked to events during chromosome segregation at meiosis, DNA replication and repair (Jeffreys *et. al.* 1988a), and spontaneous changes resulting from exposure to chemicals (Strachan and Read 1998).

According to the Mendelian Law of Segregation, an individual inherits two copies of the genome, one from the mother and one from the father. At each specific location on the chromosome, known as a locus, an individual may have a different genetic sequence. Alternative forms of a genetic locus are known as alleles and can be characterised by measuring their frequencies within a given population. If an allelic variant occurs at a frequency greater than 0.01 within a population then it is classified as a polymorphism, as the probability of it resulting from a chance recurrent mutation is low hence it is more likely to have been inherited (Strachan and Read 1998). Polymorphisms are of interest for forensic purposes as they can be used to distinguish individuals.

Variation within a coding DNA sequence can cause an alteration in the function of a particular protein (Strachan and Read 1998; Venter *et. al.* 2001). Mutations found in coding regions take on a number of different forms including nonsense mutations, where a difference in one base will cause a STOP codon leading to a shortened protein structure; missense mutations, causing one amino acid in a chain to be replaced by another; and silent mutations, a base change having no effect on the resulting amino acid encoded. These are all forms of base substitutions, also known as point mutations. There are two types of point mutation, depending on the nature of the base change (Lewin 1998): a transition, when a pyrimidine is changed to a pyrimidine or a purine to a purine (e.g. G or C base is exchanged with an A or T base respectively); or a transversion, when a purine changes to a pyrimidine or a pyrimidine to a purine (e.g. an A/T becomes a C/G). Transitions are the most common form of polymorphism as they produce the least marked change to the DNA sequence. Coding regions can also be affected by base insertions, where a number of bases are added to a sequence; base deletions, where a number of bases are deleted from a sequence; and large-scale chromosomal abnormalities.

Mutation provides the raw material for evolution to occur. In particular, mutations in coding regions may be deleterious to the affected individual, for example, causing a protein to become dysfunctional with lethal consequences. Selective pressure consequently reduces the levels of genes that are deleterious in populations. Sometimes a mutation might benefit an individual resulting in increased breeding success e.g. sickle cell anaemia in malaria-infested regions (Wood *et. al.* 1976; Hill *et. al.* 1991; Modiano *et. al.* 1996). Consequently, genes that increase fitness tend to be preserved and passed on to successive generations.

Evolutionary pressure does not work in the same way within the non-coding ("junk") regions of DNA that constitutes more than 95% of the total human genome (Ono 1972; Zuckerkandl 1992; Nowak 1994; Wong *et. al.* 2000). Recent developments in non-coding DNA research have shown that certain regions of non-coding DNA have higher levels of conservation than predicted, suggesting regulatory elements connected to genes may make up a large percentage of the "junk" DNA region (http://www.psrast.org/junkdna.htm). The rest of the non-coding DNA can demonstrate large numbers of polymorphisms, because there is less selective pressure over time. Polymorphisms mainly take the form of base substitutions and tandem repeat regions (see sections 1.1.2-1.1.5) and can be used for forensic identification purposes.

1.1.2 Tandemly repeated DNA sequences

Over 50% of the human nuclear genome contains highly repeated DNA sequences (DNA 'motifs') that appear to be largely inactive (http://www.euchromatin.org/; Wyman and White 1980). Some of these sequences are known as "tandemly repeated DNA" and vary in their size and composition to give three main subclasses of repeats: satellite DNA; minisatellite DNA (section 1.1.3); and microsatellite DNA (section 1.1.4). These motif regions are replicated with low fidelity because of a slippage that occurs between the template and the newly synthesised DNA strands during replication (Bell, Selby et al. 1982; Capon, Chen et al. 1983; Goodbourn, Higgs et al. 1983; Weller, Jeffreys et al. 1984; Stoker, Cheah et al. 1985; Tautz 1989). This slippage leads to a varying number of repeat motifs between individuals.

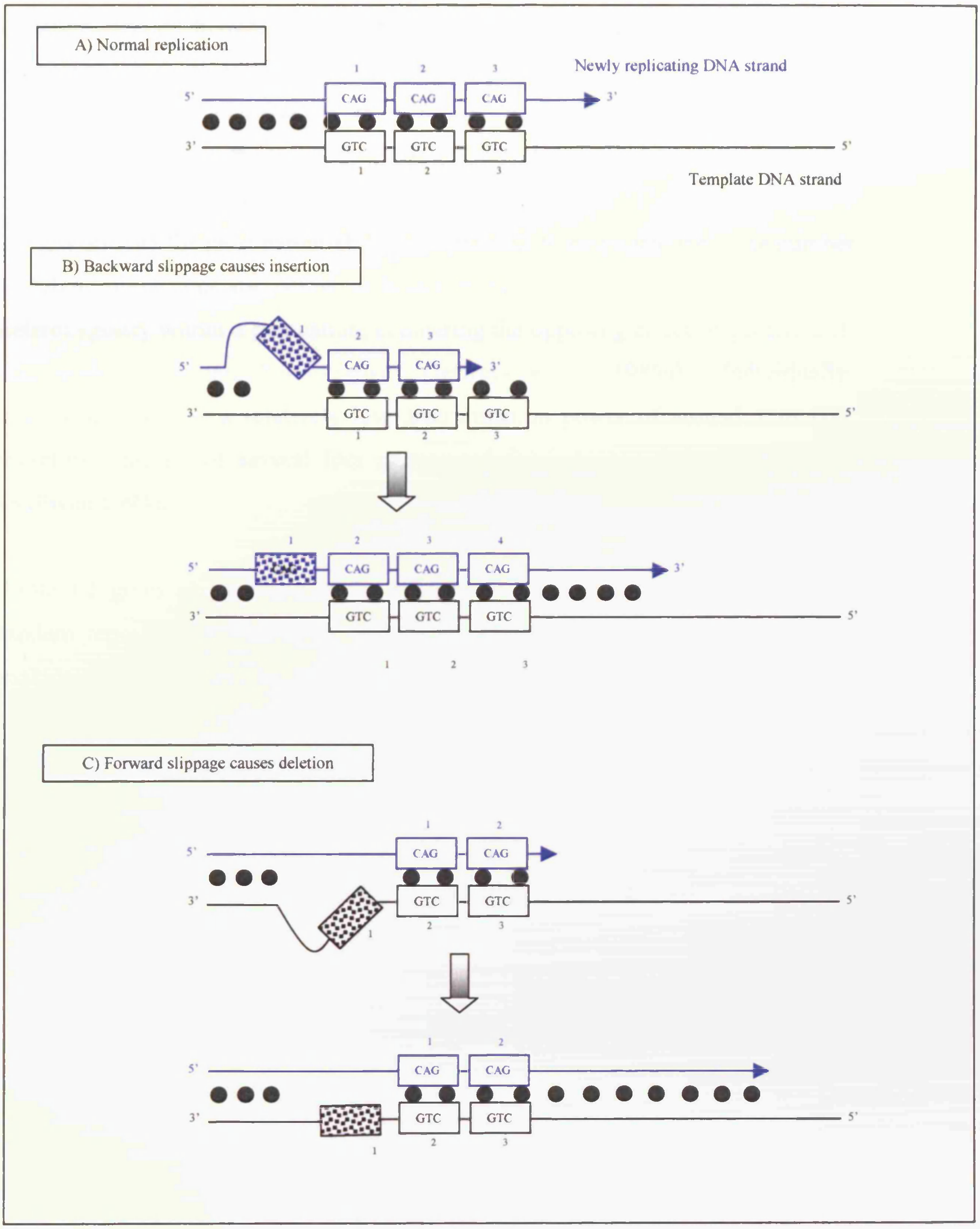

A) Normal replication
Newly replicating DNA strand
5'
3'
CAG
CAG
CAG
GTC
GTC
GTC
1
2
3
Template DNA strand
B) Backward slippage causes insertion
CAG
GTC
4
C) Forward slippage causes deletion

Figure 1.1 Slipped strand mispairing (replication slippage) during DNA replication can cause insertions or deletions. A) Normal replication leads to all three CAG repeat motifs being incorporated into the newly synthesised DNA chain. B) If all three motifs are synthesised before backward slippage occurs, a fourth motif can be added to the new strand. C) Forward slippage causes one or more repeats to be skipped giving a lower number of repeat motifs. *Taken from T. Strachan & A.P. Read (1998) "Human Molecular Genetics". BIOS Scientific Publishers Ltd, Oxford. Chapter 10 p254.*

The number of microsatellite repeats present in a DNA molecule changes by a mutation rate of between 10^{-3} and 10^{-4} per locus per gamete per generation (Weissenbach *et. al.* 1992; Weber and Wong 1993; Xu *et. al.* 2000; Huang *et. al.* 2002). This can increase up to 5 x 10^{-2} per gamete, as an extreme, in microsatellite loci (Jeffreys *et. al.* 1988a), although loci used in routine forensic analysis show rates lower than 10^{-2} (Jeffreys *et. al.* 1997). These mutation rates are low enough for most parent-child transmissions to propagate the same number of DNA motifs, but also allow sufficient mutation to maintain a high level of heterozygosity within a population, countering the opposing effect of genetic drift that tends to increase homozygosity (Jeffreys *et. al.* 1985a). Individually, microsatellites have a relatively low discrimination power of around 1 in 100, therefore analysis of several loci is required for a highly discriminating test (Sullivan 1994).

Table 1.1 gives an overview of the differences between the three subclasses of tandem repeats. Satellite DNA regions are large, spanning hundreds of bases, making them unsuitable for forensic analysis. In humans, minisatellite regions are found with greater frequency either within the telomeric regions of chromosomes or close to them. The hypervariable VNTRs (Variable Number Tandem Repeats) have been utilised for forensic identification but were superseded by microsatellite loci in the 1990s (see section 1.2). Microsatellite DNA is found across all chromosomes and is used in current DNA profiling techniques.

Class	Size of repeat unit (base pairs)	Major chromosomal location (s)
Satellite DNA (blocks often from 100kb to several Mb in length)		
Satellites 2 & 3	5	Most, possibly all, chromosomes
Satellite 1 (AT rich)	25-48	Centromeric heterochromatin
α (alphoid DNA)	171	Centromeric heterochromatin
β (*Sau*3A family)	68	Centromeric heterochromatin of 1, 9, 13, 14, 21, 22 & Y
Minisatellite DNA (blocks often within 0.1-20kb range)		
Telomeric family	6	All telomeres
Hypervariable family (VNTRs)	9-24	All chromosomes, often near telomeres
Microsatellite DNA (STRs) (blocks often less than 150bp)	1-4	All chromosomes

Table 1.1 The major classes of tandemly repeated human DNA. ***Taken from T. Strachan & A.P. Read (1998) "Human Molecular Genetics". BIOS Scientific Publishers Ltd, Oxford. Table 8.3.***

1.1.3 Variable Number of Tandem Repeats (VNTRs)

Also known as hypervariable minisatellite loci, VNTRs were the first type of DNA polymorphism described for forensic identification (Gill *et. al.* 1985a; Gill *et. al.* 1985b; Jeffreys *et. al.* 1985b; Jeffreys *et. al.* 1985c). A VNTR locus is comprised of tandemly repeated sequences, usually 9 to 80 bases in length per repeat unit, with a core sequence of GNNNNTGGG (where N can equal any nucleotide) (Bell *et. al.* 1982; Jeffreys *et. al.* 1985a; Baird *et. al.* 1986; Jarman *et. al.* 1986; Evett *et. al.* 1989). These loci can be thousands of bases in length, due to the number of repeat units, making them amenable to detection by restriction endonuclease methods (see section 1.1.3.1) (Jeffreys *et. al.* 1985a; Nakamura *et. al.* 1987). Jeffreys *et al.* demonstrated that probes designed for tandem repeats of the myoglobin locus can detect multiple hypervariable loci producing "fingerprints" when hybridisations are carried out under low stringency conditions

– using high salt concentrations in hybridisation and wash solutions (e.g. washes using 1x saline sodium citrate, SSC) (Jeffreys *et. al.* 1985b). They showed that *"variant (core)$_n$ probes can detect sets of hypervariable minisatellites to produce somatically stable DNA 'fingerprints' which are completely specific to an individual (or to his or her identical twin) and can be applied directly to problems of human identification, including parenthood testing"*. The bands produced by these multi-locus probes were shown to be randomly dispersed throughout the genome and can be considered to be independently inherited. Several hypervariable VNTR loci were discovered in the early 1980s and include sites within the insulin gene (Bell *et. al.* 1982), the Harvey ras oncogene (Capon *et. al.* 1983) and the alpha globin genes (Jarman *et. al.* 1986).

1.1.3.1 Method for forensic typing of VNTRs

Genomic DNA samples were digested using restriction endonucleases such as *Hinf*I, *Alu*I or *Hae*III that recognise well-conserved 4 base pair restriction sites flanking a specific VNTR locus. The technique was based on restriction length fragment polymorphism (RFLP) analysis methods for 3' alpha-globin (Gill *et. al.* 1985a; Jeffreys *et. al.* 1985a; Jeffreys *et. al.* 1985b; Fowler *et. al.* 1988). The VNTR locus does not contain the restriction site and therefore remains intact. Resulting restriction fragments were separated according to size by electrophoresis through an agarose gel, transferred to a nylon membrane (Southern blotting) and hybridised with probes labelled with a radioactive isotope.

Multi locus probes were superceded by single locus probes (SLP) because the former were difficult to reproduce for database purposes. SLPs were hybridised under high stringency (low salt concentrations). Each SLP used detected a unique sequence from the corresponding locus, and would only bind at this specific site (Jarman *et. al.* 1986; Wong *et. al.* 1986; Nakamura *et. al.* 1987; Wong *et. al.* 1987). The fragments were then visualised using autoradiography (Figure 1.2). Differences in sizes of the restriction fragments represented integral numbers of the tandemly repeated unit. The number of repeat sequences varied significantly between non-related individuals allowing a unique "fingerprint" to be visualised.

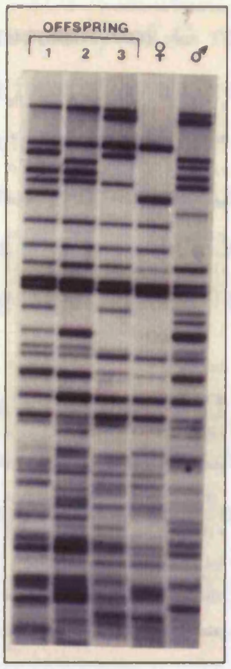


Figure 1.2 An autoradiograph example of how VNTR loci may be visualised using RFLP analysis. In this case the three offspring have alleles in common with each other and share the same alleles as either the mother or the father. The alternate size products appear due to the varying number of tandem repeats present (*photograph courtesy of Dr. J. Wetton, The Forensic Science Service™, UK*).

Most VNTR loci used for human identification exhibited more than 100 alleles within a population, meaning the typing of four markers was sufficient to differentiate between unrelated individuals with a discrimination power of 1 in 10 million (Gill *et. al.* 1991).

1.1.4 Short Tandem Repeats (STRs)

STRs, also known as microsatellites, are repetitive regions of DNA widely distributed throughout the genome, particularly found in non-coding regions of DNA (Beckman and Weber 1992). The repetitive sequences are between 1 – 6 bases in length and the number of repeats at any given locus varies, giving rise to different size loci and different allele lengths within an individual locus.

Mononucleotide repeats of A or T are very common in the human genome and make up approximately 10Mb, or 0.3% of the nuclear genome (Huang *et. al.* 2002). In the case of dinucleotide repeats, arrays of CA repeats are very common and are often highly polymorphic. CT/AG repeats are also common but CG/GC repeats are rare, due to the propensity of C residues to be methylated and deaminated into T residues when flanked by a G residue at their 3' end (Strachan and Read 1998). Trinucleotide and tetranucleotide repeats are comparatively rare but highly polymorphic and can be exploited for forensic purposes (Budowle 1999). The number of alleles at a single tetranucleotide STR locus usually ranges from 5 to 20, making the resulting target region 20 to 100 bases in length.

The use of the Polymerase Chain Reaction (PCR) (Saiki *et. al.* 1985; Mullis and Faloona 1987) for amplification of STR loci makes the amplified products larger than the target region due to the inclusion of sequences that flank the repeat region (Figure 1.3).

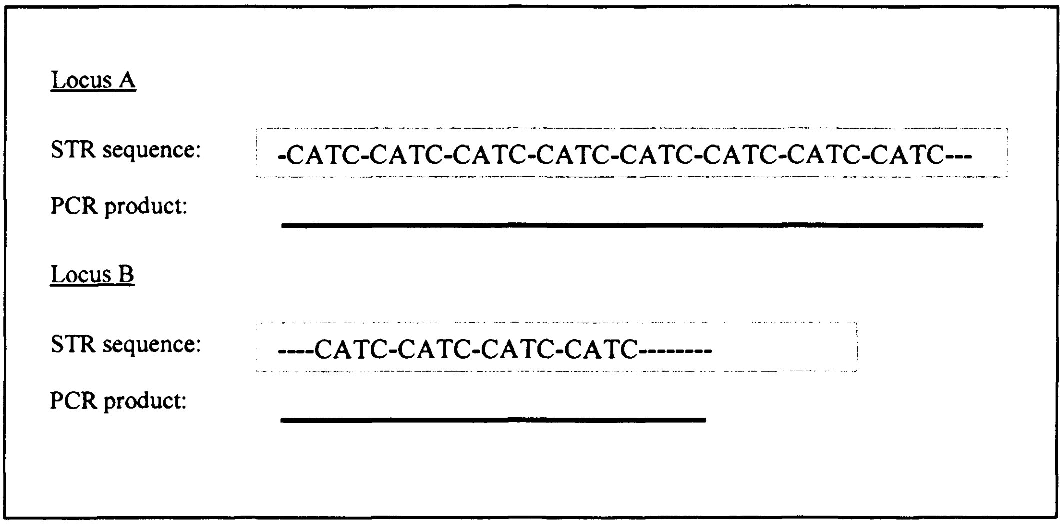

Locus A
STR sequence:
-CATC-CATC-CATC-CATC-CATC-CATC-CATC-CATC---
PCR product:
Locus B
STR sequence:
----CATC-CATC-CATC-CATC--------
PCR product:

Figure 1.3 The short tandem repeat (STR) sequence of CATC in the above example varies in the number of its repeats between locus A and locus B. By amplifying the target region using flanking primers, PCR products of varying sizes are produced. These PCR products can be visualised when run on an acrylamide gel (Figure 1.5).

PCR was first described in 1985 and enabled DNA molecules to be exponentially amplified by a series of heating and cooling reactions in the presence of dNTPs and a DNA polymerase enzyme (Saiki *et. al.* 1985; Mullis and Faloona 1987). In 1994 Sullivan explained that the development of PCR methods had allowed multiplex analysis of several loci giving a highly discriminating test, stating: "*in this regard, STRs are preferable to VNTRs because the former are more amenable to co-amplification and have narrow allelic size ranges which enable several loci* *to be chosen for co-analysis that are non-overlapping in their size ranges"* (Sullivan 1994). STRs were also preferable as a fluorescence detection system could be used for analysis of the PCR products.

The current DNA profiling system used in the United Kingdom for forensic identification utilises tetranucleotide repeat STR loci (Cotton *et. al.* 2000). An account of the development of the forensic typing systems used in the UK is given in section 1.2.

1.1.5 Single Nucleotide Polymorphisms (SNPs)

A SNP can be defined as *"a position within a DNA molecule where one base can be substituted by another"* (Strachan and Read 1998), as well as other types of DNA variations such as insertions or deletions at single positions throughout the genome (Budowle *et. al.* 2004a). SNPs occur approximately once every 1000 bases in humans (Cooper *et. al.* 1985; Kruglyak and Nickerson 2001; Venter *et. al.* 2001), making them the most abundant form of DNA variation. Like other DNA polymorphisms, SNPs can be linked back to mutations occurring from spontaneous errors in chromosome segregation at meiosis, DNA replication and repair, and spontaneous changes resulting from exposure to chemicals (Strachan and Read 1998). The use of SNPs as genetic markers is well-documented for many different applications from human and animal identification, population studies, disease associations and phylogeny (Gray *et. al.* 2000; Riley *et. al.* 2000; Schork *et. al.* 2000; Shastry 2002; Emara and Kim 2003; Schmith *et. al.* 2003).

Sections 1.5 and 1.6 describe the use of SNPs for forensic identification and the methods that can be used for their detection.

1.2 Forensic DNA Profiling

During the early 1990's DNA forensic science experienced considerable growth, instigated by the introduction of molecular techniques allowing the development of highly discriminating DNA profiling methods (Kimpton *et. al.* 1994; Lygo *et. al.* 1994; Sullivan 1994; Gill *et. al.* 1995b; Gill *et. al.* 1997; Cotton *et. al.* 2000; Grimes *et. al.* 2001; Lowe *et. al.* 2001; Lowe *et. al.* 2002; Hussain *et. al.* 2003).

The DNA profiling technique described by Jeffreys *et al.*, using specific tandem repeat (VNTR) regions of DNA (Jeffreys *et. al.* 1985a), was the first to enable a 'genetic fingerprint' of an individual to be generated. The term genetic fingerprint is no longer used and has been replaced by 'DNA profiling' as the comparison with fingerprints was not particularly helpful – forensic scientists do not use terms such as uniqueness, preferring to use match probabilities and likelihood ratios. VNTR polymorphisms are outlined in section 1.1.3. It is the variation within each locus that is exploited for use in forensic identification.

The introduction of PCR (Saiki *et. al.* 1985; Mullis and Faloona 1987) allowed new improved molecular biology methods to be used in forensic typing. PCR revolutionised many areas of DNA research and accelerated the growth of DNA analysis. This occurred primarily by use of semi-automated methods which dramatically decreased turn-round time and costs resulting in increased throughput of samples. PCR could be used to amplify much smaller quantities of DNA starting material meaning the types of cases that could be analysed expanded considerably (Hagelberg *et. al.* 1991; Jeffreys *et. al.* 1992). The VNTR method outlined by Jeffreys in 1985 required 0.5-5 μg of high molecular weight DNA to gain a significant result. The amount of offender DNA found at a crime scene was often much lower so the method was deemed unsuitable for many crime scene investigations. The introduction of PCR allowed the amount of starting DNA to be reduced to 1ng (0.001μg) as template could be exponentially amplified to a level that was easily detected. Currently, approximately 1 ng quantity of starting DNA material is used in standard profiling methods (Cotton *et. al.* 2000).

PCR methods using VNTRs were developed (Jeffreys *et. al.* 1988b) but were considered to be too time-consuming, too subjective in the interpretation of results and c.100 ng of DNA was still required to perform the SLP analysis. PCR is not as efficient at amplifying large DNA fragments (Sullivan 1994). Large VNTR products were also unsuitable for typing degraded samples, which generally have much smaller DNA fragments present (Majno and Joris 1995; Johnson and Ferris 2002) (see section 1.4).

In 1994 the first DNA profiling system used by the criminal justice system was introduced, involving four polymorphic STR loci (Kimpton *et. al.* 1994; Lygo *et. al.* 1994). This system was superseded in 1996 by a more discriminating STR system using PCR, known as the Second Generation Multiplex (SGM) (Gill *et. al.* 1995b; Gill *et. al.* 1997). This consisted of six polymorphic loci plus a non-STR sex-determining locus – the X-Y homologous amelogenin genes. Amelogenin primers flank a 6 base pair (bp) deletion within intron 1 of the X homologue, resulting in 106 bp and 112 bp PCR products from the X and Y chromosomes respectively (Sullivan *et. al.* 1993; Mannucci *et. al.* 1994). The introduction of the Applied Biosystems (AB) AMP*Fl*STR® SGM plus™ system (SGM+) in 1999, with an additional 4 STR loci added to the original 6 used in SGM (Cotton *et. al.* 2000), increased the discrimination power from approximately 1 in 50 million (SGM) to 1 in 1,000 million. DNA profiling for the UK National DNA Database (NDNAD®) is carried out using SGM+.

1.2.1 Method for forensic typing of STRs

SGM+ PCR amplifies 10 hypervariable STR loci (Table 1.2) and Amelogenin (for X/Y chromosome sex discrimination) using dye-labelled locus-specific primers, allowing fragments to be detected by the use of polyacrylamide gel electrophoresis (PAGE) (Figure 1.5) or, more recently, capillary gel electrophoresis (CE). The use of three fluorescent dyes (JOE, FAM & NED) enables loci with overlapping allele sizes to be labelled with different colours so they are easily distinguishable from each other.

Locus ID	Repeat motif	Approx. size range (base pairs)	Dye label used for analysis	Chromosome location
Amelogenin	N/A	X = 106; Y = 112	JOE	X/Y
D3S1358 (D3)	[TCTG][TCTA]	114-142	FAM	3q21.31
HUMVWF31/A (vWA)	TCTR*	157-209	FAM	12p13.31
D16S539 (D16)	GATA	234-274	FAM	16q24.1
D2S1338 (D2)	TRCC*	289-341	FAM	2q35
D8S1179 (D8)	TCTR*	128-172	JOE	8q24.13
D21S11 (D21)	[TCTA][TCTG]	187-243	JOE	21q21.1
D18S51 (D18)	AGAA	265-345	JOE	1821.33
D19S433 (D19)	AAGG	106-140	NED	19q12
HUMTHO1 (THO1)	TCAT	165-204	NED	11p15.5
HUMFIBRA (FGA)	CYKY*	215-353	NED	4q31.3
	* R = A or G; Y = C or T; K = G or T			

Table 1.2 The 10 STR loci used in the current AMP*Fl*STR® SGM plus™ DNA profiling system, plus Amelogenin (Butler *et. al.* 2004). Each locus has a different repeat motif and a variable number of repeats. Some loci give a better discrimination than others but, multiplexed, the system has a discrimination power of approximately 1 in 1,000 million, between non-related individuals.

The fluorescent dye labels present on one of the pair of flanking primers are incorporated into the newly synthesised DNA products during the PCR process, allowing them to be visualised when run on a polyacrylamide gel or CE instrument (Figure 1.4).

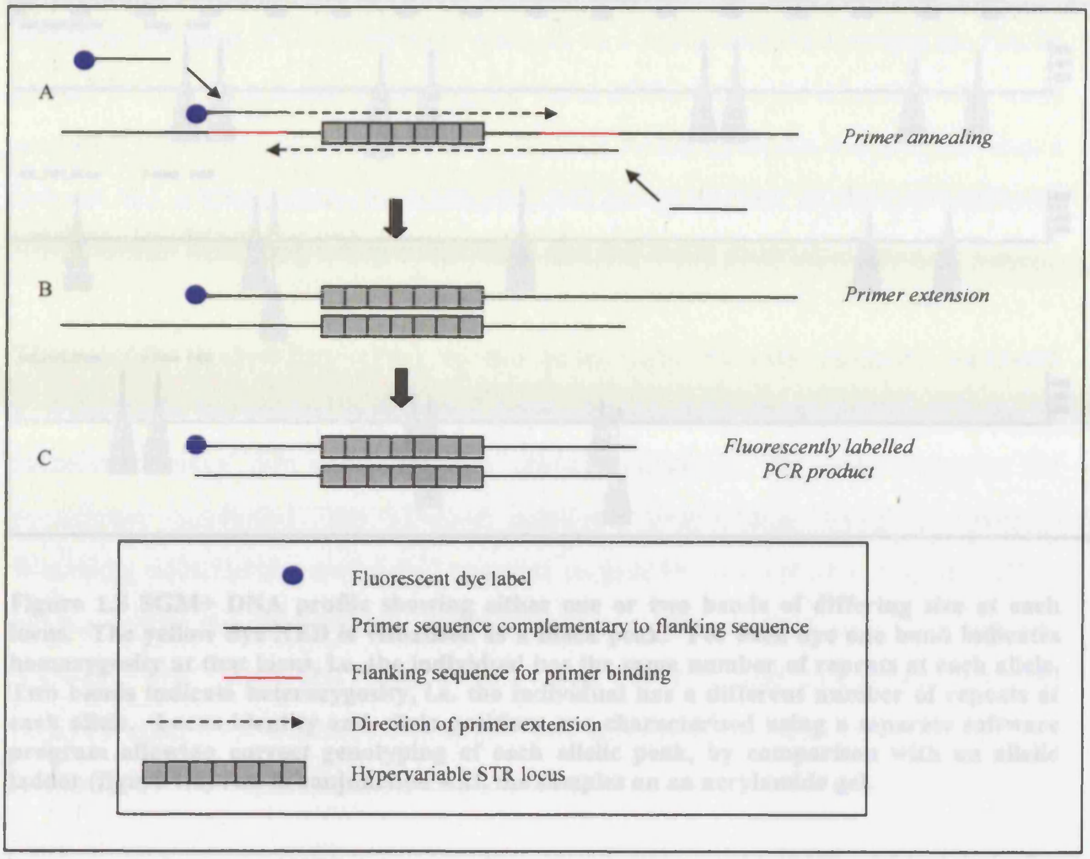

A
Primer annealing
B
Primer extension
C
Fluorescently labelled PCR product
Fluorescent dye label
Primer sequence complementary to flanking sequence
Flanking sequence for primer binding
Direction of primer extension
Hypervariable STR locus

Figure 1.4 Diagrammatical representation of the PCR process used to incorporate fluorescent labels into the targeted STR loci. A) primers are designed complementary to the STR flanking regions and are able to bind to the DNA template during the annealing stage of PCR. B) the primers extend the new DNA strand making it complementary to the target DNA region, exponentially creating new double stranded DNA molecules. C) the resulting DNA product has fluorescent dye labels incorporated into its 5' ends, allowing it to be detected by PAGE.

Data collection generates an SGM+ DNA profile (Figure 1.5). The number of repeat sequences within each locus is directly proportional to the size of the PCR product. STR fragments are given a numerical designation by comparison against a control allelic ladder run on the same gel (Figure 1.6).

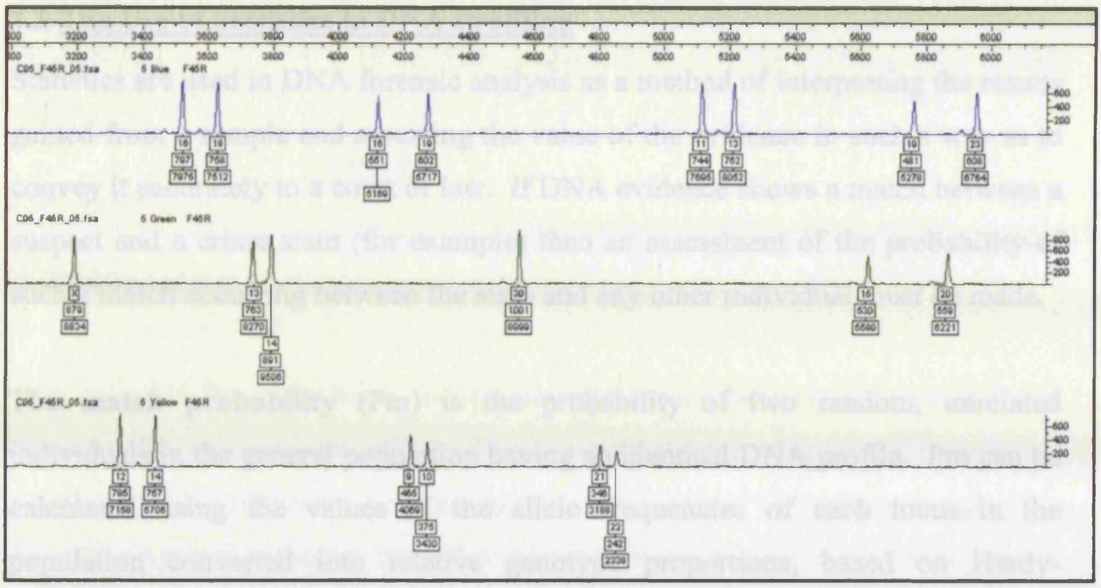


Figure 1.5 SGM+ DNA profile showing either one or two bands of differing size at each locus. The yellow dye NED is visualised as a black peak. For each dye one band indicates homozygosity at that locus, i.e. the individual has the same number of repeats at each allele. Two bands indicate heterozygosity, i.e. the individual has a different number of repeats at each allele. Locus identity and allele positions are characterised using a separate software program allowing correct genotyping of each allelic peak, by comparison with an allelic ladder (figure 1.6) run in conjunction with the samples on an acrylamide gel.

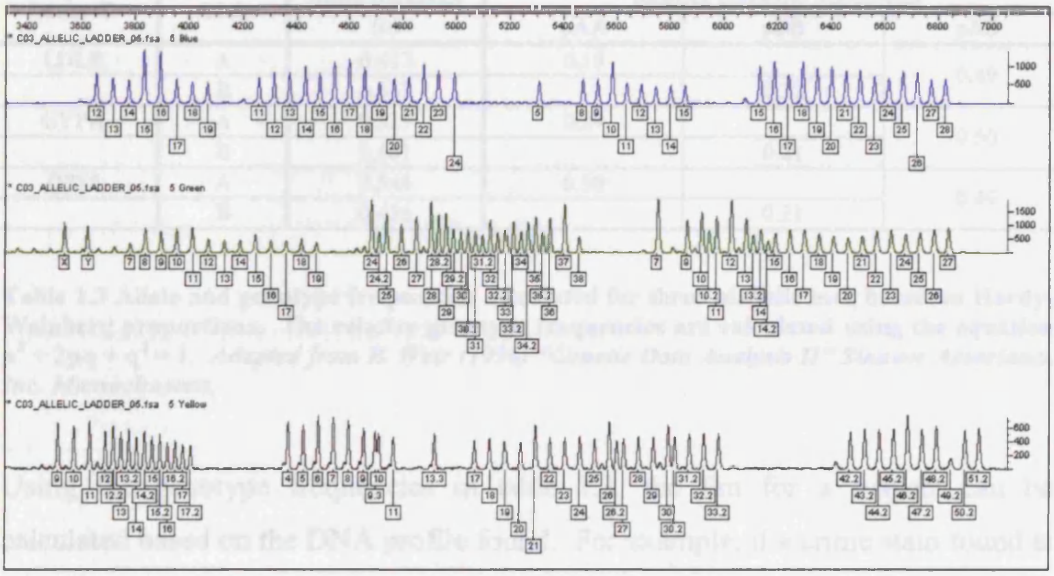


Figure 1.6 Allelic ladder profiles used to correctly score each sample using an automated process. Each peak for each locus represents an STR with a different number of repeat motifs.

1.3 The Use of Statistics in DNA Profiling

Statistics are used in DNA forensic analysis as a method of interpreting the results gained from a sample and assessing the value of the evidence in such a way as to convey it accurately to a court of law. If DNA evidence shows a match between a suspect and a crime stain (for example) then an assessment of the probability of such a match occurring between the stain and any other individual must be made.

The **match probability** (Pm) is the probability of two random, unrelated individuals in the general population having an identical DNA profile. Pm can be calculated using the values of the allele frequencies of each locus in the population converted into relative genotype proportions, based on Hardy-Weinberg equilibrium, multiplied together to give the chance of seeing this DNA profile within the population. This is known as the *product rule* (Balding and Nichols 1994; Evett and Weir 1998). The product rule calculation assumes independence both within and between loci and relies on each locus conforming to Hardy-Weinberg proportions (section 1.8).

Locus ID	Allele	Allele frequency (p)	Relative genotype frequencies		
			pAA	pBB	pAB
LDLR	A	0.437	0.19		0.49
	B	0.563		0.32	
GYPA	A	0.539	0.29		0.50
	B	0.461		0.21	
D7S8	A	0.544	0.30		0.49
	B	0.456		0.21	

Table 1.3 Allele and genotype frequencies calculated for three biallelic loci, based on Hardy-Weinberg proportions. The relative genotype frequencies are calculated using the equation $p^2 + 2pq + q^2 = 1$. ***Adapted from B. Weir (1996) "Genetic Data Analysis II" Sinauer Associates, Inc. Massachusetts.***

Using the genotype frequencies in table 1.3, the Pm for a suspect can be calculated based on the DNA profile found. For example, if a crime stain found at a scene had the profile LDLR A/A; GYPA A/B; D7S8 B/B, Pm would be calculated as:

Product rule = 0.19 (LDLR pAA) x 0.50 (GYPA pAB) x 0.21 (D7S8 pBB)

= 0.01995

this is the probability of a chance match of the profile with another random, unrelated sample.

This 'simple' Pm calculation makes assumptions of independence of loci and doesn't take into account effects from sampling error, related individuals, or population sub-structures i.e. it assumes random mating. The population genetics imposing correction factors upon the calculations are explained and examined in more detail in section 1.8.

For more complex situations a **likelihood ratio** can be calculated. *"A likelihood ratio (LR) involves a comparison of the probabilities of the evidence under two alternative propositions"* (Butler 2005b). The two alternative hypotheses seen in forensic situations are:

> H_p = "the DNA profile at the crime scene came from the suspect" i.e. the prosecution hypothesis;
> H_d = "the DNA profile at the crime scene came from another, unknown individual" i.e. the defence hypothesis.

The LR is calculated from the following equation:

$$LR = \frac{\Pr(E|H_p)}{\Pr(E|H_d)}$$

Where Pr(E|H_p) is calculated from the probability of the crime sample and the suspect sample matching given that the prosecution hypothesis is true, i.e. in simple scenarios this is equal to 1. Pr(E|H_d) is the match probability calculation, i.e. the probability of the profile given that the defence hypothesis is true. This is the same as the probability of observing the profile in the general population.

The difference between the two calculations can be explained as:

> "What is the probability of observing a particular profile?" [the match probability]

and

> "Given that I have observed this profile, what is the probability that another (unprofiled) individual will also have it?" [the likelihood ratio] (Balding 2005).

1.4 DNA Packaging & Degradation

1.4.1 DNA packaging in the nucleosome

In the nucleus of mammalian cells, chromatin is organised into subunits that consist of lengths of DNA wrapped around a histone octamer (Kornberg 1974; Bina-Stein and Simpson 1977; Finch *et. al.* 1977; Moss *et. al.* 1977; Richards *et. al.* 1977) (Figure 1.7). The octamer consists of two copies of each of the core histone proteins H2A, H2B, H3 and H4 encapsulated by a 146 base pair length of double-stranded DNA, giving rise to a nucleosome (Kornberg 1974; van Holde *et. al.* 1975; Bina-Stein and Simpson 1977; Noll and Kornberg 1977; Noll 1978; Read *et. al.* 1985a).

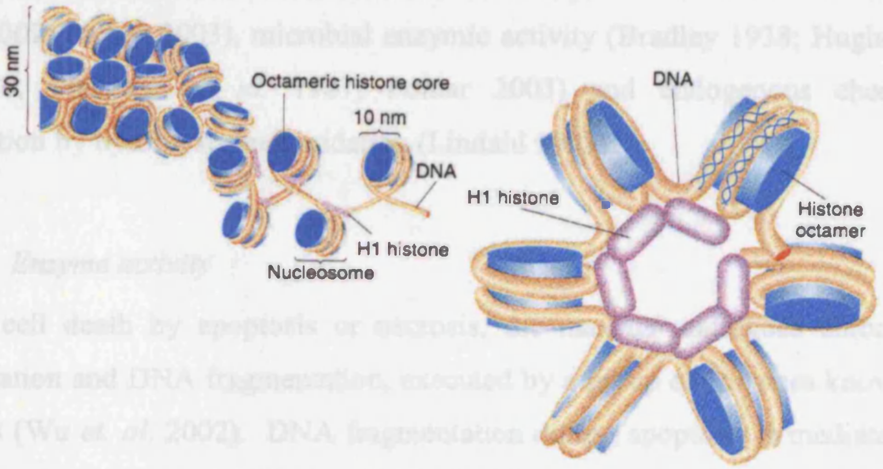


Figure 1.7 Picture of the organisation of DNA around a histone core, forming a nucleosome. The nucleosome comprises two copies of core histone proteins H2A, H2B, H3 & H4, combined with a 146 base pair length of DNA. ***Taken from "An Introduction to Genetic Analysis". W.H. Freeman & Co. New York.*** **(Griffiths *et. al.* 1998).**

Extensive bonding exists between histones and nucleosomal DNA via hydrogen bonding with DNA phosphates, hydrophobic interactions and salt linkages, protecting the core length of DNA (Luger *et. al.* 1997; Kornberg and Lorch 1999). No interactions are seen between the histones and DNA bases, allowing the histones to package any length of DNA regardless of sequence specificity. Nucleosomes are connected to each other by linker DNA (Spadafora *et. al.* 1976; Richards *et. al.* 1977; Read *et. al.* 1985a; Kornberg and Lorch 1999) forming "beads on a string" – the first level of chromosome packing. Linker DNA varies in length, an important feature for gene regulation (Spadafora *et. al.* 1976), allowing the nucleosomes to coil and fold into a chromatin fibre. Nucleosomes are more confined to location by physical barriers such as DNA-binding proteins along the length of the DNA and sequence-specific bending characteristics (Luger *et. al.* 1997). As a consequence of this physical limitation, nucleosomes are often found close to promoter regions and regulatory elements (Simpson 1991; Thoma 1992). The organisation of DNA around a octameric histone core confers some protection onto the nucleosomal DNA, making it less susceptible to attack from cellular nucleases (van Holde *et. al.* 1975; Noll and Kornberg 1977).

1.4.2 DNA degradation

DNA degradation occurs *in vivo* by a number of different mechanisms including cellular enzymic activity (Bär *et. al.* 1988; Suck 1992; Robertson *et. al.* 2000; Wu *et. al.* 2002; Poinar 2003), microbial enzymic activity (Bradley 1938; Hughes *et. al.* 1986; Madisen *et. al.* 1987; Poinar 2003) and endogenous chemical degradation by hydrolysis and oxidation (Lindahl 1993).

1.4.2.1 Enzyme activity

During cell death by apoptosis or necrosis, the nucleus undergoes chromatin condensation and DNA fragmentation, executed by a group of enzymes known as caspases (Wu *et. al.* 2002). DNA fragmentation during apoptosis is mediated by CAD[1] (caspase-activated DNase) / DFF-40 (DNA fragmentation factor) (Rudel and Bokoch 1997; Sakahira *et. al.* 1998), although other enzymes, specifically nucleases, are required for complete histone release (Robertson *et. al.* 2000; Hengartner 2001; Li *et. al.* 2001; Parrish *et. al.* 2001). Endonucleases function by hydrolysing the phosphodiester bond in the phosphate-ribose backbone structure (Suck 1992), causing fragmentation of the DNA molecules. The endonucleases first target the unprotected linker DNA, leaving monomeric nucleosomes comprising 146 base pairs of protected DNA. Exonucleases detach single nucleotides from the terminal end of the DNA strand, gradually shortening the molecule (Bär *et. al.* 1988).

1.4.2.2 Microbial enzyme activity

DNA can be further degraded by bacterial, fungal and insect interaction (Eglington and Logan 1991), an effective but often incomplete process.

1.4.2.3 Chemical degradation

Hydrolysis and oxidation of bonds within the DNA structure occurs *in vivo* and is counteracted by DNA repair mechanisms using endogenous cellular enzymes (Lindahl 1993; Friedberg *et. al.* 1995). The glycosidic base – sugar bond is the main target of direct hydrolytic attack and leads to loss of the base (Figure 1.8). The abasic site is then vulnerable to single-strand cleavage of the phosphodiester bond and the strand is sheared, unless repaired by endogenous endonucleases, phosphodiesterases, DNA polymerase and DNA ligase (Lindahl 1976; Friedberg *et. al.* 1995).

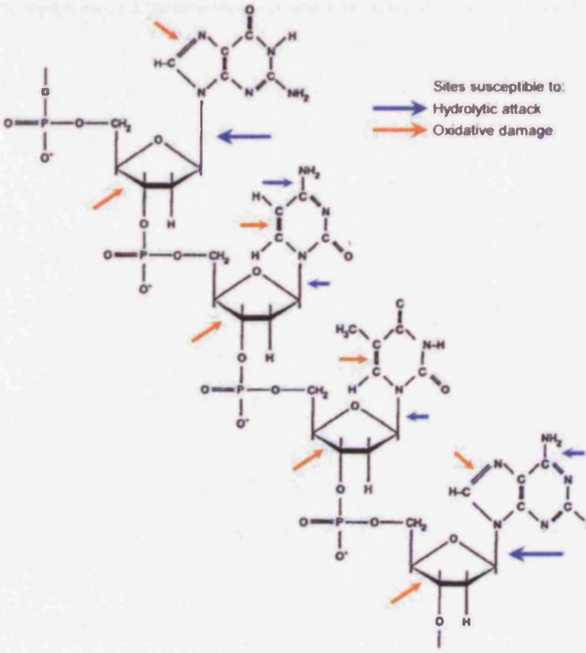


Figure 1.8 Target sites for intracellular decay. A section of one strand of the DNA double helix is shown with the four bases (from top: guanine, cytosine, thymine, adenine). Blue arrows indicate sites susceptible to hydrolytic attack and orange arrows indicate oxidative damage. The magnitude of the arrows reflects the potential for activity at each site (not to scale). ***Adapted from T. Lindahl (1993) "Instability and decay of the primary structure of DNA" Nature 362: page 713.***

Exposure to active oxygen can also damage the DNA by oxidative attack across the 3'-4' carbon bond of the deoxyribose leading to ring fragmentation and strand scission (Friedberg *et. al.* 1995; Poinar 2003). Oxidation can also occur in the ring structure of the bases (Figure 1.8). Lindahl postulates that *"deprived of the [DNA] repair mechanisms provided in living cells, fully hydrated DNA is spontaneously degraded to short fragments over a time period"*.

1.5 Single Nucleotide Polymorphisms (SNPs)

1.5.1 The history of SNPs for forensic purposes

The use of single nucleotide polymorphisms (SNPs) for forensic identification purposes was first described in 1993 (Syvänen *et. al.* 1993). The group used a method known as 'solid-phase minisequencing' (Syvänen *et. al.* 1990) to amplify (using PCR), and detect, twelve SNP loci located on different chromosomes. Analysis of two paternity cases and one murder case suggested *"in all three cases the result was consistent with that obtained by routine methods, which include typing of three VNTR loci"*. Biallelic SNPs only consist of two variant bases (Figure 1.9), allowing detection of either base by a range of different techniques (Kostrikis *et. al.* 1998; Tyagi *et. al.* 1998; Berlin and Gut 1999; Howell *et. al.* 1999; Li *et. al.* 1999; Hall *et. al.* 2000; Mei *et. al.* 2000; Petkovski *et. al.* 2003; Inagaki *et. al.* 2004; Budowle *et. al.* 2004a).

Allele 1	**A-C-T-G-G-G-C-A-C-T-C-T-A-C-G-T-A-C-C**
Allele 2	**A-C-T-G-G-G-C-A-T-T-C-T-A-C-G-T-A-C-C**
Individual A	**A-C-T-G-G-G-C-A-C-T-C-T-A-C-G-T-A-C-C**
(homozygote C)	**A-C-T-G-G-G-C-A-C-T-C-T-A-C-G-T-A-C-C**
Individual B	**A-C-T-G-G-G-C-A-T-T-C-T-A-C-G-T-A-C-C**
(homozygote T)	**A-C-T-G-G-G-C-A-T-T-C-T-A-C-G-T-A-C-C**
Individual C	**A-C-T-G-G-G-C-A-C-T-C-T-A-C-G-T-A-C-C**
(heterozygote C/T)	**A-C-T-G-G-G-C-A-T-T-C-T-A-C-G-T-A-C-C**

Figure 1.9 This diagram illustrates that one biallelic SNP site can give three possible genotypes. An individual may have one of the two possible alleles, making them a homozygote for that particular locus (as shown with individuals A & B), or an individual may have both alleles, making them a heterozygote at that locus (individual C).

STRs are currently the preferred method of DNA profiling used in forensic biology as these proved to be a discriminating form of polymorphism in lower numbers, and early methods of detection were more reliable and more amenable to high-throughput techniques (sections 1.1.4 & 1.2). The use of SNPs as a forensic tool continued to be researched during the 1990s and in 1996 Delahunty reported on the feasibility of typing SNPs using a semi-automated PCR method (Delahunty *et. al.* 1996). Although less informative than STRs, Delahunty outlined the advantages of using SNPs for identification purposes - including the frequency of the polymorphisms throughout the genome; the ease of calculating allelic frequencies due to the biallelic nature of the SNPs; the reliability of PCR amplification and the ability to automate the process. By this time, automation techniques had become increasingly more sophisticated and new methods such as microarray analysis were paving the way for new detection strategies.

The advent of the Human Genome Project (Kruglyak and Nickerson 2001; Sachidanandam *et. al.* 2001; Venter *et. al.* 2001) allowed the sequences flanking each SNP to be identified, making the process of SNP selection and primer design much simpler. These sequences became available to the public domain via Internet websites (section 1.5.2) during the late 1990s (http://snp.cshl.org; Thorisson and Stein 2003). The Human Genome Project also highlighted the sheer volume of these polymorphisms throughout the genome in both coding regions (designated cSNPs) and non-coding regions. The SNPs occurring in non-coding regions are those readily selected for forensic identification purposes, although some cSNPs may be used for intelligence work involving physical characteristics (Grimes *et. al.* 2001).

In 1999 SNPs were assessed for their power of discrimination against traditionally used STRs (Chakraborty *et. al.* 1999). Chakraborty *et al.* asked the question – *"how many SNP loci would equal the power of the [sic] combined 13 STR loci?"*. It was determined that 25-45 biallelic SNP loci would be required to give a likelihood ratio (LR) (the reciprocal of the Pm) equalling that of an STR system using 13 STR loci (CSF1PO, TH01, TPOX, FGA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11 and vWA), assuming an allelic frequency distribution of 0.3, 0.7 for the SNPs. A *"more asymmetric allele* *frequency distribution"*, i.e. SNP loci ranging in frequency from 0.1 to 0.9, would require more loci to give a similar LR as the power of each locus increases with a frequency closer to 0.5. The ease of automation and miniaturisation of detection techniques had brought SNPs back into the research domain. Chakraborty concluded that SNPs could only be used as a supplement to STRs and would not be able to resolve more complex cases, as the number of SNPs required to equal the efficiency of the 13 STR loci was a lot higher. He noted *"SNP loci had to be selected very carefully for intra- and inter-locus independence of alleles and all SNP loci must be co-amplifiable to remove any systematic bias"*.

In 2001, Gill described the use of SNPs for forensic identification purposes, particularly with respect to mixture interpretation (Gill 2001a). He determined an array of 50 SNPs would give the same, or better, discrimination as approximately [sic] twelve STRs, using a basic assumption that allelic frequency is constant across the set (i.e. 0.2-0.8) allowing match probabilities to be easily calculated. Figure 1.10 shows plots using LRs calculated across the allelic frequency range of 0.1 to 0.9 for 50 SNPs, 100 SNPs and 150 SNPs respectively. Each point is based on all loci being at a constant frequency across the array. An approximate LR of 1.0E+12 is given for STRs based on calculations using SGM+ loci.

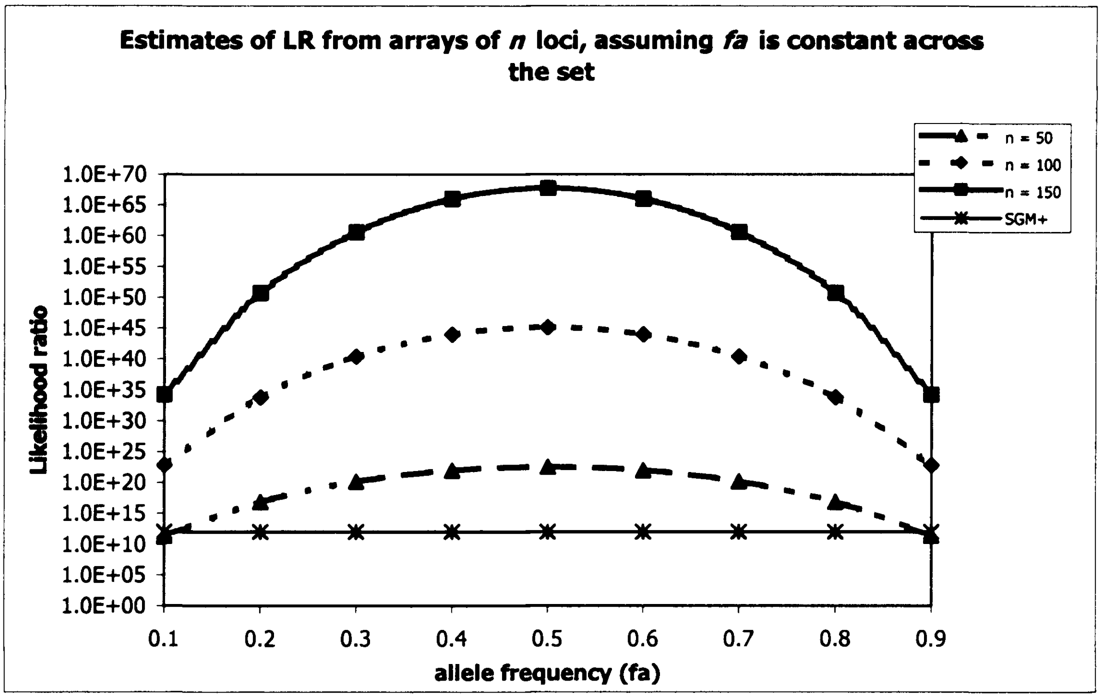


Figure 1.10 A line graph showing estimated likelihood ratios from arrays of n loci, assuming the allelic frequency is constant across the set. The approximate LR for SGM+ is shown as 1 x 10^{12} across the set. ***Adapted from P.Gill 'An assessment of the utility of single nucleotide polymorphisms (SNPs) for forensic purposes' Int J Legal Med (2001) 114:204-210.***

Gill demonstrated that it would be possible to detect mixtures by use of simple algorithms, assuming that accurate quantitative detection methods had been developed. He also showed that relatively small biallelic arrays could be used to distinguish between closely related individuals such as brothers, using relatedness formulae devised by Weir *et al.* (Weir *et. al.* 1997). The different alternatives; C = the DNA profile originated from the suspect against *C* = the DNA profile originated from a relative of the suspect; were tested.

Using SGM+ DNA profiling, high molecular weight STRs can fail to amplify in degraded samples – typically affecting any locus greater than 200 nucleotides in length (Golenberg *et. al.* 1996). As nucleases preferentially target linker DNA, the amount of DNA bound to the histone protein core may be closely linked to the size of the DNA fragments left after degradation (~146 nucleotides plus some linker DNA nucleotides) (Finch *et. al.* 1977; Richards *et. al.* 1977; Trifonov 1978; Read *et. al.* 1985a; Wu *et. al.* 2002). The single-base nature of SNPs allows small fragments of DNA to be targeted for analysis, giving a higher likelihood of detection in samples containing fragmented degraded DNA.

In August 2002, Orchid Biosciences Inc. issued a press release detailing the use of a SNP multiplex system, SNPstream™ Ultra High Throughput System (Orchid-Gene Screen, Dallas, US), in the identification of World Trade Centre terrorism victims. The company had been awarded a contract to help determine the identity of remains that had failed to give a full DNA profile by current STR detection methods. This was the first instance of SNPs being used as a forensic tool, in conjunction with STR methods.

SNPstream™ is a multiplex assay carried out in a flat-bottom microplate in which each well contains a total of 16 individual anti-tag sequences for 12 SNPs and four controls in a gridded array (Budowle 2004b). The system combines solid-phase chip array technology with a primer extension assay and universal tags. Each PCR primer comprises a 25bp segment complementary to the region immediately upstream from the SNP site plus a 20bp 'tag' sequence complementary to the anti-tag sequence attached to the bottom of the well. After PCR and primer extension, the SNP extension product is transferred to a well and allowed to hybridise to its complementary anti-tag. Typing of the two possible SNP alleles is achieved by detection of a fluorescent dye attached to the incorporated ddNTP terminator. The SNP assay used in the identification of World Trade Centre victims used 70 autosomal SNPs in five wells of the microplate.

1.5.2 The SNP Consortium (TSC) and SNP multiplex design

The SNP Consortium (TSC) was established in 1999 as a website collaboration between several companies to produce a public resource of SNPs in the human genome (Thorisson and Stein 2003). By the end of 2001, data for 1.4 million SNPs had been released into the public domain. Data on SNPs has been submitted to the TSC Data Co-ordinating Centre (http://snp.cshl.org) by participating laboratories including flanking sequences, contigs used in the SNP discovery, submitting laboratory references and other information about the alleles. More recently, results can be found pertaining to allele frequencies in several different population groups. All data is backed up and checked by the Co-ordinating Centre before being released into the public domain.

The Forensic Science Service® Ltd. has developed SNP multiplexes containing up to 26 SNPs plus an amelogenin sex-determining locus as detailed in chapters 3-7 (Hussain *et. al.* 2003; Dixon *et. al.* 2005a). The SNP consortium website (http://snp.cshl.org) was used to select SNP loci for use in this multiplex system. SNPs were selected based on the G-C content of the flanking regions, the biallelic bases present [later selections utilised A-T polymorphisms as these were thought to cause less primer-dimer interactions] and the position of the SNP on the chromosome away from the telomeres.

1.6 Methods for Genotyping SNPs

In 2001 Kwok reviewed some of the many techniques available for SNP genotyping (Kwok 2001), a summary of which is given below. He described an ideal genotyping method as possessing the following attributes: (a) The assay must be easily and quickly developed from sequence information; (b) the cost of the assay development must be low; (c) the reaction must be robust, such that even sub-optimal DNA samples will yield results; (d) the assay must be easily automated; (e) the data analysis must be simple with automated, accurate genotype calling; and (f) the reaction format must be flexible and scalable, capable of performing a few hundred to a million assays per day.

1.6.1 Allele-specific hybridisation

The hybridisation approach uses two allele-specific probes designed to hybridise to a target sequence. Each probe is identical in sequence, except for one base difference at the SNP site (Figure 1.11). Under optimised conditions, this one base mismatch will be enough to sufficiently destabilise the non-complementary target probe allowing only the correct probe to hybridise. Different assays use different methods of reporting the hybridisation event. These include the use of fluorescent probes or fluorescent DNA target sequences, allowing the hybridised DNA to be visualised (Kostrikis *et. al.* 1998; Howell *et. al.* 1999; Jobs *et. al.* 2003; Hosking *et. al.* 2004).

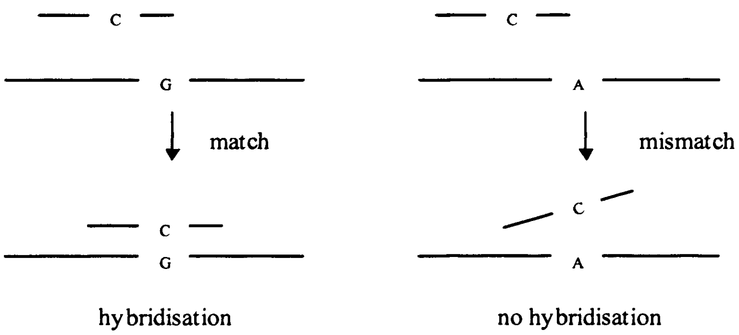


Figure 1.11 Allele-specific hybridisation. Probes are designed to hybridise to a target sequence, each probe being identical in sequence except for a one base difference at the SNP target site. Only the probe complementary to the target sequence present will hybridise.

1.6.2 Primer extension

Primer extension assays are based on the ability of DNA polymerase to incorporate specific deoxyribonucleotides complementary to the DNA template sequence. These assays either use a sequencing approach, whereby the identity of the polymorphic base in the DNA is determined and detected by a further analysis method (such as mass spectrometry or fluorescence resonance energy transfer (FRET)) (Kostrikis *et. al.* 1998; Berlin and Gut 1999; Carey and Mitnik 2002), or an allele-specific PCR approach (Syvänen *et. al.* 1993; Pastinen *et. al.* 1997; Grimes *et. al.* 2001; Bell *et. al.* 2002; Inagaki *et. al.* 2004; Quintans *et. al.* 2004). In this instance the DNA polymerase will only amplify the target DNA if the PCR primers are perfectly complementary to the DNA sequence, or the PCR product is used as a template and a primer extension probe is used which will only extend if the 3' base complements the SNP allele present in the target sequence (Figure 1.12).

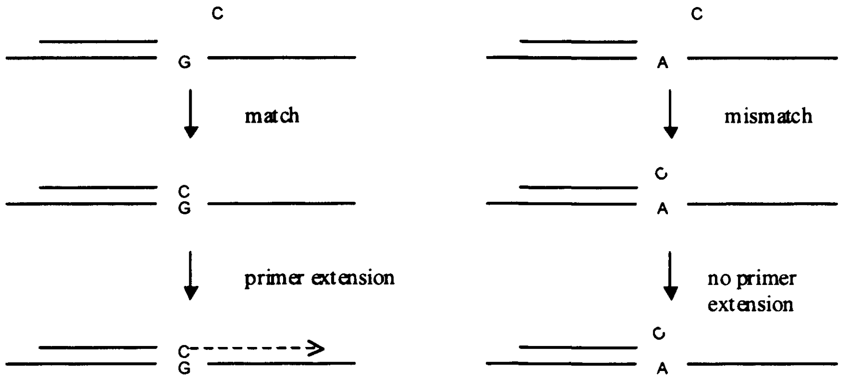


Figure 1.12 Allele-specific primer extension. A primer is designed complementary to the sequence directly upstream from the target SNP site. Only by incorporation of a complementary deoxyribonucleotide will primer extension be successful (adapted from Kwok, 2001).

1.6.3 DNA ligation

When two adjacent oligonucleotides are annealed to a DNA template can only be efficiently ligated together by a DNA ligase enzyme if they perfectly match the template at the junction (Figure 1.13). Allele-specific oligonucleotides can be used to infer the allele present in the target DNA by determining whether ligation has occurred (Lizardi *et. al.* 1998). Ligation is a highly specific technique but has a slow reaction time and requires a large number of modified probes in most instances.

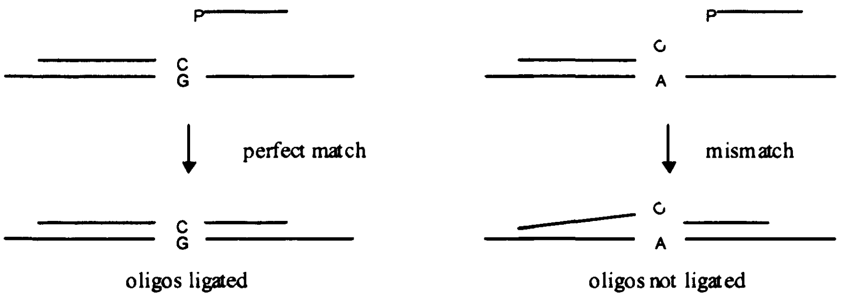


Figure 1.13 Allele-specific oligonucleotide ligation. Primers bind to the target template DNA both 3' and 5' to the SNP locus. Only if the primers match at the SNP site will ligation occur. Ligation has the potential to genotype without previously amplifying the target DNA by PCR.

1.6.4 Invasive cleavage

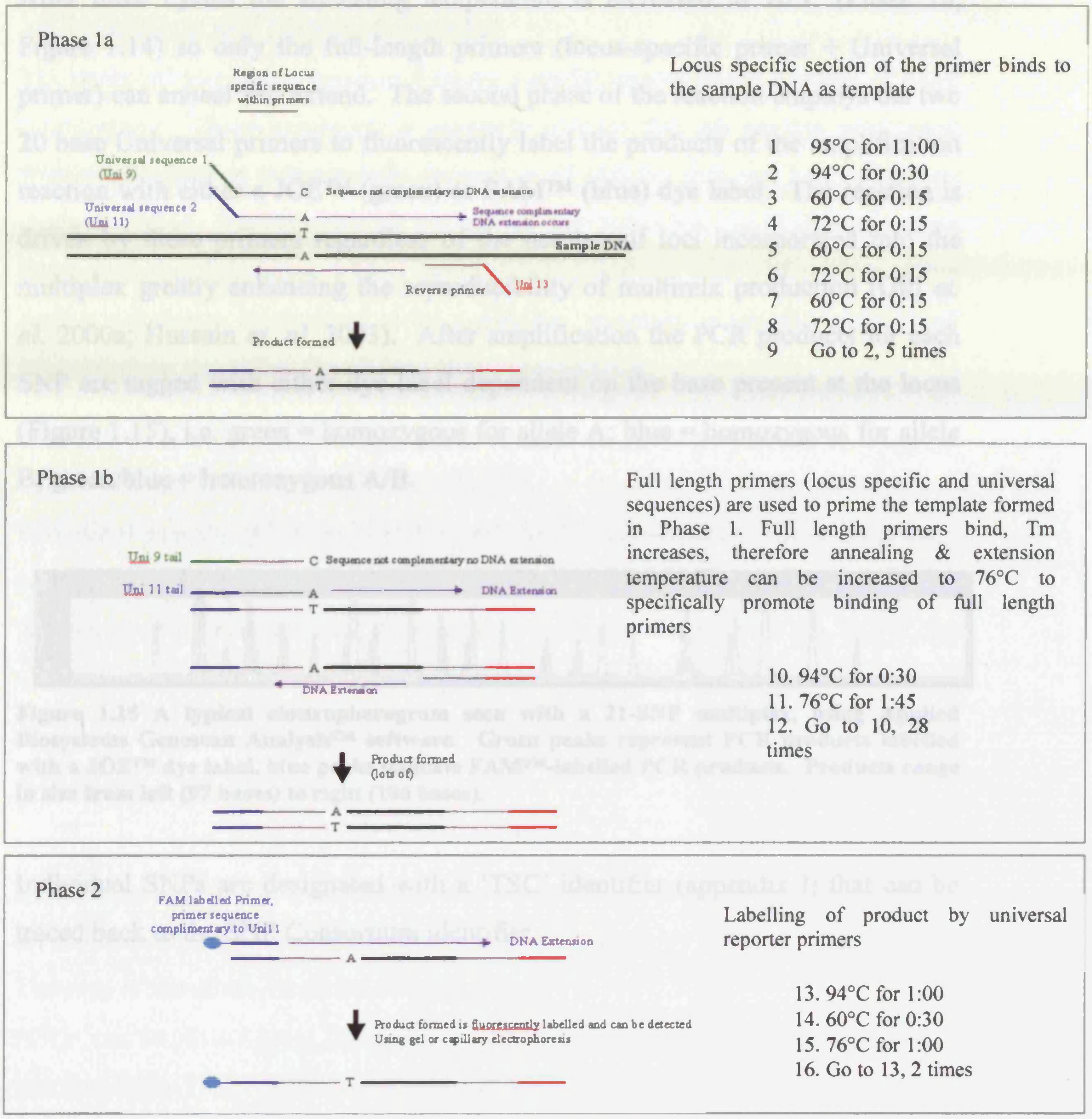
Structure-specific enzymes cleave a complex formed by the hybridisation of overlapping oligonucleotide probes. Probes can be designed to have the polymorphic SNP site at the point of overlap, meaning the correct overlapping structure is only formed when the allele-specific probe but not the probe with a one base mismatch is present (Lyamichev and Neri 2003). This method of genotyping has advantages as it uses an isothermal reaction to cleave the molecules and gives the potential for genotyping without PCR amplification. At present there are technical issues involved with the method that need refining to make the procedure more suitable for SNP genotyping in multiplexes.

Many SNP multiplexing strategies have been developed for use in a clinical or research setting, but these methods cannot be transferred to a forensic setting due to the large amount of DNA starting material required, often in excess of 100 ng. Forensic research has to develop techniques that can be adapted to the low amounts of starting DNA often found at crime scenes, these samples often give DNA quantification values of under 1 ng/μL (Lygo *et. al.* 1994; Sullivan 1994; Gill 2001a; Gill 2001b; Butler *et. al.* 2003).

1.7 The Universal Reporter Primer (URP) Principle

In the late 1990s a method of PCR amplification was developed by the Forensic Science Service® which allowed design of biallelic SNP multiplex assays (Gill *et. al.* 2000a; Hussain *et. al.* 2003). This method of amplification has become known as The Universal Reporter Primer Principle (URP principle) and has enabled simultaneous detection of up to 26 autosomal SNP loci plus the amelogenin sex-determination locus. PCR amplification exploits the Amplification Refractory Mutation System (ARMS) (Newton *et. al.* 1989) and the URP principle to amplify DNA fragments ranging from 57 to 211 base pairs in length. During amplification, two 20bp Universal reporter primer sequences are incorporated onto the ends of the DNA strand giving PCR products 40 bases longer than the original genome target size (Figure 1.14). The ARMS principle was developed in 1989 (Newton *et. al.* 1989) based on the observation that *"oligonucleotides with a mis-matched 3'-residue will not function as primers in the PCR under appropriate conditions"*. URP biochemistry comprises two phases, within a single-tube reaction (Figure 1.14).

There are two locus-specific primers (~40mer) for each SNP targeted, each carrying a different base (complementary to the biallelic SNP) at its 3' end and a different 5' Universal tail, dependent on the base present. The allele-specific reverse primer also carries a Universal tail, to balance the system. All three Universal tails are identical for each SNP. The multimix also comprises two fluorescently labelled Universal primers (20mer) complementary to the two Universal tails of the locus-specific primers. This allows the PCR to be driven by only two primers in the second phase. The first phase uses low concentrations of the locus-specific Universal primers to amplify targets to equivalent levels, whilst simultaneously incorporating universal tags. This is carried out by a two-phase cycling regime.


Phase 1a
Region of Locus specific sequence within primers
Universal sequence 1 (Uni 9)
Universal sequence 2 (Uni 11)
C
Sequence not complementary no DNA extension
A
Sequence complimentary DNA extension occurs
T
Sample DNA
A
Reverse primer
Uni 13
Product formed
A
T
Phase 1b
Uni 9 tail
Uni 11 tail
C
Sequence not complementary no DNA extension
A
DNA Extension
T
A
DNA Extension
Product formed (lots of)
A
T
Phase 2
FAM labelled Primer, primer sequence complimentary to Uni11
DNA Extension
A
Product formed is fluorescently labelled and can be detected Using gel or capillary electrophoresis
T

Locus specific section of the primer binds to the sample DNA as template

95°C for 11:00
2 94°C for 0:30
3 60°C for 0:15
4 72°C for 0:15
5 60°C for 0:15
6 72°C for 0:15
7 60°C for 0:15
8 72°C for 0:15
9 Go to 2, 5 times

Full length primers (locus specific and universal sequences) are used to prime the template formed in Phase 1. Full length primers bind, Tm increases, therefore annealing & extension temperature can be increased to 76°C to specifically promote binding of full length primers

10. 94°C for 0:30
11. 76°C for 1:45
12. Go to 10, 28 times

Labelling of product by universal reporter primers

13. 94°C for 1:00
14. 60°C for 0:30
15. 76°C for 1:00
16. Go to 13, 2 times

Figure 1.14 Diagrammatical representation of the Universal Reporter Primer / ARMS Principle. The amplification technique has two distinct phases: phase 1a uses the locus-specific portion of the ~40-mer primers to provide sufficient template with Universal tails for amplification in phase 2. The increase in Tm seen in phase 1b allows the whole length of the long primers to bind to the template, dependent on the Universal tail present. By phase 2 all long primers have been exhausted and the annealing temperature is reduced to allow the 20-mer fluorescently labelled Universal primers to anneal and extend.

Firstly, annealing temperatures are low (60°C) (Phase 1a, Figure 1.14) to allow the locus-specific portion of the primers to attach to the target template, regardless of the Universal tail. This allows Universal tails to be incorporated into the extending DNA chain, creating new DNA template for the second part of phase 1.

After three cycles the annealing temperature is increased to 76°C (Phase 1b, Figure 1.14) so only the full-length primers (locus-specific primer + Universal primer) can anneal and extend. The second phase of the reaction employs the two 20 base Universal primers to fluorescently label the products of the amplification reaction with either a JOE™ (green) or FAM™ (blue) dye label. The reaction is driven by these primers regardless of the number of loci incorporated into the multiplex greatly enhancing the reproducibility of multimix production (Gill *et. al.* 2000a; Hussain *et. al.* 2003). After amplification the PCR products for each SNP are tagged with either dye label dependent on the base present at the locus (Figure 1.15), i.e. green = homozygous for allele A; blue = homozygous for allele B; green/blue = heterozygous A/B.

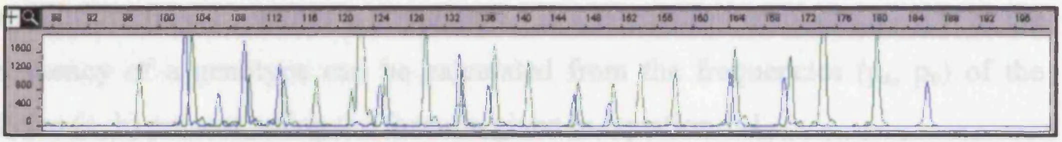


Figure 1.15 A typical electropherogram seen with a 21-SNP multiplex, using Applied Biosystems Genescan Analysis™ software. Green peaks represent PCR products labelled with a JOE™ dye label, blue peaks indicate FAM™-labelled PCR products. Products range in size from left (97 bases) to right (186 bases).

Individual SNPs are designated with a 'TSC' identifier (appendix I) that can be traced back to the SNP Consortium identifier.

1.8 Populations and Statistical Genetics

The study of population genetics follows a model incorporating a series of basic assumptions. These assumptions generally suggest that the starting population from which data is subsequently derived is of infinite size and has undergone random mating. Random mating means that any individual could choose a mate from any other individual within the population without bias. Migration within such a population is assumed to be negligible, mutation is ignored and natural selection does not affect the alleles under consideration.

1.8.1 Hardy-Weinberg Equilibrium (HWE)

The 'ideal population' model can be used for the calculation of Hardy-Weinberg equilibrium (HWE) within a population. If a population conforms to HWE, the frequency of a genotype can be calculated from the frequencies (p_a, p_b) of the alleles (a, b) present, using the formula given in equation 1-1.

$$p_a^2 + 2p_a p_b + p_b^2 = 1 \qquad \textit{Equation 1-1}$$

For a heterozygote (AB), the frequency of the genotype is given by $2p_ap_b$, whereas for the homozygotes, AA or BB, the frequency is given by p_a^2 or p_b^2 respectively. The sum of the allele frequencies is equal to 1.

HWE can be investigated using the Goodness-of-Fit Chi-Squared (χ^2) statistical test (equation 1-2). This test examines the relationship between the expected genotypes against the observed genotypes (based on the allele frequency) using the value of χ^2 as an indication of the probability of the data conforming to the *ideal population* rules.

$$\chi^2 = \sum \frac{(observed - \exp ected)^2}{\exp ected} \qquad \textit{Equation 1-2}$$

A null hypothesis is used as the basis of the χ^2 test. In the case of HWE, the null hypothesis suggests that there is no significant difference between the observed and expected genotypes derived from the allele frequencies. This would indicate that the locus conforms to HWE.

The value of χ^2 is compared to a probability table based on the number of degrees of freedom (*df*) associated with the data. *df* is equal to the number of classes of data minus the number of parameters estimated from the data minus 1. For SNP loci, *df* = 1 as there are three genotype possibilities and one parameter (p) therefore 3 – 1 – 1 = 1 *df*. A 95% confidence level is used for HWE calculations, therefore for 1 *df*, a χ^2 value greater than 3.84 would occur with a probability of less than 0.05 if the null hypothesis were true.

The probability value associated with a particular χ^2 test has the following interpretation: *"it is the probability that chance alone could produce a deviation between the expected and observed values at least as great as the deviation actually realised. Thus, if the probability is large, it means that chance alone could account for the deviation, and it strengthens our confidence in the validity of the model used"* (Hartl and Clark 1997).

Deviations from HWE suggest the alleles present at the locus under investigation are influenced by factors outside of the ideal characteristics of a random mating, infinite population, such as mutation, natural selection and migration. The identification of loci showing deviations from HWE is important in forensic research, loci behaving differently to expectations must be investigated as they can adversely influence the calculation of match probabilities.

1.8.2 Exact tests

Conventional statistical tests such as Chi-squared are useful for identifying loci that do not conform to HWE, however they are not sensitive enough to highlight small deviations in genotype frequencies that may arise especially when the allele counts available for analysis are small.

A method of avoiding spurious results calculated by less powerful statistical techniques is to use an Exact test (also known as a Probability test) (Fisher 1935). Exact tests *"assume the hypothesis is true and calculate the probability of the observed outcome or a more extreme (less probable) outcome"* (Evett and Weir 1998). Low values of this probability suggest that the null hypothesis is not true.

The tests work by using a cumulative probability based on a number of tests giving a multinomial distribution. The probability value (P-value) of the observed data is calculated and the null hypothesis can be rejected if the probability belongs to the smallest (5%) of possible values.

1.8.2.1 Exact tests for Hardy-Weinberg Equilibrium

The use of Exact tests in statistical analysis has been made more amenable by the availability of genetic analysis software, such as Genetic Data Analysis (GDA) (Weir 1996; Lewis and Zaykin 2001) and GENEPOP (Raymond and Rousset 1995). HWE can be calculated using Exact tests, allowing a more thorough investigation of the data to be undertaken. The Exact test for HWE looks at all *"possible sets of genotypic frequencies for the observed set of allele frequencies and rejects the hypothesis of HWE if the observed genotypic frequencies turn out to be unusual under HWE"* (Weir 1996).

The formulae for the calculation of Exact tests for HWE is derived from the probability of the observed genotypic frequencies (aa, ab, bb), assuming HWE, conditional on the observed allele frequencies (a, b) to give:

$$\Pr(n_{aa}, n_{ab}, n_{bb} \mid n_a, n_b) = \frac{n!\,n_a!\,n_b!\,2^{n_{ab}}}{(2n)!\,n_{aa}!\,n_{ab}!\,n_{bb}!} \qquad \textit{Equation 1-3}$$

The expression given in equation 1-3 (adapted from Evett & Weir 1998) is used to perform an Exact test for HWE, giving a probability value (P-value) that relates to the significance level of the test.

1.8.2.2 Exact tests for linkage disequilibrium

As well as a study into the associations between alleles at a single locus, it is also necessary to test for associations between the frequencies of alleles at different loci. An association between alleles could be detrimental towards the calculation of match probabilities using the product rule (section 1.3). Any associations found between alleles are referred to as *linkage disequilibrium*, although the loci being investigated are not necessarily physically linked.

Linkage disequilibrium is calculated by looking at the frequencies of the alleles at all the loci under investigation, using genotypic data (Weir 1996) to assess the Chi-squared statistic. Calculations are cumbersome and time-consuming and have been made more amenable by the use of software again, such as GDA (Lewis and Zaykin 2001) and GENEPOP (Raymond and Rousset 1995). Exact tests can be performed by comparing the observed two-locus genotypic counts with the values expected under various hypotheses (Zaykin *et. al.* 1995). This gives the significance of the association as a probability value (p-value) that can be used to assess the linkage disequilibrium seen between the two loci under investigation, using the assumption of 95% (0.05) significance.

Associations between alleles at different loci can be indicative of a population not conforming to the assumptions of an ideal population, i.e. there is non-random mating and the population is of a finite size. Sub-population effects may cause significant associations and these need to be further defined in order to properly calculate match probabilities.

1.8.3 The effects of genetic drift within a population

The population genetics statistics discussed so far have focused on an 'ideal population' scenario, where a population is infinite and mating is random. In reality, populations cannot be infinite, due to geographical location and random mating cannot occur due to the physical distance between individuals. The evolutionary processes of mutation, migration and selection have an effect on the resulting genetic make-up of a population and it is impossible to study complete populations due to limitations of size and sampling methods. Observations are made on *n* individuals who have been randomly sampled from within a population. Due to the inferences and assumptions made regarding a sampled population, a method of defining the limitations of the data is necessary. An estimate of genetic drift and/or inbreeding within a population is essential to be able to apply a probability to the data obtained from forensic DNA analysis.

"Individuals with common ancestors are said to be related, and their children are inbred. If no further qualifications are made then all humans are both inbred and related to everyone else simply because the population is finite" (Evett and Weir 1998). The genetic consequences of inbreeding follow Mendelian principles, whereby an individual receives one allele from each parent and thereby transmits one of the two alleles to their subsequent offspring. As related individuals share ancestors, there is a chance that the two alleles received from an individual's parents are copies from the same common ancestor (Figure 1.16). In this case, an allele is known as *identical by descent* (ibd).

It becomes necessary with forensic genetics to give a probability value to the likelihood of an allele within a DNA profile being identical by descent. These calculations are worked out using *F* statistics. *F* is the probability that (using the notation in figure 1.16) **A ≡ A**, given that A and A are the two alleles of a person chosen at random from a population. Expanding from this is the calculation for F_{ST}, also known as θ. F_{ST} is the probability that two alleles are IBD in one sub-population compared to the population as a whole.

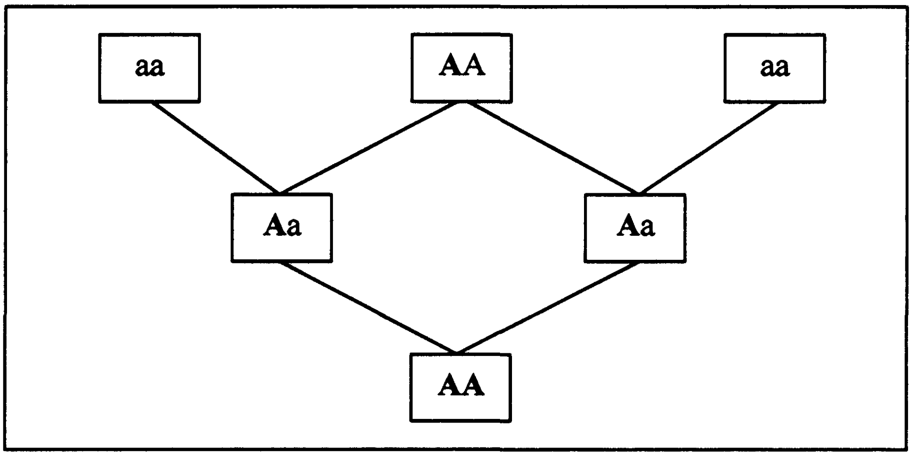


Figure 1.16 A simple depiction of alleles inherited within an inbred population. Each copy of allele A within the third generation becomes 'identical by descent' as each allele is inherited from the same common ancestor.

F_{ST} is a measure of the average progress of sub-populations towards fixation, and is also known as a 'fixation index' (Wright 1951; Balding 2005). $\theta = 1$ implies that all sub-populations have reached fixation at a locus, i.e. in figure 1.16 all individuals would have an AA genotype, conversely $\theta = 0$ implies that allele proportions are the same in all sub-populations, and so the population is homogenous, i.e. there are no alleles that are IBD. An F_{ST} correction factor is used when calculating statistical data for STR loci (Nichols and Balding 1991; Balding *et. al.* 1996; Gill *et. al.* 2003), to allow for deviations in the expected data set. This means likelihood ratio statistics are biased towards the defence, as the calculation decreases the relative allele frequencies used for calculations.

An assessment of F_{ST} for SNPs was performed, to allow correction factors to be applied to the data obtained from the SNP multiplex used in this study.

1.8.4 Kinship analysis for body identification

The SNP multiplex was developed as a forensic intelligence tool, to give information about samples that may have been subjected to high levels of fragmentation. The use of a SNP multiplex using 20 SNPs plus Amelogenin gives an LR of approximately 1 in 4.5 million, i.e. there is a one in 4.5 million chance of seeing the same SNP profile in a randomly selected, unrelated individual. Samples are compared to a reference sample obtained from personal items belonging to an individual. Items that can be submitted as a reference sample include: toothbrushes, razors and hairbrushes, along with saliva samples from bedding, cigarette butts and handkerchiefs. Reference samples can give varying levels of DNA dependent on the source of the sample; a razor is more likely to give a good yield of DNA compared to saliva from a pillowcase.

In cases of mass disaster, or old cases that have been re-opened, there may not be a suitable reference sample for the deceased individual. In these cases, kinship analysis (also referred to as pedigree analysis) can be used to ascertain the identity of the individual, using a modified formulae for calculating a likelihood ratio (LR) (Weir 1996; Leclair *et. al.* 2004; Buckleton *et. al.* 2005).

The scenarios considered in this study were one parent and two parent pedigrees. The formulae are derived using two basic hypotheses:

Hp = The body is the biological child of M and/or F

Hd = The body is an unknown, unrelated individual

To give the following equation:

$$LR = \frac{\Pr(E|H_p)}{\Pr(E|H_d)}$$ *Equation 1-4*

In both scenarios, the allele frequencies of the SNP loci are used to calculate the likelihood of the two hypotheses based on Hardy-Weinberg expectations. The formulae derived for both two parent and one parent scenarios are shown Table 1.4.

Parent 1	Parent 2	Body	Likelihood ratio calculation
aa	aa	**aa**	$1/a^2$
aa	aa	**aF**	1/(a x 1) = 1/a
aa	ab	**aa**	$1/2a^2$
aa	ab	**aF**	1/(2a x 1) = 1/2a
aa	ab	**ab**	1/4ab
ab	ab	**ab**	1/4ab
ab	ab	**aF**	1/(4a x 1) = 1/4a
ab	ab	**Fb**	1/(1 x 4b) = 1/4b
ab	ab	**aa**	$1/4a^2$
ab	ab	**bb**	$1/4b^2$
ab	ab	**bF**	1/(4b x 1) = 1/4b
aa	ab	**Fb**	1/(4b x 1) = 1/4b
aa	bb	**ab**	1/2ab
aa	bb	**aF**	1/(2a x 1) = 1/2a
aa	bb	**Fb**	1/(1 x 2b) = 1/2b
ab	bb	**ab**	1/4ab
ab	bb	**aF**	1/(4a x 1) = 1/4a
ab	bb	**Fb**	1/(1 x 4b) = 1/4b
ab	bb	**bb**	$1/2b^2$
bb	bb	**bb**	$1/b^2$
bb	bb	**bF**	1/(b x 1) = 1/b
aa	-	**aa**	1/a
aa	-	**ab**	1/2a
aa	-	**aF**	1/2a
aa	-	**Fb**	1
ab	-	**aa**	1/2a
ab	-	**ab**	(a + b)/4ab
ab	-	**bb**	1/2b
ab	-	**aF**	1/4a
ab	-	**Fb**	1/4b
bb	-	**ab**	1/2b
bb	-	**bb**	1/b
bb	-	**aF**	1

Table 1.4 Formulae for the likelihood ratio in situations where genotypes of either both parents or one parent of the deceased individual are available as reference samples. Alleles have been simplified to *a* and *b*, where *a* = allele 1 (green peak) and *b* = allele 2 (blue peak). *Adapted from J. Buckleton (2005) "Forensic DNA Interpretation" CRC Press, Florida, tables 11.1 & 11.2.*

1.8.5 Paternity testing using SNPs

In cases of disputed paternity it is possible to use DNA profiling to calculate a probability of an alleged father being the biological parent of a questioned child. Calculations assume the DNA profile of the mother and child are known and the DNA profile of the alleged father is compared to these. Calculations can be carried out using two methods: probability of parentage (the *paternity index* PI); and paternity exclusion (the *exclusion probability*).

"The determination of parentage is made based on whether or not alleles are shared between a child and an alleged father when a number of genetic markers are examined" (Butler 2005b). Paternity can be assessed by inclusion or exclusion of alleles at a locus, based on Mendelian inheritance, which assumes one allele is inherited from the mother and the other from the father.

PI is calculated using a likelihood ratio $LR = \frac{\Pr(E|H_p)}{\Pr(E|H_d)}$ based on the following hypotheses:

> H_p = *the alleged father is the father of the child*
> H_d = *another unknown individual is the father of the child.*

The likelihood ratio then provides a value indicating how many times more likely it is to see the evidence under the first hypothesis compared to the second hypothesis. PI is calculated for each locus and each individual PI value is multiplied together for all loci to give a combined PI for the entire set of genetic loci examined. For *inclusion* of paternity, a minimum value of 100 is required. A PI of 100 correlates to the probability that the alleged father has a 99 to 1 better chance of being the father than a random male in the population.

The exclusion probability is calculated from allele frequencies within a population and does not depend on the genotypes seen in any particular case. It is calculated from the combined frequencies of all the genotypes that would be excluded if the pedigree relationships were true assuming Hardy-Weinberg equilibrium (Weir 1996; Evett and Weir 1998; Ayres 2005; Butler 2005b).

By using allele frequency data for the SNPs selected for use in this study, an assessment of the utility of these SNPs for paternity testing could be carried out.

1.9 Aims

The overall aim of this study was to investigate the use of single nucleotide polymorphisms (SNPs) for forensic identification purposes. In addition, the chosen technique would need to be validated for use in a casework environment by studying population genetics, comparison with existing techniques and effective DNA profiling of true casework samples. The aims were:

1) To identify and develop a technique for genotyping selected SNP loci;
2) To develop a computer program for rapid analysis of SNP data, dependent on the technique selected for detection;
3) Construction of SNP loci population databases for the major ethnic classifications of the United Kingdom, namely White Caucasian, British Afro-Caribbean and Indian sub-continent;
4) To study the population genetics of SNP loci, including an assessment of Hardy-Weinberg equilibrium, linkage disequilibrium, physical linkage and the effects of population structure;
5) An evaluation of SNPs compared to current forensic DNA profiling techniques for genotyping of low copy number and degraded sample types, including mock samples and true casework samples;
6) To determine the most effective method for amplification and analysis of degraded sample types through casework studies and collaboration with European forensic laboratories.

2 Microarray Technology

2.1 Introduction

Methods of genotyping SNPs have been widely reviewed in the last few years (Syvänen 1999; Kwok 2001; Heller 2002). Most systems use fluorescent detection methods in order to correctly genotype samples (Delahunty *et. al.* 1996; Bell *et. al.* 2002; Ye *et. al.* 2002; Jobs *et. al.* 2003; Lyamichev and Neri 2003; Martinez-Garcia *et. al.* 2004; Quintans *et. al.* 2004; Budowle 2004b) but whereas much research has been carried out to develop such systems, there has been far less progress to automate the genotyping of the samples. In part this may be because of the variability between different samples and analytical runs, which in itself varies depending on the technology used. Computer algorithms must make allowance for variability between different analyses. The assay method used is standard across all systems using fluorescence detection for biallelic SNP genotyping - one of the dyes will fluoresce for homozygous alleles and both will fluoresce for heterozygotes (Heil *et. al.* 2002); this information is used to determine the genotype of the sample.

Many of the major companies interested in SNP detection have developed genotyping software to complement the technology they provide. Applied Biosystems™ developed a SNPlex™ Genotyping System which uses an oligonucleotide ligation assay (OLA) to analyse SNP genotypes using capillary electrophoresis (Delahunty *et. al.* 1996; Wenz *et. al.* 2005). In conjunction with this technology, Genemapper software was also developed to automatically genotype samples. This software creates a genotype plot for each SNP (based on the peak height data) and uses a clustering algorithm (unpublished) to assign genotypes. The algorithm calculates a genotype based on the peak height data and the user can manually set a threshold level above which genotypes can be reliably designated. Genemapper displays data as both a Cartesian plot and a Polar plot, allowing each locus to be manually viewed. The Cartesian plot measures the intensity of both peaks relative to each other by plotting the relative fluorescence data on both the x and y axis whilst the Polar plot measures the intensity of the peaks on the x axis and the ratio of both peak heights on the y axis.

SNPstream® instrumentation supplied by Beckman Coulter, Inc. (Bell *et. al.* 2002; Budowle 2004b) allows genotyping of up to 1,000,000 SNP genotypes per day, using a high throughput primer extension assay. Associated software, GetGenos™, was developed to allow automated genotyping of the samples using a set of algorithms where the parameters can be manually altered by the user (Huang *et. al.* 2004). The software uses cluster geometry to genotype a sample by analysis of fluorescent signal data into three clusters, according to the ratio of intensities from two fluorescent colours in the SNP spot.

Microarray technology was developed to carry out DNA analysis using high-throughput processing (Southern 1995; Southern 1996b; Southern 2001). A variety of DNA microarray systems have been developed and commercialised, allowing *"DNA and/or RNA hybridisation analysis to be carried out in microminiaturized highly parallel formats"* (Heller 2002). Thousands of DNA samples can be processed at one time and, by being automated, there is minimum scope for both operator and sampling error. DNA microarrays are mainly used for gene expression profiling in biological samples (Schena 1996; Watson *et. al.* 1998; Golub *et. al.* 1999; Granjeaud *et. al.* 1999; Alizadeh *et. al.* 2000; Jain *et. al.* 2002), although there are many other applications including pharmacogenetics (Service 1998; Debouck and Goodfellow 1999), infectious & genetic disease diagnostics and mutation analysis (Ravine 1999).

The use of microarray technology predominantly for gene expression profiling has led to the development of automated software capable of estimating gene expression levels. Software is available both commercially and as 'freeware' from the Internet[1]. Most software options use hierarchical clustering to give information on gene clusters and gene expression levels in the test samples. Data is first standardised relative to background levels of fluorescence calculated from the amount of fluorescence in negative control samples. The conversion of normalised data into ratios varies between the different programs, with some deriving a simple ratio from the fluorescence of one allele compared to another

[1] GeneMaths XT; http://research.nhgri.nih.gov/microarray/main.html; http://www.bio.davidson.edu/projects/GCAT/GCATprotocols.html; http://www.bio.davidson.edu/projects/magic/magic.html; http://www.tigr.org/software/; ImaGene

allele, whilst others use the data from one allele divided by the total fluorescent data for the gene being interrogated. Jain *et. al.* 2002 described one such microarray analysis program known as 'UCSF Spot', available as freeware to academic research bodies via the internet (Jain *et. al.* 2002). This program was developed as there were *"a number of methods available for the quantification of images, [however] many of the software systems in wide use either encourage or require extensive human interaction at the level of individual spots on arrays"*. 'UCSF Spot' automatically locates and segments microarray spots and estimates ratios based on either two or three-colour fluorescent images. These data can then be carried through for more in-depth analyses of expression profiling and DNA copy number profiling (Jain *et. al.* 2002).

The availability of software for gene expression profiling has maximised the effectiveness of microarray technology in the research environment, however there was a lack of available software for simple SNP genotype calling based on microarray fluorescence data. For forensic purposes it was necessary to develop a software program capable of accurately genotyping samples for a number of SNP loci, whilst maintaining a set of interpretation criteria allowing any anomalous samples to be identified. Whereas most existing SNP genotyping programs attain 99% accuracy, it was imperative that any software used in the forensic context had a better accuracy rate to reduce the chance of false positive or false negative results. In this study a new automated genotyping method was developed that was suitable for forensic genotyping. This was achieved by producing a population of negative controls, positive controls and a set of unknown samples (of the same origin). This strategy enabled a more robust assessment of genotypes than had been previously been achieved.

2.2 Materials and Methods

2.2.1 The Generation III Microarray System (Amersham Biosciences)

The Generation III Microarray System comprised the Array Spotter, the Array Scanner and the Analysis Workstation. The adaptability of the system using automated spotting, detection and analysis of samples made it ideal for use with SNP technology for gene mapping and gene discovery, as massive parallel analyses could be carried out simultaneously. Whereas genome studies have the benefit of virtually unlimited amounts of DNA to analyse, in forensic applications the amount is limited, hence a different strategy is required.

2.2.1.1 Array spotter

The spotter facilitated the deposition of minute quantities of amplified DNA (200pL) onto glass slides (Figure 2.1). The instrument used 12 spotting pens to deposit the PCR product and could be programmed to spot a maximum of 12 microplates, each with 384 wells, onto 36 glass slides. The glass slides were coated with a layer of epoxysilane that allowed binding and attachment of the DNA to the slide. Before spotting, each sample was mixed 50:50 with dimethylsulphoxide (DMSO) to minimise evaporation and ensure an even spot morphology. The slides were subsequently hybridised with labelled probes that attached to the DNA spots on the glass surface, via specific target sites. The SNPs used were biallelic therefore two slides were needed for each individual locus, in order to target each allele separately.

2.2.1.2 Array scanner

The Array Scanner detected fluorescently labelled probes using two lasers (green 532nm and red 633nm) to scan the slides and Array Scanner Control software to collect the data. The two fluorescent labels used were Cy™3 and Cy™5 which fluoresce at the 575nm and 675nm respectively. The Cy3 label was attached to locus-specific probes and would only be detected if that locus was present on the microarray slide. The Cy5 label was attached to allele-specific probes and was detected if a sample had the specific allele for the SNP being tested.

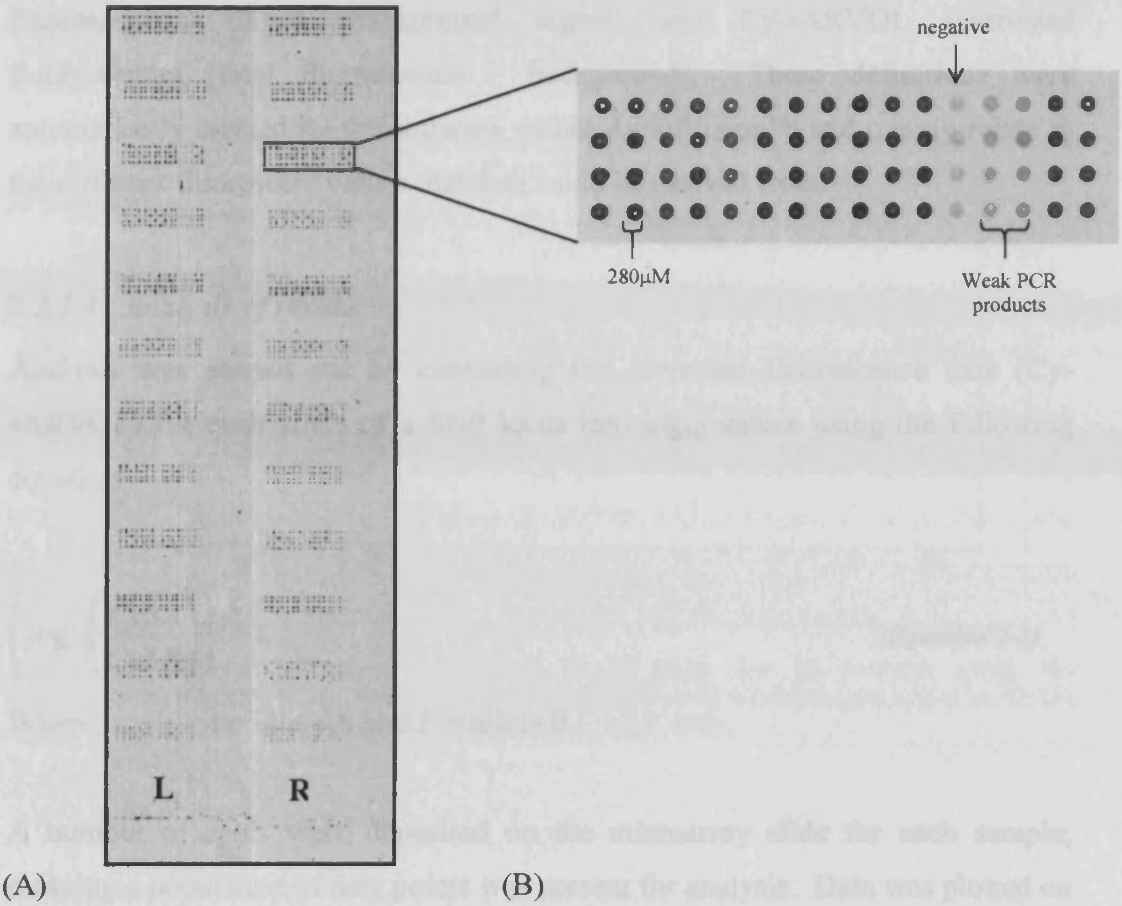


Figure 2.1 Cy3 fluorescent images of a post-spotted, hybridised glass slide derived from ImageQuant™. (A) = duplicated spots (L & R) in 12 separate blocks, each block being spotted by a different pen from the 12-pen set. (B) = one block enlarged to show the individual spots, each one indicating a single PCR product.

The presence of a Cy3 signal was indicative of PCR product for the targeted SNP locus being present. A Cy5 signal would only be present if the specific allele was present on the microarray slide.

2.2.1.3 Analysis workstation

The Analysis Workstation was connected to the Array Scanner computer enabling data to be transferred between them. ImageQuant™ software was used initially to assess slide and spot quality. The Cy3 and Cy5 images for each slide were created in separate files and Figure 2.1 shows a typical Cy3 slide image seen.

The image files were transferred into ArrayVision™ software that used set criteria to locate spots, quantify fluorescence and generate numerical data for each Cy-dye. This data was exported into an Excel spreadsheet as Cy-ARVOL-RFU (total fluorescence), Bkgrd (background signal) and Cy-sARVOL (corrected fluorescence) [total fluorescence – background]. These definitions were automatically created by the software within ArrayVision™ and merely relate to the different fluorescent values that data could be derived from.

2.2.1.4 Analysis of results

Analysis was carried out by converting the corrected fluorescence data (Cy-sARVOL) for each allele of a SNP locus into $\log_{10}$ values using the following equation:

$$\left(\log_{10}\left(\frac{Cy5A}{Cy5B}\right)\right) \qquad \textbf{\textit{(Equation 2-1)}}$$

Where: A = allele A and B = allele B

A number of spots were deposited on the microarray slide for each sample, meaning a population of data points was present for analysis. Data was plotted on a scattergraph, in clusters for each sample, to visually characterise the sample genotype (Figure 2.2). Data points lay around +1 for samples homozygous for allele A, -1 for samples homozygous for allele B and zero for heterozygous samples. Genotyping was manually performed by designation of the clusters depending on where they lay on the graph.

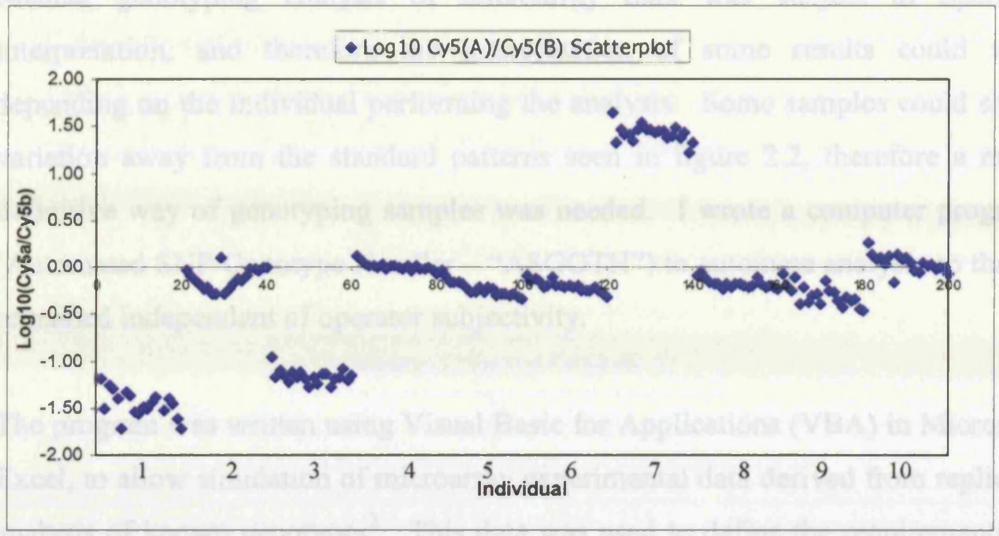


Figure 2.2 Scattergraph showing clusters of 20 spots for 10 samples using the $\log_{10}$(Cy5A/Cy5B) values. In this example the samples 1 to 10 would be genotyped as: HOM B, HET, HOM B, HET, HET, HET, HOM A, HET, HET, HET.

2.2.2 The computer program – ASGOTH

Manual genotyping analysis of microarray data was subject to operator interpretation, and therefore the interpretation of some results could vary depending on the individual performing the analysis. Some samples could show variation away from the standard patterns seen in figure 2.2, therefore a more definitive way of genotyping samples was needed. I wrote a computer program (Automated SNP Genotype Handler – "ASGOTH") to automate analysis so that it remained independent of operator subjectivity.

The program was written using Visual Basic for Applications (VBA) in Microsoft Excel, to allow simulation of microarray experimental data derived from replicate analysis of known genotypes[2]. This data was used to define the requirements of the process, especially to include: the ability to recognise failed samples; assessment of negative controls; and the ability to correctly genotype unknown samples.

The most accurate way to measure any parameter in an experimental protocol is by using a population and calculating the median or mean of the data. In microarray technology, three parameters are important: the negative threshold (*T*), control sample data (*C*) and unknown sample data (*U*). Populations were obtained by spotting each sample type onto a microarray slide numerous times and analysing the data obtained from this. One of the important questions to be answered by validation of the program was – how many spots are needed for each parameter in order to correctly genotype samples.

The design of the program allowed ASGOTH to genotype unknown samples analysed by the microarray by comparison to a negative threshold and control samples (see appendix II). The program used the Log_{10} (Cy5A/CyB) values, calculated from corrected fluorescent data, to indicate sample genotype based on the values observed in the control samples.

[2] European and US patent pending: P208010WO

ASGOTH was divided into three main sections (Figure 2.3):

- Calculation of the negative threshold (*T*)
- Utilising positive controls to calculate control bins
- Determination of genotypes for the unknown samples

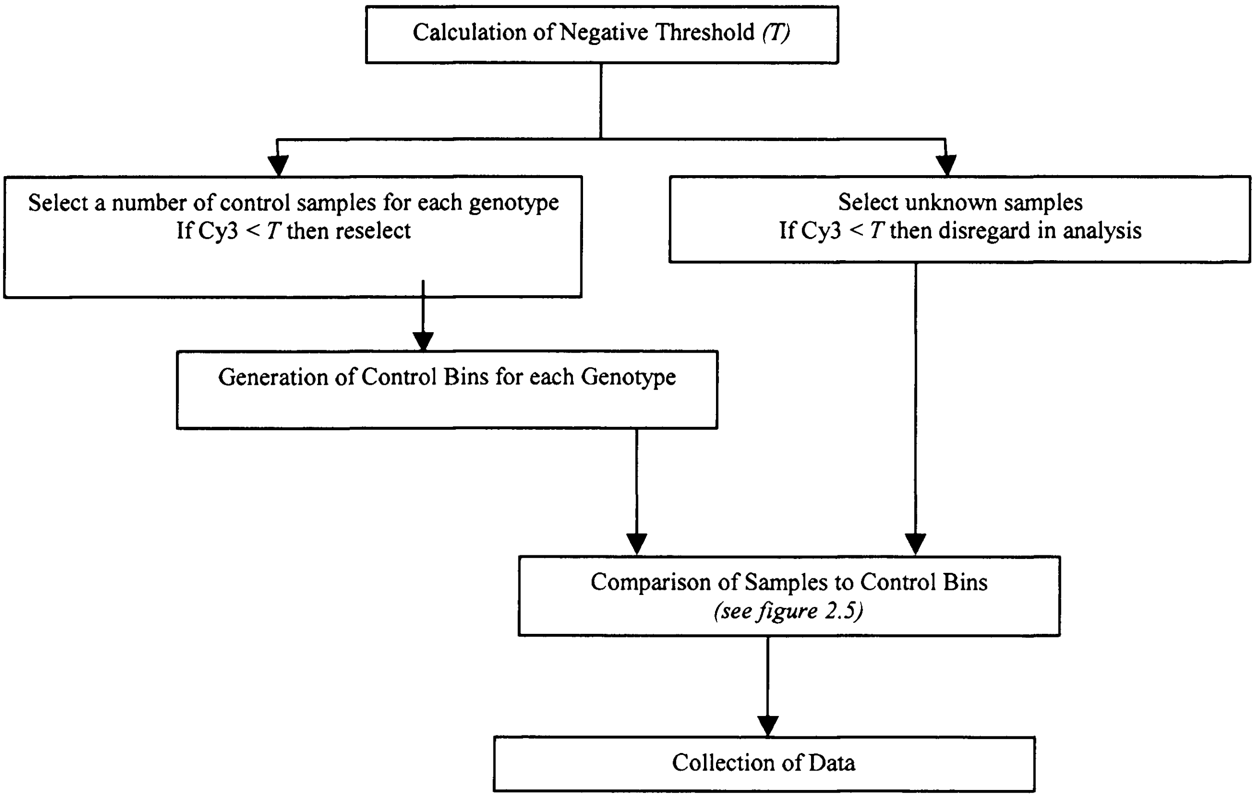

Calculation of Negative Threshold *(T)*
Select a number of control samples for each genotype
If Cy3 < *T* then reselect
Select unknown samples
If Cy3 < *T* then disregard in analysis
Generation of Control Bins for each Genotype
Comparison of Samples to Control Bins
(see figure 2.5)
Collection of Data

Figure 2.3 Flow diagram illustrating the path ASGOTH follows in order to correctly genotype samples.

2.3 Microarray Computer Program Validation

2.3.1 Calculation of the negative threshold (*T*)

It was important to calculate a negative threshold value (*T*) to allow identification of spots where hybridisation had failed or where DNA levels were too low to give a reliable result. Negative controls consisting of sterile water in place of sample DNA were run with each batch of samples taken through the microarray process, in order to obtain data values from which *T* could be calculated.

The average Cy3-sARVOL ($\bar{x}$) and standard deviation (SD) of the 24 negative controls were used to calculate *T* as:

$$T = \bar{x} + (6 \times \mathrm{SD}) \qquad \textit{(Equation 2-2)}$$

SD: $\sqrt{\dfrac{\sum\left(x-\bar{x}\right)^2}{n-1}}$ *where:* *n = number of observations*

6 SDs were used in the calculation as experimentation had shown that fluorescent values from the 24 negative controls were always less than this (section 2.4.1.2). 6 SDs approximates to about 99.7% of the range of a normal distribution.

Successful amplification of a locus was characterised by a Cy3 signal for each allele. If the Cy3 value for a specific spot fell below *T* it suggested there had been insufficient hybridisation of probe to that spot. Reasons for spot failure included failure of samples to amplify by PCR, failure of specific alleles to amplify by PCR, poor spot morphology, poor slide quality and lack of deposition of the sample onto the slide.

Cy3-sARVOL was used to calculate *T* and after subtraction of background fluorescence, some Cy3 fluorescence commonly remained in the negative control sample (Figure 2.1). Fluorescence varied depending on the SNP locus being investigated. A probable cause was non-specific binding of the Cy3 probe to primer-dimer formations, alternatively a small quantity of probe may have become bound to the slide surface during hybridisation.

2.3.2 Utilising positive controls to calculate control bins

Control samples of known genotype were used to define control bins encompassed by C_{min} and C_{max} where:

$$C_{min} = \mathit{median}\left(\log_{10}\left(\frac{Cy5A}{Cy5B}\right)\right) - \mathit{(n)SDs} \text{ and}$$

$$C_{max} = \mathit{median}\left(\log_{10}\left(\frac{Cy5A}{Cy5B}\right)\right) + \mathit{(n)SDs}$$

where A = allele A and B = allele B and n = the optimal number of SDs (defined by experimentation) for each control bin. Three different positive controls were required for each locus:

1 – C_{AA}	(Homozygote - *AA*)
2 – C_{AB}	(Heterozygote –*AB*)
3 – C_{BB}	(Homozygote – *BB*)

The control sample data were subject to two filters to ensure that all of the data used to create the control bins were of appropriate quality. Firstly, for each fluorescent spot, the program compared the Cy3 fluorescence to *T* (Figure 2.3). If the Cy3 value for a specific spot fell below *T* then it was not selected as it could not be distinguished from the negative controls. Secondly, C was evaluated, using the $\log_{10}$ calculation set out in equation 3.1. The hybridisation efficiency of Cy5A and Cy5B could never be absolutely the same, therefore C $\neq$ 0. Consequently, if a result was obtained where C = 0, this was used as an indicator of hybridisation failure as a zero value was indicative of no signal. This allowed the assumption that there was no product at that spot position and that specific spot would not be used in the calculation of the control bins.

The control bins were established using the following equations, with optimum SDs derived from experimental data outlined in section 2.4.1.1:

Type 1:	[$C_{AArange}$]	C_{min} – 3 SD
		C_{max} > median C
Type 2:	[$C_{ABrange}$]	C_{min} – 4 SD
		C_{max} + 4 SD
Type 3:	[$C_{BBrange}$]	C_{min} < median C
		C_{max} = median C + 3 SD

Figure 2.4 indicates the positioning of these control bin ranges on a Log_{10} graph.

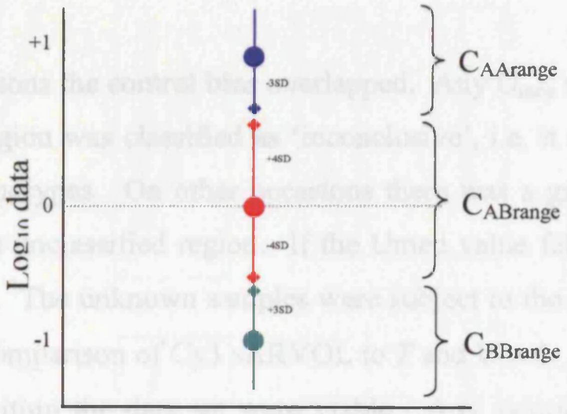


Figure 2.4 Diagrammatical representation of control bins produced from log_{10} Cy5A/Cy5B data

2.3.3 Determination of genotypes for the unknown samples

Data for unknown samples was obtained from the relative fluorescence of allele A and allele B and was given an identifier of U, where:

$$\text{U value} = \left(\log_{10}\left(\frac{Cy5A}{Cy5B}\right)\right)$$ for each spot for unknown samples and

U_{med} = median of U for each unknown sample.

For each unknown sample U was calculated and compared to $C_{AArange}$, $C_{ABrange}$ and $C_{BBrange}$ generated by the first part of the program (Figure 2.5). To be genotyped correctly, each sample must fall into one of the three control bins.

On most occasions the control bins overlapped. Any U_{med} value falling within the overlapping region was classified as 'inconclusive', i.e. it could have been either one of two genotypes. On other occasions there was a gap between the control bins, giving an unclassified region. If the Umed value fell between two C bins, then it 'failed'. The unknown samples were subject to the same two filters as the control data: comparison of Cy3 sARVOL to T and $U \neq 0$. Samples failed if none of the spots within the data set were viable. Any samples giving an incorrect genotype were scored as 'wrong' answers.

A correctly scored sample was defined as either:

a correct genotype (type 1, 2 or 3); or
a failure to score the sample (total Cy3: sARVOL < T, U = 0); or
an inconclusive result ($C_{AArange} > U < C_{ABrange}$, $C_{ABrange} > U < C_{BBrange}$).

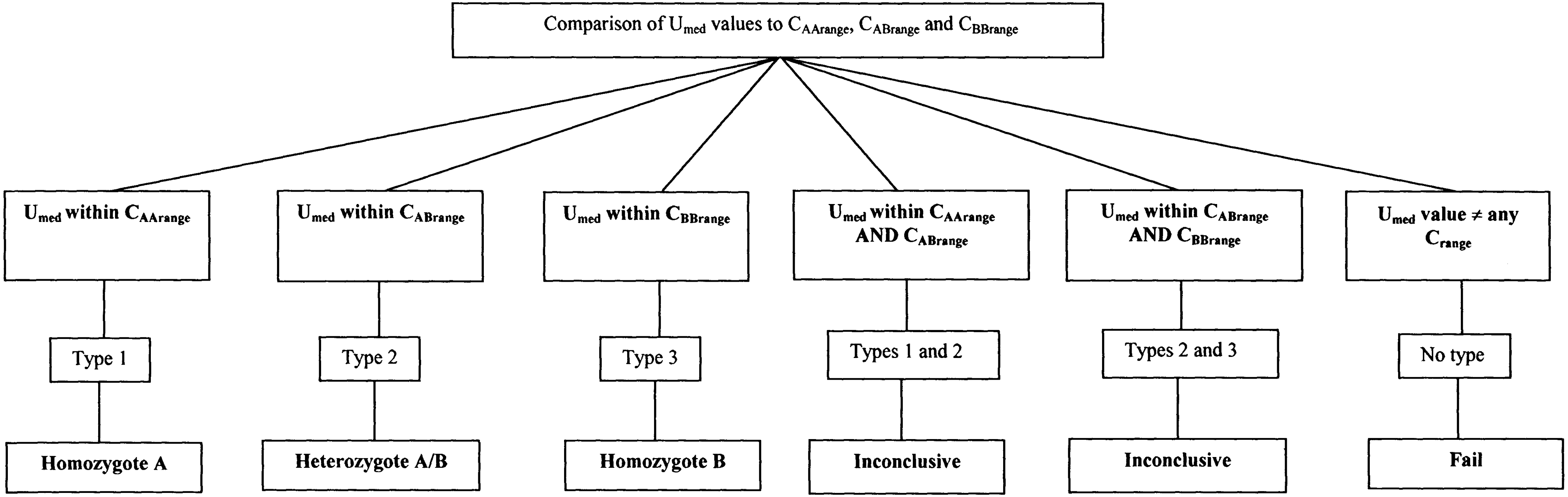

Comparison of U_{med} values to $C_{AArange}$, $C_{ABrange}$ and $C_{BBrange}$
U_{med} within $C_{AArange}$
U_{med} within $C_{ABrange}$
U_{med} within $C_{BBrange}$
U_{med} within $C_{AArange}$ AND $C_{ABrange}$
U_{med} within $C_{ABrange}$ AND $C_{BBrange}$
U_{med} value ≠ any C_{range}
Type 1
Type 2
Type 3
Types 1 and 2
Types 2 and 3
No type
Homozygote A
Heterozygote A/B
Homozygote B
Inconclusive
Inconclusive
Fail

Figure 2.5 Flow diagram depicting allocation of genotypes to unknown samples based on comparison to C (expanded from figure 2.3)

2.4 ASGOTH – A Simulation Program

31 samples of known genotype, plus one negative control, were taken through the microarray process to generate data that could be used to determine the following factors, to ensure correct genotyping of unknown samples:

How many standard deviations were required for both *T* and the control bins?
How many control samples were needed to define the control bins?
How many spots were needed per sample for both controls and unknowns?

Bootstrapping, with replacement, was used to select a series of data sets that contained values for known samples for the SNP TSCO Z2. Mock "unknown" samples were generated from the same data sets to make sure the generated genotypes were correct before running true unknown samples.

The program was designed to simulate 1000 experiments, using randomly chosen samples (with replacement) from the same data set each time. Each stage of the program is outlined in Figure 2.6, from calculation of *T* to analysis of simulated data.

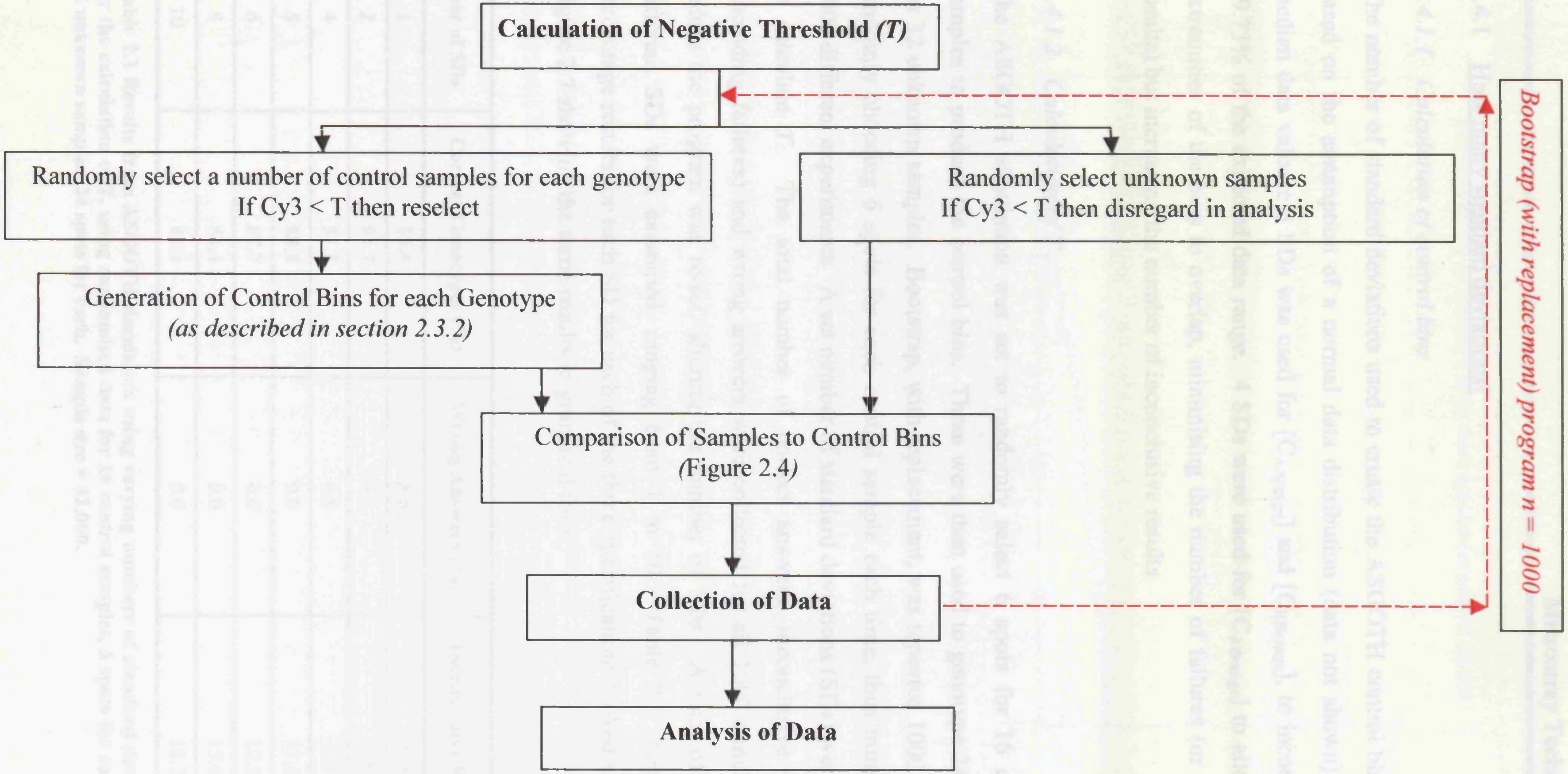

Calculation of Negative Threshold (T)
Randomly select a number of control samples for each genotype
If Cy3 < T then reselect
Randomly select unknown samples
If Cy3 < T then disregard in analysis
Generation of Control Bins for each Genotype
(as described in section 2.3.2)
Comparison of Samples to Control Bins
(Figure 2.4)
Collection of Data
Analysis of Data
Bootstrap (with replacement) program n = 1000

Figure 2.6 Flow diagram depicting the path of the validation bootstrapping program. Samples are randomly selected (with replacement) and the program was repeated 1000 times.

2.4.1 How many standard deviations?

2.4.1.1 Calculation of control bins

The number of standard deviations used to create the ASGOTH control bins was based on the assumption of a normal data distribution (data not shown). The median data value ± 3 SDs was used for [$C_{AArange}$] and [$C_{BBrange}$], to incorporate 99.73% of the expected data range. 4 SDs were used for [$C_{ABrange}$] to allow the extremities of the bins to overlap, minimising the number of failures (or wrong results) but increasing the number of inconclusive results.

2.4.1.2 Calculation of T

The ASGOTH simulation was set to randomly select 6 spots for 16 control samples to produce the control bins. These were then used to genotype 24 spots for 32 unknown samples. Bootstrap, with replacement, was repeated 1000 times, randomly choosing 6 spots for each control sample each time, thus mimicking 1000 different experiments. A set number of standard deviations (SDs) were used to calculate *T*. The total number of correct answers, inconclusive results (including failures) and wrong answers were collected for all 1000 simulations before the program was re-set, altering the number of SDs. A total of seven different SDs were examined, ranging from 1 to 10. Table 2.1 shows the percentage results for each SD for each of the three specifications looked at, with Figure 2.7 showing the same results in graphical form.

Number of SDs	Correct Genotypes (%)	Wrong Answers (%)	Inconclusive Results (%)
1	85.4	8.6	6.0
2	91.7	4.8	3.5
4	91.8	0.8	7.3
5	88.4	0.0	11.6
6	87.7	0.0	12.3
8	84.4	0.0	15.6
10	81.3	0.0	18.7

Table 2.1 Results from ASGOTH simulation using varying numbers of standard deviations for the calculation of *T*, using randomised data for 16 control samples, 6 spots for each and 32 unknown samples, 24 spots for each. Sample size = 32,000.

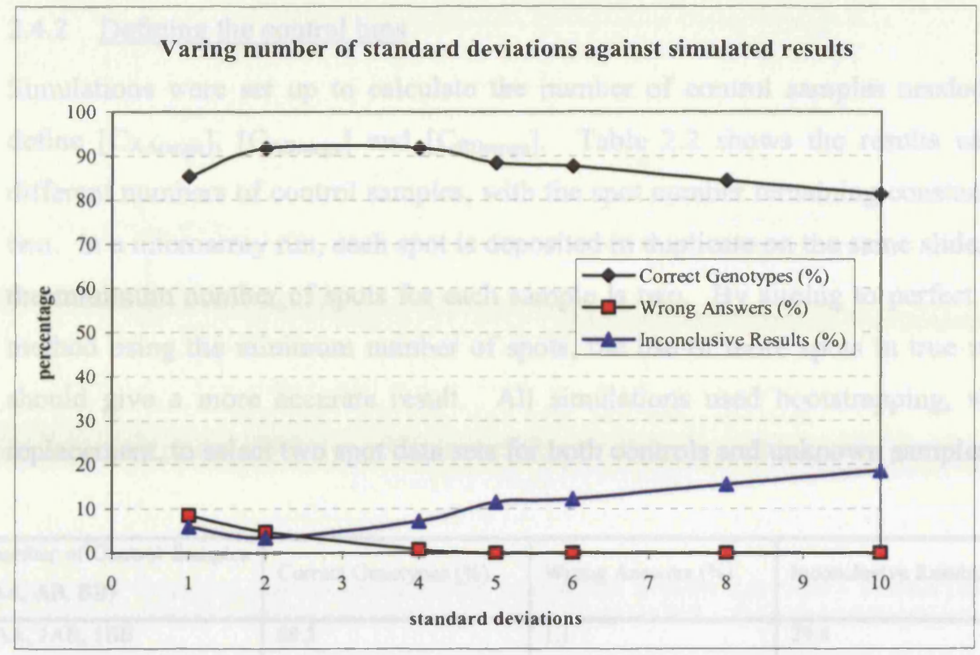


Figure 2.7 Graphical representation of the data shown in table 2.1. No wrong answers were seen when a standard deviation above 5 was used to calculate *T*. However, as the number of SDs was increased from 5 to 10, the number of correct genotypes scored declined and the number of inconclusive results rose. Sample size = 32,000.

Results from these preliminary simulations suggested that (T + ≥5 SDs) would be sufficient to eliminate all ambiguous data resulting from spot failure. Using an SD of 5 or over gave no wrong answers on the ASGOTH simulation. However, as the SD was increased above 5 the number of correctly genotyped samples fell and the number of inconclusive results rose (Figure 2.7). This phenomenon would have been a consequence of samples failing as Cy-sARVOL-RFU values fell below T. A decision was made to use 6 SDs in all further validation studies, to maximise the number of correctly genotyped samples whilst minimising the number of inconclusive results.

2.4.2 Defining the control bins

Simulations were set up to calculate the number of control samples needed to define [$C_{AArange}$], [$C_{ABrange}$] and [$C_{BBrange}$]. Table 2.2 shows the results using different numbers of control samples, with the spot number remaining constant at two. In a microarray run, each spot is deposited in duplicate on the same slide, so the minimum number of spots for each sample is two. By aiming to perfect the method using the minimum number of spots, the use of more spots in true runs should give a more accurate result. All simulations used bootstrapping, with replacement, to select two spot data sets for both controls and unknown samples.

Number of Control Samples (AA, AB, BB)	Correct Genotypes (%)	Wrong Answers (%)	Inconclusive Results (%)
1AA, 1AB, 1BB	69.5	1.1	29.4
2AA, 2AB, 2BB	79.4	0.7	20.0
4AA, 4AB, 4BB	81.6	0.3	18.2
6AA, 6AB, 6BB	81.8	0.3	17.9
8AA, 8AB, 8BB	81.9	0.3	17.9

Table 2.2 Results of ASGOTH simulation using varying numbers of control samples. All simulations were carried out using 2 random spots from each control AND each unknown sample. All results are shown as percentages of actual data. Sample size = 1000 simulations of 32 samples.

The number of samples scored correctly and the number of wrong answers observed reached a plateau as the number of control samples was increased above four, suggesting little variation in data between control samples of the same genotype (Figure 2.8). This indicated that it was the number of spots used in a data set as opposed to the number of different samples that defined the scope of the control bins. By using two random spots for each simulation the number of wrong answers had steadily declined to 0.3% but never fell to zero.

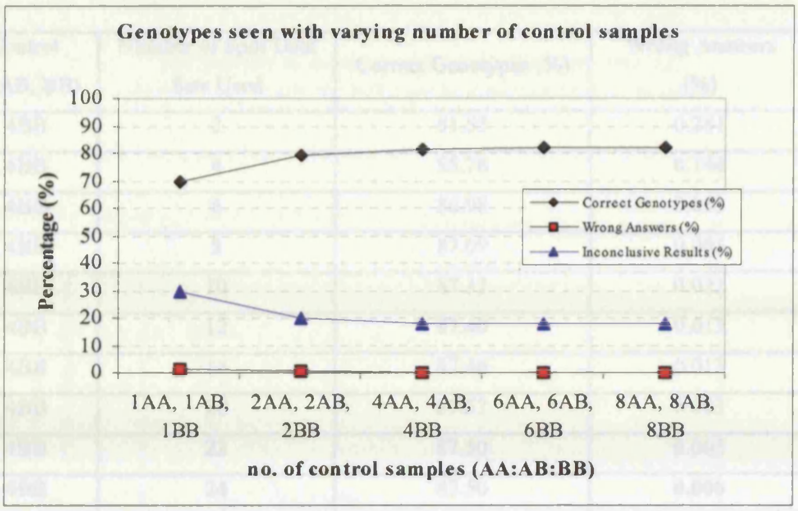


Figure 2.8 Graphical representation of the data shown in table 2.2. AA = control sample with a homozygous A genotype; AB = heterozygous control; BB = homozygous B control.

It was important to show that ASGOTH could fully genotype samples without giving any false results, therefore another set of simulations were set up using four control samples per genotype and increasing the number of spot data sets used. By increasing the numbers of spots used per sample, it was hoped that the control bins would become more defined leading to improved scoring of each unknown sample. The results of these simulations are shown in table 2.3, along with results for simulations using six control samples per genotype.

Number of Control Samples (AA, AB, BB)	Number of Spot Data Sets Used	Correct Genotypes (%)	Wrong Answers (%)	Inconclusive Results (%)
4AA, 4AB, 4BB	2	81.55	0.281	18.17
4AA, 4AB, 4BB	4	85.76	0.144	14.09
4AA, 4AB, 4BB	6	86.98	0.053	12.97
4AA, 4AB, 4BB	8	87.09	0.063	12.85
4AA, 4AB, 4BB	10	87.31	0.022	12.67
4AA, 4AB, 4BB	12	87.40	0.013	12.59
4AA, 4AB, 4BB	14	87.46	0.013	12.53
4AA, 4AB, 4BB	20	87.52	0.003	12.48
4AA, 4AB, 4BB	22	87.50	0.003	12.50
4AA, 4AB, 4BB	**24**	87.50	**0.000**	12.50
6AA, 6ABB, 6BB	2	81.83	0.288	17.88
6AA, 6ABB, 6BB	6	86.97	0.050	12.98
6AA, 6ABB, 6BB	10	87.47	0.022	12.51
6AA, 6ABB, 6BB	12	87.43	0.013	12.56
6AA, 6ABB, 6BB	**14**	87.48	**0.000**	12.52

Table 2.3 Results of ASGOTH simulations using increasing numbers of spot data sets for both 4 control samples per genotype and 6 control samples per genotype. All results are shown as percentages of actual data. Sample size = 1000 simulations of 32 samples.

After performing the first set of simulations using four control samples per genotype, the data illustrated the need to use a total of 24 spot data sets per sample in order to get no wrong answers. More simulations were carried out using an increased number of control samples per genotype to see if fewer spots would be required. Table 2.3 and Figure 2.9 show the number of wrong answers falling to zero with 14 spot data sets, as opposed to 24, using six control samples per genotype. This demonstrates the benefit of using a larger number of control samples to further define the control bins.

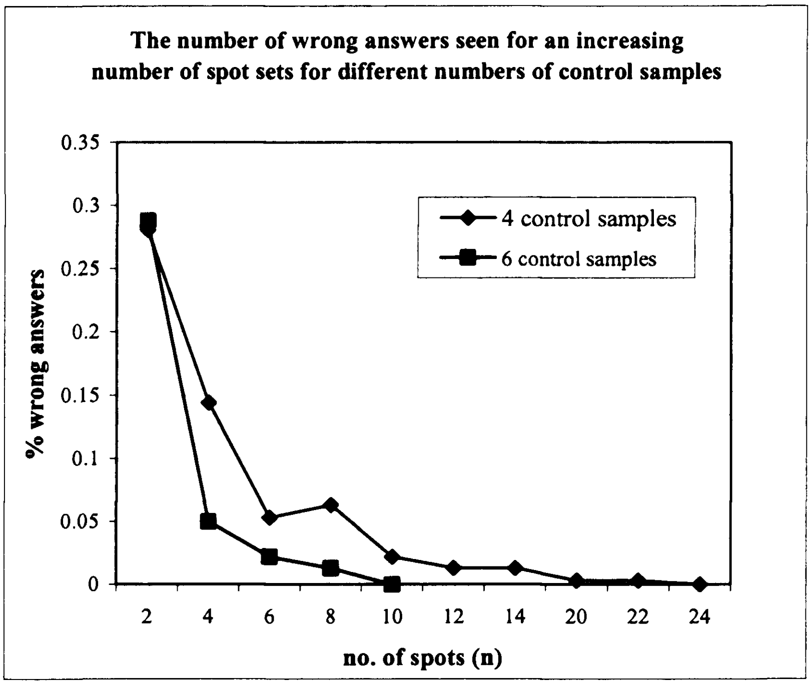


Figure 2.9 Graphical representation of the percentage of wrong answers seen when using a different number of control samples per genotype and varying numbers of spot data sets. Sample size = 32,000.

The results of these simulations also showed little variation in the number of inconclusive results gained when using six or more spot data sets. The 'inconclusive results' group consisted of both samples falling within two control bins and sample failures. The lack of variation within this group as the number of spots increased suggested the group to be mainly composed of failing samples. As the control bins became more defined with increasing numbers of data sets, the samples originally falling within two control bins would have been shifted into one bin or the other, increasing the number of correct answers.

2.5 How Representative Is The Data Set?

2.5.1 Same controls, different unknowns

A simulation was set up to test whether the parameters designed for one experiment could be used to genotype samples from a different experiment. The same control samples were used as those described in section 2.3 but a different unknown sample data set was used, consisting of 16 samples plus one negative control. Both sets of data were randomised, using 6 control samples per genotype and 14 spots for both controls and unknown samples. 1000 simulations gave a sample size of 17,000.

82% of samples were scored with the correct genotype, a further 18% gave an inconclusive result and no wrong answers were seen with these parameters. The increased percentage of inconclusive results seen suggested that the control bins were not well enough defined for this different data set, causing an increased number of samples to fall with two control bins or to fail completely. Also, the *T* value may not have been high enough to guarantee all outliers to be taken out of the data set, again increasing the number of inconclusive results.

This brief experiment highlighted the need to run control samples on each separate microarray run. Variations in different experiments could occur during PCR amplification, during microarray spotting, through slide quality, probe hybridisation and operator differences. By using control samples on each run, these variations would be translated into the control bins and samples could be genotyped accordingly.

2.5.2 Using the same parameters for a different SNP – TSCO D

A simulation was set up using the same control parameters on an entirely different data set using a different SNP – TSCO D, to test the limitations of the ASGOTH system. Only five control samples per genotype were available to create the control bins for TSCO D and 14 spot data sets were used for both controls and unknowns. All data were randomised using a random number generator, giving a sample size of 16,000.

The same parameters as in section 2.4, but with 15 control samples, generated the following results when using TSCO D:

Correct results = 93.73%
Inconclusive results = 6.25%
Wrong answers = 0.02%

Only 2 out of 1000 simulations generated the three wrong answers seen with this SNP. It is most likely the wrong answers were generated by outlier samples that had managed to pass the negative threshold filter. The amount of fluorescence seen for each individual SNP varied greatly due to the presence of primer-dimer formations. Hybridisation probes could bind to the negative control spots if primer-dimer was present, giving an enhanced fluorescence reading. This emphasised the need to optimise the system for each separate SNP used on the microarray system. Optimisation would involve the parameter testing used in section 2.4 however, the ability to type almost 94% of samples without optimisation showed the robustness of the ASGOTH automated system.

2.6 Discussion

The use of microarrays in the research environment has increased significantly in the last decade as arrays have become more miniaturised and biochemistry has developed to enable millions of SNPs to be accurately genotyped with very high-throughput and low error (Southern 1995; Schena 1996; Bowtell 1999; Brown and Botstein 1999; Southern 2001; Heller 2002; Holloway *et. al.* 2002; Simon *et. al.* 2003). Analysis of microarray data uses the same methods as other fluorescent detection systems, based on relative fluorescence data obtained from samples compared to controls, however most of the specialised software, such as ImageQuant™ and ArrayVision™ (Amersham Biosciences), was unsuitable for automated genotyping of SNPs.

Due to the increased use of microarray technology within gene expression research, a number of analysis programs were developed to cope with the massive amounts of data obtained from gene expression experiments. Bowtell (1999) reviewed the main microarray platforms and software available for data analysis, with a further, updated, review carried out three years later (Bowtell 1999; Holloway *et. al.* 2002). All software programs developed have focussed on methods to improve the sensitivity and classification of samples in gene expression experiments, highlighting a need for a simpler method of microarray analysis that could be translated to the biallelic genotyping required for forensic use. The main difference between the needs of the different systems being that gene expression microarrays use pairwise comparisons to assess the amount of gene expression present in a given gene against a housekeeping control gene; for forensic purposes there was a requirement to compare a single allele in a biallelic SNP locus to a single allele in a control sample.

Methods used for gene expression analysis of microarray data could be translated into a format for use with the data derived from our low-throughput genotyping work, for example; normalisation of data (Chen *et. al.* 2002; Park *et. al.* 2003; Suzuki *et. al.* 2003; Munir *et. al.* 2004), use of reference samples and genotyping based on fluorescence data (Schena 1996; Watson *et. al.* 1998; Granjeaud *et. al.* 1999; Syvänen 1999; Jain *et. al.* 2002).

Normalisation of data is defined as *"the process of removing some sources of variation which affect the measured [gene expression levels]"* (Park *et. al.* 2003), i.e. any fluorescent data collated from a microarray analysis is subject to variation due to background levels of fluorescence, different affinities of the various dyes used, differences in samples and differences in operators. Normalisation was carried out prior to use with ASGOTH software by subtraction of background fluorescence (Bkgrd) from total fluorescence (Cy-ARVOL-RFU) to give a corrected fluorescence value (Cy-sARVOL), a calculation automatically performed by ArrayVision™ software. There have been a number of articles reviewing the benefits and drawbacks of using such a calculation in gene expression analyses as the subtraction of background fluorescence can vary due to the algorithms used in different software programs (Brown *et. al.* 2001; Simon *et. al.* 2003; Scharpf *et. al.* 2005). Background can arise from a number of sources, including incomplete washing of the slide after hybridisation, features of the slide that bind dye (such as the epoxysilane layer) and imprecision of the spot grid during image acquisition (Schuchhardt *et. al.* 2000). Due to these factors it was decided that for our purposes it would be better to err on the side of caution and use background fluorescence subtractions rather than risk gaining false positive results from inaccurate background readings. A second normalisation procedure – converting data into log ratios - was performed on the data before importing into ASGOTH.

Log_{10} ratios were used to standardise the data obtained using our system, as fluorescence for each allele was obtained from two separate slides spotted with the same PCR products. Each of the two slides was investigated using a different probe labelled with the same fluorophore, so hybridisation would only occur if the sequence complementary to the probe sequence was present within the DNA sample. Variations in slide quality and hybridisation affinities meant that it was necessary to normalise the data to directly compare one allele to the other. By carrying out a log transformation, the data obtained could be standardised across the set, regardless of slide or hybridisation variability (Thygesen and Zwinderman 2004).

Positive control samples were essential to provide a basis for defining control bins by which to analyse data from unknown samples. In gene expression analysis, housekeeping genes (for which expression levels are known) are used as references and patterns of expression are compared to them (Schena 1996; Bowtell 1999; Alizadeh *et. al.* 2000; Southern 2001; Holloway *et. al.* 2002; Jain *et. al.* 2002; Suzuki *et. al.* 2003). Statistical analyses are carried out by hierarchical clustering, using software written for gene expression profiling. Clustering analysis was not applicable to the samples used in our study, as sample sizes were small and information needed to be derived from a single sample for a single SNP being interrogated, therefore a more precise method of analysis was required. Negative controls, using sterile distilled water samples taken through the whole microarray process, were used to assess the amount of fluorescence seen in a sample without DNA. This fluorescence data (*T*) formed the basis of the primary interrogation of data for each spot in the experiments.

The initial ASGOTH program was developed to assess how many microarray features would be required per sample, for both controls and experimental samples, in order to allow automated genotyping of samples with little operator intervention. These guidelines could then be used as the basis for all microarray experimentation, minimising errors and increasing the throughput of the system. An error rate of up to 1% is seen in microarray technology used for gene expression experiments, due to the ultra-high throughput of the system and the less stringent guidelines in place for determining the level of gene expression (Heil *et. al.* 2002; Huang *et. al.* 2004; Wenz *et. al.* 2005). For forensic purposes a system needs to be close to 100% efficient, therefore the rules governing the calling of alleles needs to be infallible. A method of achieving this was developed by using populations of spots on a microarray, as opposed to single spots. This allowed median analysis (±SD) to be used to create both control bins and medians for unknown samples, and the two could then be further compared.

A lack of available data for further SNPs limited the amount of validation that could be performed using the ASGOTH program. Nevertheless a comprehensive framework was developed that could be used to evaluate any SNP system. Initial studies suggested the use of Cy3 fluorescence in negative control spots could be used to filter any ambiguous results resulting from experimental variations in slide quality, spotting and probe hybridisation.

Random number generator simulations (Monte-Carlo simulations) were used to test the limits of the ASGOTH system by imitating 1000 different experimental results. Monte-Carlo simulations have been used in many different research areas to generate datasets when only a minimal amount of data is initially present (Triggs and Curran 1995; Chen *et. al.* 2002; Kimbrough 2004; Gill and Kirkham 2004b; Gill *et. al.* 2005b). This type of simulation randomly generates values for uncertain variables over and over to simulate a model and, by defining the characteristics required for the simulation, any number of experiments can be performed using a sub-optimal size dataset. For use with the ASGOTH program, simulations were performed to repeat the experimental outcomes without the need for re-running the laboratory work, giving a dataset that could be easily interpreted for validation purposes.

Control bins generated using median data and SD values allowed correct genotyping of 27994 out of 32000 samples (over 87%) with an unknown genotype, in an optimised system. No wrong answers were seen when using an increased number of spots and samples to generate the control bins. Inconclusive results consisted of samples which either fell into two control bins or which had failed. Spots not passing the negative threshold, for reasons such as low PCR amplification or poor spotting quality, would have caused failures. The program was therefore shown to be 100% efficient in calling alleles, either with a genotype or with an inconclusive / fail marker.

The ASGOTH system could be adapted for use with other platforms (e.g. Luminex™, capillary electrophoresis) as well as other biochemical methods on the microarray, such as reverse dot-blot whereby specific probes are spotted onto the microarray slide and PCR products are added to the slide for hybridisation. Further experimental work using SNPs on the microarray was ceased, due to the inability to produce large multiplexes easily on such a platform. Future work looked at developing the SNP multiplex for use on a capillary electrophoresis platform, where products could be separated by size, thereby minimising the effects of primer-dimer formations.

3 Degradation Studies

3.1 Introduction

DNA damage, arising from enzymatic activity and errors encountered during replication, is a relatively common event in metabolically active cells (Lindahl 1993). DNA repair mechanisms are in place that can reverse the vast majority of changes made to the DNA molecule allowing little irreversible damage to the cells. In inactive cells (i.e. dead or dormant cells), the mechanisms of DNA damage persist but the repair mechanisms have been disabled, leading to DNA degradation (Pääbo 1989; Lindahl 1993; Hofreiter *et. al.* 2001; Willerslev and Cooper 2005). Spontaneous hydrolysis and oxidation of DNA leads to single-strand breaks, baseless sites (depurination), miscoding lesions and cross-links, all of which decrease the chance of successfully amplifying the DNA molecules (Lindahl 1993; Willerslev and Cooper 2005). The mechanisms of DNA degradation are described in more detail in chapter 1.

The ancient DNA research community has carried out a great deal of work on the degradation of DNA. As described by Willerslev & Cooper (2005), *"the post-mortem instability of nucleic acids is central to the methodological problems inherent to ancient DNA research"*, and the same can be said when analysing degraded DNA for forensic purposes. The hydrolytic and oxidative damage seen in ancient DNA samples has been shown to correlate with the temperature of the recovery site as opposed to the age of the samples, *"thus, while forensic samples are much younger than ancient samples, these will presumably contain similar types of [DNA] damage"* (Poinar 2003). Bär *et. al.* (1988) observed the post-mortem stability of DNA in various human organs and tissues, using both direct agarose gel analysis and RFLP analysis with minisatellite probe 33.15. The rate of DNA fragmentation was found to be variable between the different sample types, with total degradation of the high molecular weight fragments (15-23 kb) appearing between five days and three weeks.

With the introduction of the PCR amplification method, a smaller quantity of initial starting DNA template was needed for forensic analysis, as outlined in chapter 1. The fragmentation of DNA following degradation reduces the efficiency of the PCR reaction, in particular it is expected that *"(i) PCR of ancient* *or degraded DNA should only amplify small fragments; (ii) the amount of amplified product should be small compared with similar reactions with (modern) DNA"* (Golenberg *et. al.* 1996). Pääbo (1989) used PCR techniques for analysis of several ancient DNA samples ranging in age from four years to 13,000 years. He found that amplification of a 121bp mitochondrial DNA sequence was possible from aged samples but when the size of the DNA target was increased, the amount of product generated rapidly diminished, with no amplification of ancient DNA sequences longer than 140bp (Pääbo 1989).

In the early 1990s, the introduction of STR systems for forensic DNA profiling increased the success of gaining a result over the previous RFLP methods. The STR systems amplified shorter target sequences (<500bp) so increased success rates in fragmented samples (Kimpton *et. al.* 1994; Lygo *et. al.* 1994; Gill *et. al.* 1995b; Wiegand and Kleiber 2001; Tsukada *et. al.* 2002). As the sensitivity of the DNA profiling techniques increased, it was observed that the higher molecular weight STRs would fail to amplify when DNA was degraded or starting DNA template was sub-optimal (Whitaker *et. al.* 1995; Gill *et. al.* 2000b; Whitaker *et. al.* 2001; Butler *et. al.* 2003; Chung *et. al.* 2004). It was hypothesised that this was primarily due to fragmentation of the DNA molecule, leaving only small fragments of DNA that had been protected at the primary level of chromosome packing, the nucleosome, as outlined in chapter 1.

Forensic DNA samples are subject to varying levels of both DNA degradation and copy number. Amplification of sub-optimal amounts of DNA can result in partial profiles, allele dropout, unbalanced loci and failure to generate any profile (Whitaker *et. al.* 2001; Gill 2001b; Gill 2002). In the UK, low copy number (LCN) DNA analysis may be carried out on samples containing sub-optimal quantities of DNA (generally <100 ng template) to try and increase the likelihood of gaining a reportable profile. The LCN technique uses SGM+ profiling 34 as opposed to the standard 28 amplification cycles (Gill *et. al.* 2000b).

Some sample types, such as bone, teeth and hair shafts, naturally have little nuclear DNA. For this reason, analysis of these sample types is generally carried out using mitochondrial DNA (mtDNA) sequencing. MtDNA profiling is also carried out when the sample is suspected to contain little or no DNA, due to the circumstances surrounding the acquirement of the sample, e.g. the biological sample may have come from a skeleton or a sample from a high temperature mass disaster (Budowle *et. al.* 2005; Graham 2005). MtDNA analysis is routinely carried out in the United States, as opposed to LCN STR analysis, as it is thought to be less subject to issues surrounding LCN analysis, i.e. contamination through secondary transfer (Lowe *et. al.* 2002), presence of mixtures, allele dropout and allele drop-in, as well as the reliability of the DNA source (http://www.ncjrs.gov/pdffiles1/nij/grants/203971.pdf). The main drawback of mtDNA analysis is the linear inheritance of the DNA material through the maternal line. This decreases the discrimination power of the test to approximately 1 in 100, as it cannot distinguish between mothers, siblings and further progeny. However, in cases where maternal relatives are the only source of reference, an analysis of the mtDNA sequence can be advantageous (http://www.forensic.gov.uk/forensic/foi/foi_docs/Mito.pdf; Crespillo *et. al.* 2000; Just *et. al.* 2004).

The nucleosome unit was defined in the 1970s as eight histone molecules interacting with approximately 200bp DNA (Kornberg 1974). The nucleosome units were attached to each other by lengths of linker DNA, forming a 'beads on a string' effect. Further research used micrococcal nuclease digestion methods to determine the exact length of the DNA interacting with the histone octamer. The micrococcal nucleases preferentially targeted the unprotected linker DNA, leaving 146bp of DNA attached to the nucleosome core particle (van Holde *et. al.* 1975; Noll and Kornberg 1977). A further ten base pairs protruding from each end of the nucleosome were shown to be more readily digestible than those protected within the histone octamer, leaving approximately 125bp lengths of DNA protected by the nucleosome structure (Read and Crane-Robinson 1985b).

To assess the DNA profiling techniques used in forensic laboratories it was necessary to artificially degrade a set of samples that could be used for analysis of fragmentation. A number of different methods have been proposed that degrade DNA *in situ*, including the use of enzymes such as DNase I & II or micrococcal nuclease (Wilcox and Smith 1976; Golenberg *et. al.* 1996; Wu *et. al.* 2000;

Cousins *et. al.* 2004); boiling (Stroop and Schaefer 1989); subjecting DNA to UV light or microwaves (Stroop and Schaefer 1989); storing samples for periods of time at room temperature (Tsukada *et. al.* 2002; Butler *et. al.* 2003); and storing samples in a humid environment (Dixon *et. al.* 2005a). Other studies on DNA degradation have used samples from animal tissue post-mortem (Johnson and Ferris 2002); ancient DNA samples (Pääbo 1989; Burger *et. al.* 1999; von Wurmb-Schwark *et. al.* 2003; Willerslev and Cooper 2005); and boiled bones (von Wurmb-Schwark *et. al.* 2003).

A comparison study was set up, using DNA artificially degraded by two different methods, to test the hypothesis that the nucleosome was protecting the small fragments of DNA. One set of samples was degraded by boiling extracted DNA in solution in a waterbath for a set time course of five hours. The DNA extraction process would have removed all proteins, including the nucleosome, and enzymes from the samples prior to boiling. The other set of samples was obtained from a previous project whereby blood, semen and saliva samples had been deposited on cotton squares and left in a humid environment at 37°C (to mimic the optimal temperature for enzyme and bacterial activity) for a period of up to 243 days, with samples being taken out and stored at -20°C at set periods. DNA was extracted from the body fluid samples after degradation therefore enzymes and proteins would still have been present in the samples during the degradation period. By comparing these two degradation methods, the nucleosome degradation theory and the pattern of DNA fragmentation could be assessed. Both sets of samples were DNA profiled using SGM+ STR analysis and the SNP 27-plex method to a) assess the size of the fragments surviving in the degraded samples and b) assess the efficiency of the two techniques to amplify degraded DNA.

Initial studies suggested an increased amplification efficiency of samples when smaller amplicons were used for DNA profiling. As a consequence of this work the SNP 27-plex was redesigned to only include amplicons less than 186 bases in length. This allowed fragment sizes of 146 base pairs and lower to be targeted, incorporating an extra 20 bases at each end of the amplicon in the form of universal tails. The number of loci that could be detected within the smaller size range had to be reduced to twenty SNPs plus Amelogenin. This new 21-SNP multiplex allowed products from 96 bases to 186 bases to be amplified and detected in a single-tube reaction. Work was carried out to validate the technique for casework situations and to assess the suitability of SNPs for use in circumstances where limited DNA or DNA of poor quality was available.

3.2 Materials and Methods

3.2.1 Boiling DNA samples

3.2.1.1 DNA extraction

DNA from five reference samples (CAS, DRJ, HER, SHM, ST) was extracted from 1 mL of liquid blood using the Qiagen™ Genomic-Tip system (Cat no. 10223, 20/G tips) according to the manufacturer's protocol to obtain between 5-15 ng/µL DNA suspended in 2 mL 1 x TE Buffer (ABD). Liquid blood had been stored frozen at -20°C and was defrosted at 37°C in a shaking incubator, prior to DNA extraction. Samples were quantified using a UV spectrophotometer according to the manufacturer's protocol (Ultrospec 3100 *pro* UV/Visible spectrophotometer, Biochrom Ltd, UK).

3.2.1.2 Boiling of DNA extract

1 mL of each stock DNA solution was aliquoted into Nunc™ tubes and placed in a boiling waterbath. DNA extracts were boiled for 5 hours to provide 14 aliquots per control sample, each 50 µL aliquot taken from the water-bath at different time intervals (15m, 30m, 45m, 1h, 1h15m, 1h30m, 1h45m, 2h, 2h30m, 3h, 3h30m, 4h, 5h). Aliquots were left to cool to room temperature before being stored in a refrigerator at 4°C, ready for PCR amplification.

3.2.2 Artificially degraded body fluid samples

3.2.2.1 Preparation of artificially degraded samples

Blood, saliva and semen samples had been previously degraded for other research projects. Degradation was carried out by spotting the samples onto cotton squares before placing in a pipette tip box partly filled with water. This 'humidity box' was stored in an incubator at 37°C (normal body temperature) for a period of approximately 8 months (243 days). At specific time intervals, a number of cotton squares for each sample were removed from the incubator and stored in the freezer until the degradation period was complete for all samples. The water level in the pipette box was kept filled to maintain humidity levels.

3.2.2.2 DNA extraction of artificially degraded samples

Degraded DNA from the cotton squares was extracted using the Qiagen™ QiaAmp Mini-Kit (Cat no. 51306). Samples had been stored frozen at -20°C and were defrosted at room temperature prior to DNA extraction. The manufacturer's protocol for each sample type was used to obtain 0-2 ng/μL DNA, suspended in 150 μL 1 x TE Buffer (ABD). Samples were quantified using Picogreen methodology (Ahn *et. al.* 1996) (Table 3.1).

	Days in humidifier	Picogreen quant (ng/μL)
Saliva	0	0.60
	42	0.06
	62	0.05
	84	0.04
	147	0.04
	243	0.03
Semen	0	2.20
	42	2.00
	62	1.70
	84	0.70
	147	0.50
	243	0.50
Blood	0	0.50
	42	0.50
	62	0.40
	84	0.30
	147	0.03
	243	0.02

Table 3.1 Picogreen DNA quantification values for artificially degraded saliva, semen and blood samples.

3.2.2.3 Dilution series of control DNA samples

A dilution series of five control samples (CAS, DRJ, HER, SHM, ST) was set up to give a series of DNA extracts with concentrations ranging from 1 ng/μL down to 16 pg/μL. These dilution series were used for amplification of the 21-SNP multiplex.

3.2.3 DNA amplification

The amount of DNA sample added to each PCR reaction varied depending on the sample type and the length of degradation.

Boiled samples were diluted to 1 ng/μL using DNA quant values given for the stock DNA solution of each control sample. Samples from time intervals between 0 minutes and 90 minutes were added to both the SNP 27-plex and SGM+

multimixes in 1μL volumes, as these were shown to over-amplify if higher volumes of DNA were added (data not shown). Samples boiled for >90 minutes were added at maximum volumes (14 μL for SNP 27-plex; 20 μL for SGM+).

Artificially degraded sample DNA was added to the multimixes at a concentration of 1 ng/μL, using data obtained from Picogreen quantification. Samples that had DNA concentrations of <0.1 ng/μL were added at maximum volumes (14 μL for the SNP 27-plex; 12 μL for the SNP 21-plex; 20 μL for SGM+).

For the dilution series experiments using the SNP 21-plex, DNA was added at 1 μL per PCR reaction.

3.2.3.1 SNP multiplex amplification

Both the SNP 27-plex and the SNP 21-plex were used for degradation experiments. The amplification multimixes consisted of oligonucleotide primers (synthesised by IBA, Germany) at varying concentrations, 0.4 μg bovine serum albumin (Boehringer Manheim, Germany), 225 μM dNTPs (dATP, dCTP, dTTP, dGTP; Boehringer Mannheim, Germany), 1 x PCR Buffer II containing 1.5mM $MgCl_2$ (Applied Biosystems™, UK) and 5 units Amplitaq Gold® (Applied Biosystems, UK). Primer sequences and concentrations per reaction are outlined in appendix III and IV.

Samples were amplified in strips of eight 0.2 mL tubes, without mineral oil, on a thermal cycler (Applied Biosystems™ GeneAmp PCR system 9600) using the conditions given in appendix V.

3.2.3.2 SGM+ amplification

SGM+ amplification kits (Cat. No. 4307133) were used, following the manufacturer's protocol. Samples were amplified in strips of eight 0.2 mL tubes, without mineral oil, on a thermal cycler (Applied Biosystems™ GeneAmp PCR system 9600) using the parameters given in appendix V for low copy number conditions, i.e. 34 cycles amplification.

3.2.4 Capillary electrophoresis detection

1.1 μL of each PCR product (SNPs/SGM+) + 10 μL GS-HD400 ROX (Applied Biosystems™ Part no. 402985):HI-DI Formamide (Applied Biosystems™) [ratio 1:37] was added to a 96-well micro-titre plate and heat-denatured at 95°C for 2 minutes before running on a capillary electrophoresis (CE) sequencer (AB model 3100) using ABI Collection software v1.1. SGM+ PCR products were run at a single injection time of 22 seconds whilst SNP 27-plex products were injected for 20 seconds. The SNP 21-plex was injected for 12 seconds.

3.2.5 Analysis of results

Sample data were analysed using ABI Prism™ Genescan Analysis v3.7.1 and ABI Prism™ Genotyper software v3.7 NT. SGM+ data were interpreted using STRIPE™ (in-house computer program) to gain likelihood ratios for each sample. SNP data extracted from Genotyper™ (peak height, peak area, scan number, size in bases) were transformed into *.csv format and analysed by Celestial™ (chapter 4) to give profile data and likelihood ratios. SGM+ data for each sample were extracted from Genotyper™ (peak height, peak area, allele designation) and tabulated.

3.3 Results

For all three profiling techniques (SNPs and SGM+), over-amplification of loci was seen with the less degraded samples, i.e. samples boiled for less than 30 minutes or kept for less than 42 days in the incubator. An excess of pull-up peaks and split peaks made genotyping difficult, however full sequencing of each SNP had been carried out previously for reference samples and the correct SGM+ profiles for all five individuals were known. Comparison of all results to these control results verified the profiles that were obtained in these experiments.

The SNP 27-plex failed to give a full profile, even when using optimal amounts of DNA (i.e. 1 ng). It is possible that degradation of the multiplex occurred whilst in storage, as the aliquot volumes were small enough (10 μL) to allow freeze-thawing when subjected to an increase in temperature for a matter of seconds. The presence of 27 sets of three primers, plus Universal primers, would have further decreased the efficiency of the multiplex.

For all analyses results were divided into full profiles, low molecular weight (LMW) profiles and high molecular weight (HMW) profiles, to ascertain whether there was a significant difference in the amplification efficiency of these loci. The process of DNA fragmentation by nucleases indicates that linker DNA is targeted first, leaving fragments of approximately 146 bases in length. During PCR amplification using the SNP 27-plex, 20 base 'Universal' primers were added to the 3' and 5' ends of each SNP locus, making each SNP amplicon 40 bases longer than its original base pair sequence, i.e. a locus sequence of 120 bases would give a product of 160 bases in length, therefore LMW SNPs were deemed to be loci less than 186 bases in length, as opposed to less than 146bp.

The 21-SNP multiplex was designed as a consequence of the initial boiling and degradation experiments. A comparison of the 21-SNP multiplex is given for the artificially degraded samples.

3.3.1 Boiled DNA samples (SNP 27-plex vs. SGM+)

Amplification of boiled DNA samples was performed using 1 μL dilutions for the first seven samples (reference samples – 90 minutes boiling); followed by maximum volumes for the remaining samples (105 minutes – 300 minutes). These volumes were determined from previous results obtained that showed over-amplification of samples boiled for 90 minutes or less when using increased amounts of DNA. Using 1 μL of these DNA dilutions was sufficient to produce a full profile. Evaporation of some samples during the boiling process meant that there was insufficient volume of extract left to assess degradation after 5 hours. In these instances, any remaining extract was amplified using the SNP 27-plex instead of SGM+.

SNP profiles were generated by Celestial™, using a set of interpretation rules for the designation of each SNP, based on a 20-second injection time (Table 3.2 & Table 3.3). The SNP profiles demonstrated allele dropout in the HMW loci over 190 bases in length at earlier time intervals to the LMW loci.

The SGM+ profiles, generated using Genotyper™ software showed the same pattern of high molecular weight dropout as the SNP 27-plex (Table 3.4 & Table 3.5).

	SNP (size in bp)	Amelo	D	B6	N4	Y3	A4	O6	Z2	K3	J2	Y6	J8	X	F	G	L2	W3	H8	L6	K4	X7	U6	W5	U5	V4	P7	P5
FileName	Time boiled (m)	(97)	(103)	(109)	(113)	(117)	(124)	(129)	(133)	(137)	(141)	(146)	(153)	(157)	(164)	(171)	(174)	(180)	(186)	(190)	(195)	(200)	(204)	(212)	(215)	(221)	(226)	(231)
CAS_REF	0	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	A/T	G/C	A	A/T	F/A	A/T	A/T	T	T/F
CAS_1	15	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	A/T	G/C	A/F	F/T	F/A	A/T	A/T	T	T/F
CAS_2	30	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	A/T	G/C	A/F	F/T	F/A	A/T	A/T	T	T/F
CAS_3	45	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	A/T	G/C	A/F	F/T	F/A	A/T	A/T	T	T/F
CAS_4	60	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	A/T	G/C	A/F	F/T	F/A	A/T	A/T	T	T/F
CAS_5	75	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	A/T	G/C	A/F	F/T	F/A	A/T	A/T	T	T/F
CAS_6	90	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	A/T	G/C	A/F	F/T	F/A	A/T	A/T	T	T/F
CAS_7	105	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	F/T	G/C		F/T	F/A	A/F	F/T	T	T/F
CAS_8	120	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	F/T	G/C		F/T	F/A			T/F	T/F
CAS_9	150	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C	A	A	C/A	C	T	C	C	T	F/T	G/C		F/T	F/A			T/F	
CAS_10	180	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C/F	A	A	F/A	C	T	C	C	T				A/T	F/A				
CAS_11	210	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C/F	A	A	F/A	C	T	C	C	T				A/T	F/A				
CAS_12	240	X	T/C	A/T	A/T	G	C/G	A	T	G/C	C/F	A	A		C	T	C	C	T					F/A				
CAS_13	300	X	T/C	A/T	A/T	G	C/G			G/C	C/F	A	A					C	T									
DRJ_REF	0	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G	A/T	A/T	T/F	T	T	T/A	T
DRJ_1	15	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G	A/T	A/T	T/F	T	T	T/A	T
DRJ_2	30	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G	A/T	A/T	T/F	T	T	T/A	T
DRJ_3	45	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G	A/T	A/T	T/F	T	T	T/A	T
DRJ_4	60	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G	A/T	A/T	T/F	T	T	T/A	T/F
DRJ_5	75	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G	A/T	A/T	T/F	T	T	T/A	T/F
DRJ_6	90	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G	A/T	A/T	T/F	T	T	T/A	T/F
DRJ_7	105	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G		F/T	T/F	F/T	T	T/A	T/F
DRJ_8	120	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G		F/T	T/F	T	T	T/A	T/F
DRJ_9	150	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G		F/T	T/F	F/T	T	T/F	T/F
DRJ_10	180	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G/F		F/T	T/F	F/T	T		
DRJ_11	210	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T	G/F		F/T		F/T	F/T		
DRJ_12	240	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T	C/A	C	T	C	C/G	T	T			F/T		F/T			
DRJ_13	300	X/Y	T	A/T	A/T	G	C	A	C	C	C/T	T	T		C	T	C	C/G	T	T								
HER_REF	0	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	T	T	A	F/A
HER_1	15	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	T	T	A	F/A
HER_2	30	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	T	T	A	F/A
HER_3	45	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	F/T	T	A	F/A
HER_4	60	X	T	A/T	A/T	G	C/G	A/T	C	C	C/F	T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	T	T	F/A	F/A
HER_5	75	X	T	A/T	A/T	G	C/G	A/T	C	C	C/F	T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	F/T	T	F/A	F/A
HER_6	90	X	T	A/T	A/T	G	C/G	A/T	C	C		T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	F/T	F/T	F/A	
HER_7	105	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	T		A	T/A
HER_8	120	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	T		A	T/A
HER_9	150	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G		A/T	F/A	T		F/A	T/A
HER_10	180	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G/F		A/T	F/A	T		F/A	F/A
HER_11	210	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G/F		F/T	F/A	T			
HER_12	240	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	C/A	A	T	C	C	T	T	G/F		F/T		F/T			
HER_13	300	X	T	A/T	A/T	G	C/G	A/T	C	C	C	T/A	A	F/A	A	T/F	C	C	T	F/T	G/F							

Table 3.2 SNP 27-plex profiles obtained for three reference control samples (CAS, DRJ, HER) using 1 μL of diluted DNA for the first seven time intervals followed by 14 μL for the remaining time intervals. Grey boxes represent alleles that are absent from the profile. SNP loci are shown in size (bp) order from the smallest amplicon (Amelo) to the largest (P5).

	SNP (size In bp)	Amelo	D	B6	N4	Y3	A4	O6	Z2	K3	J2	Y6	J8	X	F	G	L2	W3	H8	L6	K4	X7	U6	W5	U5	V4	P7	P5
FileName	Time boiled (m)	(97)	(103)	(109)	(113)	(117)	(124)	(129)	(133)	(137)	(141)	(146)	(153)	(157)	(164)	(171)	(174)	(180)	(186)	(190)	(195)	(200)	(204)	(212)	(215)	(221)	(226)	(231)
SHM_REF	0	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C	A/T	T	T/A	A	T	T/A	T
SHM_1	15	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C	A/T	T	T/A	A	T	T/A	T
SHM_2	30	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C	A/T	T	T/A	A	T	T/A	T
SHM_3	45	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C	A/T	T	T/A	A	T	T/A	T
SHM_4	60	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C	A/T	T	T/A	A	T	T/A	T/F
SHM_5	75	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C	A/T	T	T/A	A	T	T/A	T/F
SHM_6	90	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C	A/T	T	T/A	A	T	T/A	T/F
SHM_7	105	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C	F/T	T	F/A	A	T	T/A	T/F
SHM_8	120	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C		T	T/A	A	T	T/A	T/F
SHM_9	150	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C	C/A	T/C	C	C	T	A/T	G/C	F/T	T	F/A	A	T		T/F
SHM_10	180	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C/F	C/A	T/C	C	C	T	A/F	G/C	A/T	T	F/A	A	T		
SHM_11	210	X	T/C	A/T	A/T	G	C/G	A	C/T	G/C	C	T/A	A	C/F	C/A	T/C	C	C	T	F/T	G/C		T		A			
SHM_12	240	X	T/C	A/T		G	C/G	A/F	F/T	G/F	C	T/A	A	C/F		F/C	C/F	C/F	T						A/F			
SHM_13	300	X	T/C	A/T		G	C/G		F/T	G/F	C	T/A	A						T									
ST_REF	0	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/T	T		
ST_1	15	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/T	T		
ST_2	30	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/T	T		
ST_3	45	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/T	T		
ST_4	60	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/T	T		
ST_5	75	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/T	F/T		
ST_6	90	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/F	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/F			
ST_7	105	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/T			T/F
ST_8	120	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/T			T/F
ST_9	150	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G		A/T	T/A	A/T			T/F
ST_10	180	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G/F		A/T	T/F	A/T			T/F
ST_11	210	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	T	G/F		F/T		A/T			
ST_12	240	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C	T/A	A	C/A	C	T	C	G	T	F/T	G/F				A/T			
ST_13	300	X	T/C	A/T	A/T	G/C	C	A	C	G/C	C/F	T/A	A	C/A		T	C	G	T				F/T					

Table 3.3 SNP 27-plex profiles obtained for two reference control samples (SHM, ST) using 1 μL of diluted DNA for the first 7 time intervals followed by 14 μL for the remaining time intervals. Grey boxes represent alleles that are absent from the profile. SNP loci are shown in size (bp) order from the smallest amplicon (Amelo) to the largest (P5).

FileName	Time boiled (m)	Amelo		D19		D3		D8		VWA		THO		D21		FG		D16		D18		D2	
CAS_REF	0	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_1	15	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_2	30	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_3	45	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_4	60	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_5	75	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_6	90	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_7	105	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_8	120	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_9	150	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_10	180	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13	14	13	14	24	24
CAS_11	210	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24	13		13	14	24	24
CAS_12	240	X	X	14	14	17	18	13	14	15	18	9	9	30	31	21	24		14	13	14		
DRJ_REF	0	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_1	15	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_2	30	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_3	45	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_4	60	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_5	75	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_6	90	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_7	105	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_8	120	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_9	150	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15	23	23
DRJ_10	180	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25	10	12	13	15		
DRJ_11	240	X	Y	14	14	15	17	13	15	16	16	8	9	30	30	23	25		12				
HER_REF	0	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19	23	24
HER_1	15	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19	23	24
HER_2	30	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19	23	24
HER_3	45	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19	23	24
HER_4	60	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19	23	24
HER_5	75	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19	23	24
HER_6	90	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19	23	24
HER_7	105	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19		24
HER_8	120	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19		
HER_9	150	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19		
HER_10	180	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9	12	17	19		
HER_11	210	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9			19		
HER_12	240	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22	23	9					
HER_13	300	X	X	14	16	13	16	12	12	14	18	7	9.3	30	31	22		9					

Table 3.4 SGM+ profiles obtained for three reference control samples (CAS, DRJ, HER) using 1μL of diluted DNA for the first 7 time intervals followed by 20μL for the remaining time intervals. Grey boxes represent alleles that are absent from the profile. STR loci are shown in size (bp) order from the smallest amplicon (Amelo) to the largest (D2).

FileName	Time boiled (m)	Amelo		D19		D3		D8		VWA		THO		D21		FG		D16		D18		D2	
SHM_REF	0	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	17	20
SHM_1	15	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	17	20
SHM_2	30	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	17	20
SHM_3	45	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	17	20
SHM_4	60	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	17	20
SHM_5	75	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	17	20
SHM_6	90	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	17	20
SHM_7	105	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	17	20
SHM_8	120	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	17	20
SHM_9	150	X	X	13	15	15	15	14	15	16	18	9	9	28	28	21	24	11	11	16	16	▨	▨
SHM_10	180	X	X	13	15	15	15	14	15	16	18	▨	▨	28	28	▨	▨	11	11	16	16	▨	▨
SHM_11	210	X	X	13	15	15	15	14	15	▨	18	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
SHM_12	240	X	X	13	15	▨	▨	▨	▨	▨	18	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
ST_REF	0	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	24	25
ST_1	15	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	24	25
ST_2	30	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	24	25
ST_3	45	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	24	25
ST_4	60	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	24	25
ST_5	75	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	24	25
ST_6	90	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	24	25
ST_7	105	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	24	25
ST_8	120	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	24	25
ST_9	150	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	▨	24	▨
ST_10	180	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	12	20	▨	▨
ST_11	210	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	22	12	14	▨	▨	▨	▨
ST_12	240	X	X	13	13	15	17	12	13	17	18	6	9.3	30	31.2	19	▨	12	▨	▨	▨	▨	▨
ST_13	300	X	X	13	13	15	17	12	13	17	18	6	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨

Table 3.5 SGM+ profiles obtained for two reference control samples (SHM, ST) using 1μL of diluted DNA for the first 7 time intervals followed by 20μL for the remaining time intervals. Grey boxes represent alleles that are absent from the profile. STR loci are shown in size (bp) order from the smallest amplicon (Amelo) to the largest (D2).

3.3.1.1 Analysis of variance (ANOVA) calculations[3]

Percentage profiles for both SNPs and STRs were calculated from the numbers of allele dropouts for each sample at each time interval (appendix VI) using the equation:

$$\left(\frac{a}{n}x100\right) \qquad \textit{(Equation 3-1)}$$

Where a = the number of surviving alleles and n = total number of possible alleles.

An ANOVA general linear model was performed on the percentage profile data and results were tabulated (Table 3.6). An ANOVA allows simple analysis of variance tests to be performed to test the hypothesis that means from two or more samples are equal. By using a general linear model, more than one sample for more than one group of data can be tested for variance by generating statistics for each pairwise comparison.

The results indicated a significant difference between the percentage profiles obtained for each sample, using both profiling techniques, compared to the amount of time boiled (P<0.002) (Variant 1. Table 3.6). This suggested that the DNA had been fragmented in solution causing varying percentage profiles to be obtained. No significant differences were seen in the percentage profiles obtained for different individuals for full SGM+ profiles (P=0.339), LMW SGM+ profiles (P=0.096) or HMW SGM+ profiles (P=0.518), indicating no preferential amplification of the low molecular weight loci (Variant 2. Table 3.6). There was a significant difference between individual samples for full SNP profiles (P=0.010) and HMW SNPs (P=0.001) but not for LMW SNPs (P=0.533) (Variant 2. Table 3.6), suggesting that DNA may have been less efficiently amplified in some individuals.

A two-factor crossed ANOVA model compared different individuals to the time boiled (Variant 3. Table 3.6) suggested no significant differences for the SNP 27-plex and the LMW SNPs ($P \geq 0.060$), indicating all samples performed with the

[3] All statistical analyses were carried out using Minitab Release 14™ software

same efficiency with these profiling techniques. There was a significant difference between the different percentage profiles for each sample for the different boiling times for HMW SNPs and all SGM+ profiling analyses (P<0.017), suggesting a possible variation between different individuals as well as between the different DNA profiling methods. This is discussed further in section 3.3.1.2.

Variant considered	**Analysis of variance tests (ANOVA)**	**R-sq %**	**DF**	**SS**	**F**	**P**
1) Time boiled	SNP 27-plex	72.29	1	3825.80	109.98	**<0.001**
	LMW SNPs	31.55	1	164.42	13.11	**0.001**
	HMW SNPs	80.77	1	28752.70	177.47	**<0.001**
	SGM+	60.40	1	5293.32	49.40	**<0.001**
	LMW SGM+	53.23	1	351.12	11.00	**0.002**
	HMW SGM+	62.42	1	18947.80	61.20	**<0.001**
2) Sample ID	SNP 27-plex	72.29	4	639.59	3.71	**0.010**
	LMW SNPs	31.55	4	32.13	0.80	0.533
	HMW SNPs	80.77	4	5742.80	5.81	**0.001**
	SGM+	60.40	4	1008.60	1.16	0.339
	LMW SGM+	53.23	4	611.49	2.08	0.096
	HMW SGM+	62.42	4	2210.30	0.82	0.518
3) Sample ID x Time boiled	SNP 27-plex	72.29	4	314.73	2.40	0.060
	LMW SNPs	31.55	4	106.90	2.27	0.073
	HMW SNPs	80.77	4	2044.60	3.29	**0.017**
	SGM+	60.40	4	1994.66	5.13	**0.001**
	LMW SGM+	53.23	4	1254.48	9.01	**<0.001**
	HMW SGM+	62.42	4	4100.10	3.78	**0.009**

Table 3.6 ANOVA results (P values, R-Sq %, Sum of Squares (SS) and F statistics) calculated for the SNP 27-plex and SGM+ for boiled samples.

3.3.1.2 Chi-Squared analysis using contingency tables

Chi-squared tests were carried out on the percentage profile data (appendix VI) to highlight any variation between techniques and between the different individuals. Each technique was divided into full profiles, LMW loci only and HMW loci only and all percentage profiles were compared. One out of the five control samples (SHM) showed a significant difference in the percentage data obtained for the SNP 27-plex compared to SGM+ (P=0.022) (Table 3.7 comparison 1). The SNP 27-plex gave an increased percentage profile, compared to SGM+, at longer boiling times (78% SNP profile after 3h30m : 32% SGM+ profile; 48% SNP profile after 4h : 23% SGM+ profile). All other samples showed no variation between the efficiency of the two techniques.

The most significant differences were seen between LMW and HMW loci for both SNPs and SGM+. All five individuals showed variation between results for the LMW and HMW SNPs (Table 3.7 comparison 3) and three out of five individuals showed variation between LMW and HMW SGM+ profiles (Table 3.7 comparison 5). This suggested an association between percentage profiles and the size of the target DNA sequence. This is discussed further in section 3.3.1.3.

	Two-way	**CAS**			**DRJ**			**HER**			**SHM**			**ST**		
	Goodness of Fit χ^2 Analysis	**df**	**X^2**	**P**	**df**	**X^2**	**P**	**df**	**X^2**	**P**	**df**	**X^2**	**P**	**df**	**X^2**	**P**
1	SNP 27-plex * SGM+	12	7.33	0.835	11	0.74	1.000	13	1.62	1.000	12	23.72	**0.022**	13	4.37	0.987
2	SNP 27-plex * LMW SNPs	12	7.21	0.844	11	2.75	0.994	13	3.68	0.994	12	4.74	0.966	13	2.57	0.999
3	LMW SNPs * HMW SNPs	12	158.34	**<0.001**	11	42.48	**<0.001**	13	67.41	**<0.001**	12	73.65	**<0.001**	13	66.64	**<0.001**
4	SGM+ * LMW SGM+	12	1.04	1.000	11	2.98	0.991	13	6.69	0.917	12	14.37	0.278	13	15.17	0.297
5	LMW SGM+ * HMW SGM+	12	5.19	0.951	11	16.80	0.114	13	41.11	**<0.001**	12	112.07	**<0.001**	13	111.99	**<0.001**
6	LMW SNPs * LMW SGM+	12	0.55	1.000	11	0.00	1.000	13	0.35	1.000	12	11.29	0.504	13	0.22	1.000

Table 3.7 Chi-squared Goodness-of-Fit contingency table test results.

3.3.1.3 Allelic dropout compared to fragment length

The proportion of allelic dropout seen at different amplicon sizes for SNPs and SGM+ was calculated by amalgamating results for all individual samples, disregarding the time boiled. These dropout proportions are shown graphically, compared to the fragment size, in Figure 3.1.

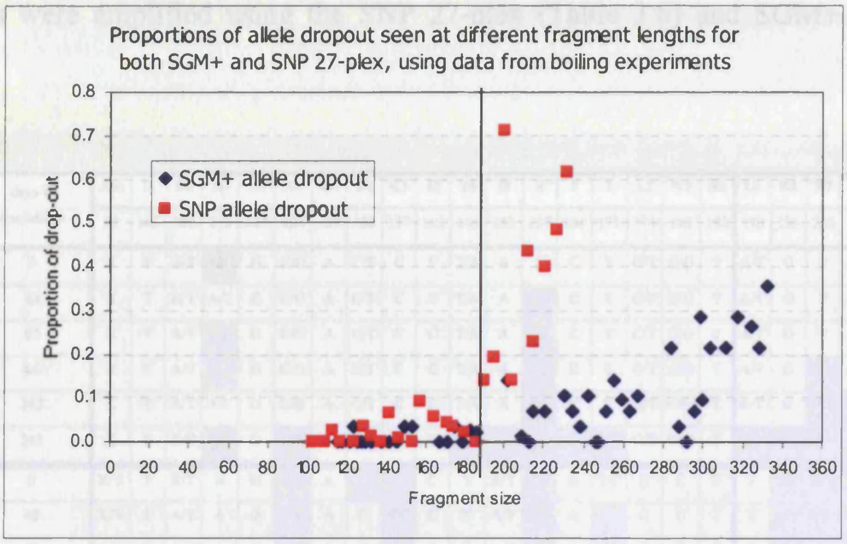


Figure 3.1 Scattergraph showing proportion of allelic dropout seen compared to fragment length size for both the SNP 27-plex and SGM+, obtained using data from boiling experiments.

The vertical line intersecting the graph at 186 bases indicates the visual boundary between high and low molecular weight loci, as discussed in section 3.3. The proportion of dropout appears to increase above 186 bases in length for both SNPs and SGM+ and there is a distinct difference between the LMW and HMW 'populations' ($P<0.001$; $df = 65$; $X^2 = 297.9$ (SGM+) and 471.7 (SNP 27-plex)).

Profiles obtained in these experiments showed typical signs of HMW degradation, using both SNPs and STRs, with over-amplification or better amplification of the LMW products less than 200bp in size.

3.3.2 Artificially degraded body fluid samples

Body fluids were degraded at 37°C (body temperature) and 100% humidity, in situ - the optimal temperature for enzyme and bacterial activity. Enzymes and proteins would still have been present in the samples during the degradation period as DNA was extracted from the body fluid samples after degradation. Samples were amplified using the SNP 27-plex (Table 3.8) and SGM+ (Table 3.9).

Sample type	days in humidifier	Am	D	B6	N4	Y3	A4	O6	Z2	K3	J2	Y6	J8	X	F	T	L2	W3	H8	L6	K4	X7	U6	W5	U5	V4	P7	P5
		97	103	109	113	117	124	129	133	137	141	146	153	157	164	171	174	180	186	190	195	200	204	212	215	221	226	231
Blood	0	X	T	A/T	F/T	G	C/G	A	C/T	C	C	T/A	A	C	C	T	C/T	C/G	T	A/T	G	T	A/T	F/A	A/T		T/F	T/F
	42	X	T	A/T	A/T	G	C/G	A	C/T	C	C	T/A	A	C/F	C	T	C/T	C/G	T	A/T	G	T	A/T	F/A	A/T	F/T	T/F	T/F
	62	X	T	A/T	F/T	G	C/G	A	C/T	C	C	T/A	A	C/F	C	T	C/T	C/G	T	A/T	G	T	A/T	F/A	A/T		T/F	T/F
	84	X	T	A/T	F/T	G	C/G	A	C/T	C	C	T/A	A	C/F	C	T	C/T	C/G	T	A/T	G	F/T	A/T	F/A	A/F			T/F
	147	X	T	A/T	A/T	G	C/G	A	C/T	C	C	T/A	A	C/F	C	T	C/T	C/G	T	A/T	G	F/T	F/T	F/A	A/F			
	243	X	T	A/T	F/T	G	C/F	A	C/T	C	C	T/F				T/F	C/T	C/G	T	F/T	G/F				A/F			
Saliva	0	X/Y	T	A/T	A	G	C/F	A	C/F		C	T	A/T	C/F	A	T/C	C	C	T	T	G/F	F/T	F/T				A	F/A
	42	X/Y	T	A/T	A	G	C/F	A	C	F/C	C	T	A/T	C/F	A		C	C	T	T	G/F	F/T						
	62	X/Y	T	A/T	A	G	C/F	A	C	F/C	C	T	A/T		A	F/C	C	C	T	T	G/F		F/T					
	84	X/Y	T	A/T	A	G	C/F	A	C	F/C	C	T/F	A/T			T/F	C	C	T	T	G/F	F/T						
	147	X/Y	T	A/T	A	G	C/F	A	C	C	C	T/F	A/T		A	T/C	C	C	T	T	G/F	F/T						
	243	F/Y	T	A/F	A/F	G/F						T/F					C/F											
Semen	0	X/Y	T	A/T	A	G	C/G	A	C	C	C	T/A	A/T	C/F	A	T/C	C	C	T	T	G	T	A/T	T/A	F/T	T	A	F/A
	42	X/Y	T	A/T	A	G	C/G	A	C	C	C	T/A	A/T	C/F	A	T/C	C	C	T	T	G	T	A/T	T/A	F/T	T	A	F/A
	62	X/Y	T	A/T	A	G	C/G	A	C	C	C	T/A	A/T	C/F	A	T/C	C	C	T	T	G	T	A/T	T/A	F/T		A	F/A
	84	X/Y	T	A/T	A	G	C/G	A	C	C	C	T/A	A/T	C/F	A	T/C	C	C	T	T	G	T	A/T	T/A	F/T		A	F/A
	147	X/Y	T	A/T	A	G	C/G	A	C	C	C	T/A	A/T	C	A	T/C	C	C	T	T	G	T	A/T	T/A	F/T		A	F/A
	243	X/Y	T	A/T	A	G	C/G	A	C	C	C	T/A	A/T	C/F	A	T/C	C	C	T	T	G/F	F/T	F/T		F/T		F/A	F/A

Table 3.8 SNP 27-plex profiles obtained for each sample type at each stage of the degradation experiment. The black line at H8 divides the plex into LMW (<186 bases) and HMW SNPs to illustrate the increased locus dropout at longer fragment lengths.

SGM+ profiles	Size range of STR loci (bp)	102-136		111-140		124-170		155-207		163-202		185-240		212-353		229-270		262-346		291-345	
	Days in humidifier	D19		D3		D8		VWA		THO1		D21		FGA		D16		D18		D2	
Saliva	0	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	F
	42	12	14	15	17	11	15	16	17	6	F										
	62	12	14	15	17	11	15	16	17	6	F										
	84	12	14	15	17	11	15	16	17	6	F										
	147			15	F	11	F														
	243																				
Semen	0	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	42	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	62	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	84	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	147	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	243	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	F
Blood	0	13	15	16	18	12	13	16	17	6	6	28	32.2	20	21	11	12	12	13	25	25
	42	13	15	16	18	12	13	16	17	6	6	28	32.2	20	21	11	12	12	F	25	F
	62	13	15	16	18	12	13	16	17	6	6	28	32.2	20	21	11	12	12	13	25	25
	84	13	15	16	18	12	13	16	17	6	6	28	32.2	20	21	11	12			25	F
	147	13	15	16	18	12	13	16	17	6	6					11	F				
	243	13	F	F	18																

Table 3.9 SGM+ profiles obtained for the artificially degraded samples for saliva, blood and semen. Grey boxes indicate alleles that have failed to amplify. Homozygous loci are designated with one allele or two dependent on the size of the peak present, i.e. the peak height must be >150rfu to be called as a true homozygote. Homozygous peaks falling below this have the second allele designated with an 'F'.

The different sample types showed varying levels of allele dropout, suggesting a variable degradation rate dependent on the sample. Saliva showed the highest level of degradation, with semen showing very little dropout, even after prolonged periods in the incubator.

The amount of allele dropout and the percentage profile data was calculated for each sample, for both SGM+ and the SNP 27-plex, as well as likelihood ratios for both full profiles and LMW profiles (Table 3.10).

days in humidifier (BLOOD)	BLOOD - SNP 27-plex			BLOOD - LMW SNPs: <186 BASES			BLOOD - SGM+			BLOOD - SGM+ <186 BASES		
	No. of alleles	% profile	LR	No. of alleles	% profile	LR	No. of alleles	% profile	LR	No. of alleles	% profile	LR
0	48	89	23,200,000	36	100	68,300	22	100	165,000,000,000	10	100	113,000
42	49	91	27,800,000	35	97	25,200	20	91	20,500,000,000	10	100	113,000
62	47	87	16,700,000	34	94	51,300	22	100	165,000,000,000	10	100	113,000
84	45	83	6,620,000	34	94	51,300	19	86	20,500,000,000	10	100	113,000
147	44	81	6,190,000	34	94	25,200	13	59	17,600,000	10	100	113,000
243	30	56	13,300	27	75	5,510	4	18	7	2	20	5
days in humidifier (SALIVA)	SALIVA - SNP 27-plex			SALIVA - LMW SNPs: <186 BASES			SALIVA - SGM+			SALIVA - SGM+ <186 BASES		
	No. of alleles	% profile	LR	No. of alleles	% profile	LR	No. of alleles	% profile	LR	No. of alleles	% profile	LR
0	39	72	65,200,000	31	86	877,000	21	95	9,010,000,000,000	10	100	367,000
42	35	65	899,000	31	86	196,000	11	50	67,600	9	90	67,600
62	35	65	1,980,000	31	86	823,000	12	55	125,000	9	90	67,600
84	32	59	40,200	28	78	8,770	11	50	67,600	9	90	67,600
147	37	69	7,110,000	33	92	1,550,000	4	18	10	2	20	10
243	8	15	5	8	22	5	2	9	10	-	-	1
days in humidifier (SEMEN)	SEMEN -SNP 27-plex			SEMEN - LMW SNPs: <186 BASES			SEMEN - SGM+			SEMEN - SGM+ <186 BASES		
	No. of alleles	% profile	LR	No. of alleles	% profile	LR	No. of alleles	% profile	LR	No. of alleles	% profile	LR
0	51	94	1,490,000,000	36	100	1,570,000	22	100	9,010,000,000,000	10	100	367,000
42	51	94	826,000,000	34	94	962,000	22	100	9,010,000,000,000	10	100	367,000
62	49	91	97,200,000	35	97	964,000	22	100	9,010,000,000,000	10	100	367,000
84	48	89	27,700,000	35	97	964,000	22	100	9,010,000,000,000	10	100	367,000
147	47	87	20,100,000	36	100	1,570,000	22	100	9,010,000,000,000	10	100	367,000
243	43	80	2,480,000	35	97	128,000	21	95	9,010,000,000,000	10	100	367,000

Table 3.10 Percentage profiles and likelihood ratios (LRs) calculated for artificially degraded samples. The table includes results for full SNP 27-plex profiles (all 26 loci plus Amelogenin), 27-plex SNPs under 186 bases in length, full SGM+ profiles and STRs under 146 bases in length.

3.3.2.1 Semen samples

The semen samples showed very little degradation in these experiments and a full SGM+ profile was obtained at all time points, except for one case of STR allele dropout at 243 days (Table 3.9). The SNP 27-plex did not perform as well as SGM+.

Within a sperm cell, DNA is associated with protamines and exists in a highly condensed state, allowing it to be packaged into a very small volume (Ward and Coffey 1991). This packaging makes it less susceptible to nuclear attack by enzymes or bacteria, an evolutionary requirement originating from the function of the mammalian sperm. In these instances, SGM+ can be used to obtain a full profile with a higher discrimination power than that gained when using SNPs.

3.3.2.2 Blood samples

The SNP 27-plex gave higher percentage profiles for degraded blood samples compared to SGM+, using both the full 27-plex results and LMW SNP results.

SGM+ dropped from a 100% profile down to only 18%, whereas SNPs still gave a 56% profile after the 8 month degradation period (Table 3.10).

The STR profiles showed a distinct degradation pattern for SGM+, with the high molecular weight loci dropping out of the profile first (Table 3.9). The SNP profile showed less dropout, although the peak heights for each allele decreased significantly with time (data not shown). The presence of 'split' peaks in the SNP profile after 8 months degradation was indicative of very low amounts of DNA starting material, i.e. LCN DNA. Split peaks consisted of two peaks with only one base pair difference in size, giving the impression of a 'split' peak. This action occurs due to the activity of the Taq polymerase enzyme used during PCR, preferentially adding an extra base to the PCR product. The presence of split peaks indicated two populations of PCR products whereby some were the expected size (*n*) and some had an extra base added (*n+1*). This should have been eliminated by the 45 minute final extension of the PCR amplification, however the phenomenon of split peaks in LCN DNA samples had been observed during development of the SNP multiplex methodology (pers. comm. P. Gill).

3.3.2.3 Saliva samples

Saliva is well known to degrade rapidly once outside of the body, both under room temperature conditions and within a humid environment. This is probably due to the presence of large numbers of bacteria and digestive enzymes that break down the proteins and cellular matter found in saliva (Benedek-Spät 1973a; Benedek-Spät 1973b). The reference sample failed to give a full profile with either SGM+ or the SNP 27-plex, suggesting that the DNA may have already been fragmented before the experiment began.

The ability to obtain a profile from an individual's saliva is dependent upon factors such as food intake, smoking preferences, alcohol consumption and caffeine intake. The amount of enzymes and bacteria present in saliva make the DNA highly susceptible to degradation and this is variable within and between individuals due to their lifestyles. For these reasons, some saliva samples may give full profiles using SNPs or SGM+ whereas others will always give sub-optimal results.

3.3.2.4 *Analysis of allelic dropout fragment length*

Table 3.8 & Table 3.9 illustrate the pattern of allele dropout seen for the artificially degraded samples for SGM+ and the SNP 27-plex. The LMW SNPs were described as those being less than 186 bases in length, highlighted by the black line dividing H8 (fragment length 186 bases) and L6 (fragment length 190 bases). The size of the amplicon was compared to the proportion of allelic dropout seen using both techniques (Figure 3.2).

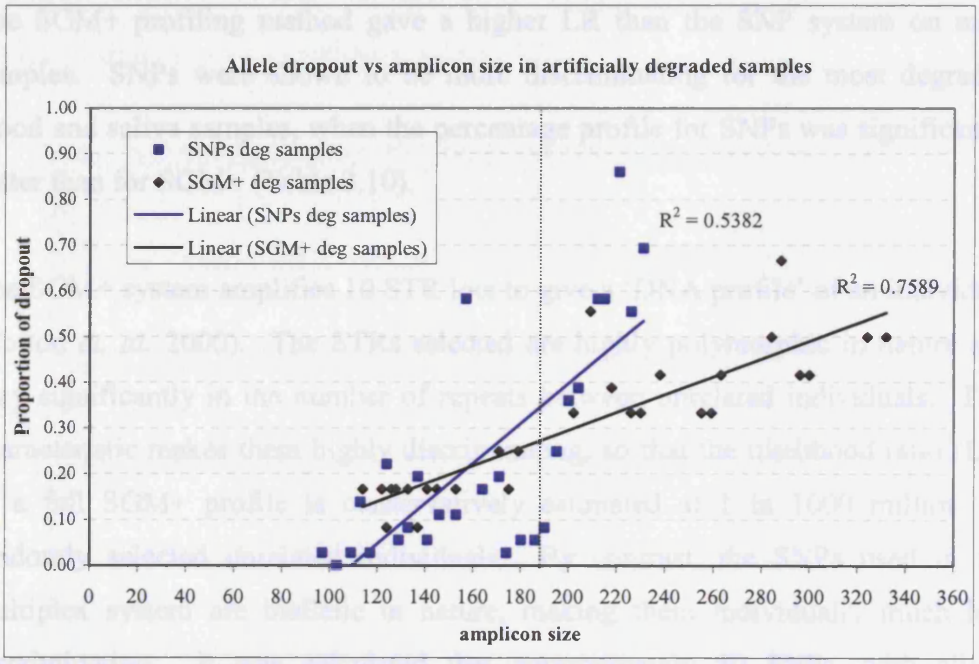


Figure 3.2 Scattergraph indicating the number of alleles that fail to amplify using both SGM+ and the SNP 27-plex compared to the size of the allele fragment. This data is based on the allelic dropout seen in tables 3.8 & 3.9 for all three degraded sample types. The dotted line indicates the division between LMW SNPs and HMW SNPs.

A positive regression was observed between the number of alleles failing to amplify and the size of the amplicon length (Figure 3.2). This suggested the nucleosome may be protecting the DNA molecule from further degradation, leaving only small fragments of DNA available for amplification, although it is possible this linear pattern would be observed even if there was random fragmentation of the DNA molecules. Bacterial and enzymatic activity brought about by the 37°C temperature and optimal humidity would have sheared the DNA to only 146 base pairs in length. The addition of universal primers to the ends of each amplicon increased the locus size to 186 bases and anything above this showed decreased amplification efficiency. The observations seen here were used in the development of a 21-SNP multiplex and have been used as an illustration of degradation patterns in low molecular weight DNA.

3.3.3 Comparison of likelihood ratios (LRs)

Likelihood ratios (LRs) were calculated for each DNA profiling technique for each sample using STRipe™ for SGM+ and Celestial™ for SNPs (Table 3.10). The SGM+ profiling method gave a higher LR than the SNP system on most samples. SNPs were shown to be more discriminating for the most degraded blood and saliva samples, when the percentage profile for SNPs was significantly better than for SGM+ (Table 3.10).

The SGM+ system amplifies 10 STR loci to give a 'DNA profile' of an individual (Cotton *et. al.* 2000). The STRs selected are highly polymorphic in nature and vary significantly in the number of repeats between unrelated individuals. This characteristic makes them highly discriminating, so that the likelihood ratio (LR) of a full SGM+ profile is conservatively estimated at 1 in 1000 million for randomly selected unrelated individuals. By contrast, the SNPs used in the multiplex system are biallelic in nature, making them individually much less discriminating. It was calculated that approximately 50 SNPs, with allelic frequencies between 0.2 and 0.8, would be needed to give a comparable LR of SGM+ (Gill 2001a). Using a population database of white Caucasian individuals (200 unrelated individuals), an LR of approximately 1 in 500 million was calculated for the SNP 27-plex. The LRs for both systems were highly variable depending on the alleles present in the profiles obtained.

3.3.4 Dilution series experiments (21-SNP multiplex)

Initial studies suggested the SNP multiplex system could be improved by the use of SNPs less likely to fail amplification. This could be done by designing a new multiplex using smaller amplicon sizes, altering the sizes of some of the primers in the current multiplex or by the addition of different loci. Further work was carried out developing a SNP multiplex using these criteria. The 21-SNP multiplex was designed with products less than 186 base pairs in length, to allow amplification of the small DNA fragments found in degraded samples.

The data generated from the dilution series experiments, using the 21-SNP multiplex, were used to estimate the limit of detection of the system. Electropherograms allowed profiles to be visualised before the data was run through further analysis programs (Figure 3.3).

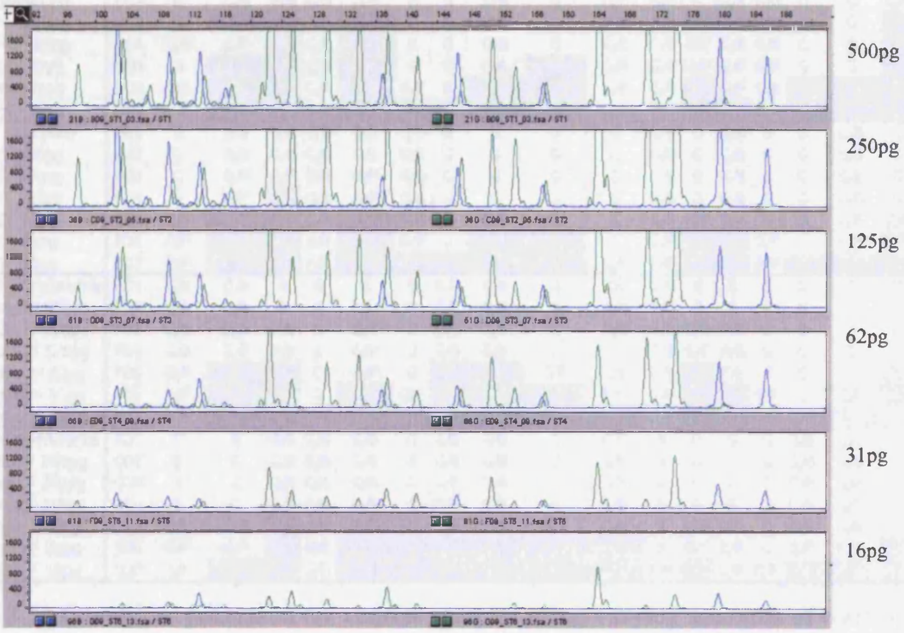
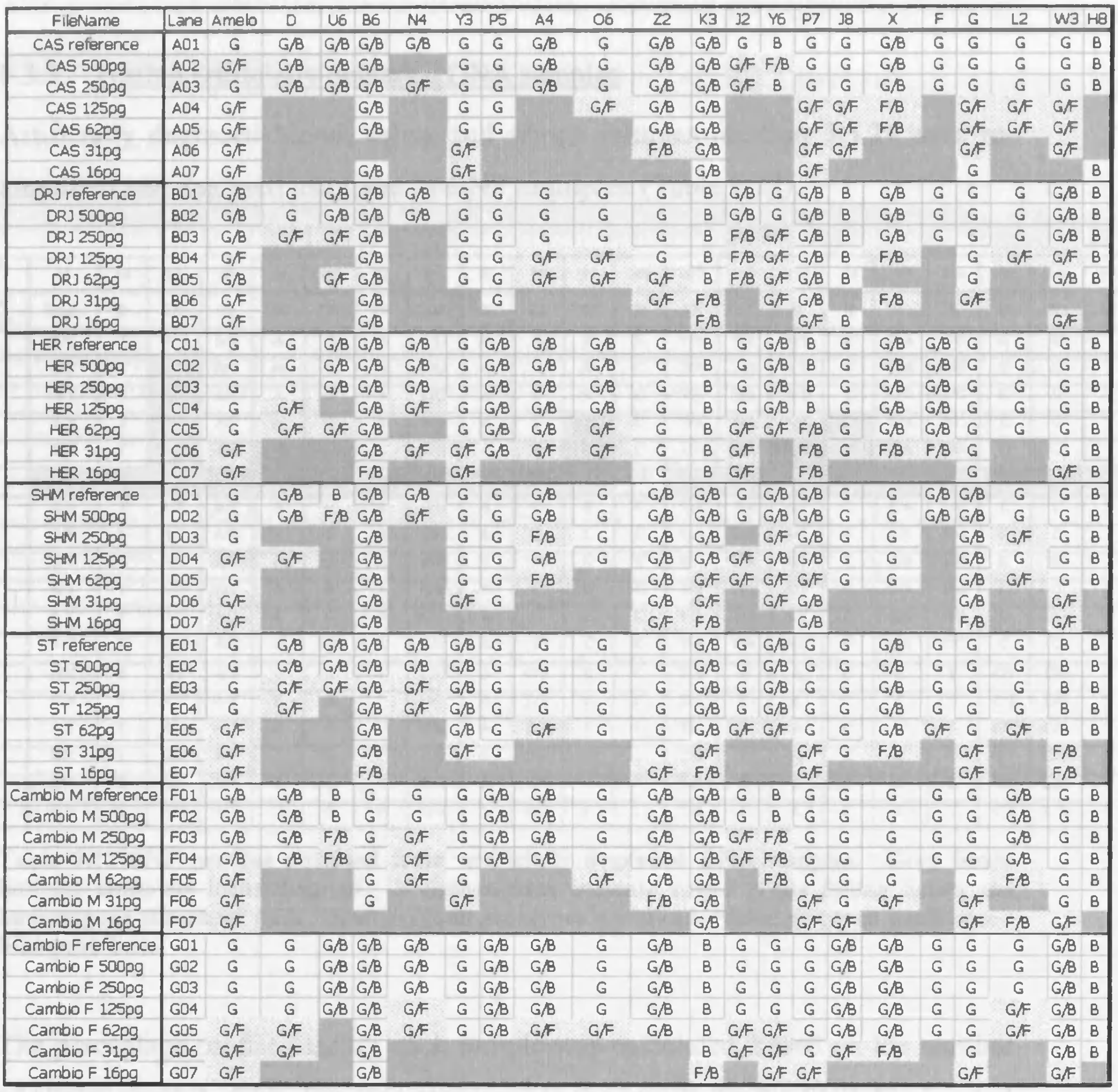


Figure 3.3 Electropherograms showing SNP profiles obtained for the dilution series of ST control DNA.

Full profiles were observed for all samples using 1 ng of DNA template and six out of seven samples gave full profiles at 500 pg DNA template (Table 3.11), although some homozygote peaks gave a peak height (rfu) less than the homozygote threshold level set in Celestial™ and were subsequently labelled with an 'F' designation (chapter 4). The control samples HER and ST showed full profiles down to 250 pg starting DNA template and Cambio™ male and female samples were correctly genotyped using 125 pg DNA template. All seven samples gave partial DNA profiles down to the lowest template level of 16 pg.



FileName	Lane	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
CAS reference	A01	G	G/B	G/B	G/B	G/B	G	G	G/B	G	G/B	G/B	G	B	G	G	G/B	G	G	G	G	B
CAS 500pg	A02	G/F	G/B	G/B	G/B		G	G	G/B	G	G/B	G/B	G/F	F/B	G	G	G/B	G	G	G	G	B
CAS 250pg	A03	G	G/B	G/B	G/B	G/F	G	G	G/B	G	G/B	G/B	G/F	B	G	G	G/B	G	G	G	G	B
CAS 125pg	A04	G/F			G/B		G	G		G/F	G/B	G/B			G/F	G/F	F/B		G/F	G/F	G/F	
CAS 62pg	A05	G/F			G/B		G	G			G/B	G/B			G/F	G/F	F/B		G/F	G/F	G/F	
CAS 31pg	A06	G/F					G/F				F/B	G/B			G/F	G/F			G/F		G/F	
CAS 16pg	A07	G/F			G/B		G/F					G/B			G/F				G			B
DRJ reference	B01	G/B	G	G/B	G/B	G/B	G	G	G	G	G	B	G/B	G	G/B	B	G/B	G	G	G	G/B	B
DRJ 500pg	B02	G/B	G	G/B	G/B	G/B	G	G	G	G	G	B	G/B	G	G/B	B	G/B	G	G	G	G/B	B
DRJ 250pg	B03	G/B	G/F	G/F	G/B		G	G	G	G	G	B	F/B	G/F	G/B	B	G/B	G	G	G	G/B	B
DRJ 125pg	B04	G/F			G/B		G	G	G/F	G/F	G	B	F/B	G/F	G/B	B	F/B		G	G/F	G/F	B
DRJ 62pg	B05	G/B		G/F	G/B		G	G	G/F	G/F	G/F	B	F/B	G/F	G/B	B			G		G/B	B
DRJ 31pg	B06	G/F			G/B			G			G/F	F/B		G/F	G/B		F/B		G/F			
DRJ 16pg	B07	G/F			G/B							F/B			G/F	B					G/F	
HER reference	C01	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 500pg	C02	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 250pg	C03	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 125pg	C04	G	G/F		G/B	G/F	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 62pg	C05	G	G/F	G/F	G/B		G	G/B	G/B	G/F	G	B	G/F	G/F	F/B	G	G/B	G/B	G	G	G	B
HER 31pg	C06	G/F			G/B	G/F	G/F	G/B	G/F	G/F	G	B	G/F		F/B	G	F/B	F/B	G		G	B
HER 16pg	C07	G/F			F/B		G/F				G	B	G/F		F/B				G		G/F	B
SHM reference	D01	G	G/B	B	G/B	G/B	G	G	G/B	G	G/B	G/B	G	G/B	G/B	G	G	G/B	G/B	G	G	B
SHM 500pg	D02	G	G/B	F/B	G/B	G/F	G	G	G/B	G	G/B	G/B	G	G/B	G/B	G	G	G/B	G/B	G	G	B
SHM 250pg	D03	G			G/B		G	G	F/B	G	G/B	G/B		G/F	G/B	G	G		G/B	G/F	G	B
SHM 125pg	D04	G/F	G/F		G/B		G	G	G/B	G	G/B	G/B	G/F	G/B	G/B	G	G		G/B	G	G	B
SHM 62pg	D05	G			G/B		G	G	F/B		G/B	G/F	G/F	G/F	G/F	G	G		G/B	G/F	G	B
SHM 31pg	D06	G/F			G/B		G/F	G			G/B	G/F		G/F	G/B				G/B		G/F	
SHM 16pg	D07	G/F			G/B						G/F	F/B			G/B				F/B		G/F	
ST reference	E01	G	G/B	G/B	G/B	G/B	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 500pg	E02	G	G/B	G/B	G/B	G/B	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 250pg	E03	G	G/F	G/F	G/B	G/F	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 125pg	E04	G	G/F		G/B	G/F	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 62pg	E05	G/F			G/B		G/B	G	G/F	G	G	G/B	G/F	G/F	G	G	G/B	G/F	G	G/F	B	B
ST 31pg	E06	G/F			G/B		G/F	G			G	G/F			G/F	G	F/B		G/F		F/B	
ST 16pg	E07	G/F			F/B						G/F	F/B			G/F				G/F		F/B	
Cambio M reference	F01	G/B	G/B	B	G	G	G	G/B	G/B	G	G/B	G/B	G	B	G	G	G	G	G	G/B	G	B
Cambio M 500pg	F02	G/B	G/B	B	G	G	G	G/B	G/B	G	G/B	G/B	G	B	G	G	G	G	G	G/B	G	B
Cambio M 250pg	F03	G/B	G/B	F/B	G	G/F	G	G/B	G/B	G	G/B	G/B	G/F	F/B	G	G	G	G	G	G/B	G	B
Cambio M 125pg	F04	G/B	G/B	F/B	G	G/F	G	G/B	G/B	G	G/B	G/B	G/F	F/B	G	G	G	G	G	G/B	G	B
Cambio M 62pg	F05	G/F			G	G/F	G			G/F	G/B	G/B		F/B	G	G	G		G	F/B	G	B
Cambio M 31pg	F06	G/F			G		G/F				F/B	G/B			G/F	G	G/F		G/F		G	B
Cambio M 16pg	F07	G/F										G/B			G/F	G/F			G/F	F/B	G/F	
Cambio F reference	G01	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 500pg	G02	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 250pg	G03	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 125pg	G04	G	G	G/B	G/B	G/F	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G/F	G/B	B
Cambio F 62pg	G05	G/F	G/F		G/B	G/F	G	G/B	G/F	G/F	G/B	B	G/F	G/F	G	G/B	G/B	G	G	G/F	G/B	B
Cambio F 31pg	G06	G/F	G/F		G/B							B	G/F	G/F	G	G/F	F/B		G		G/B	B
Cambio F 16pg	G07	G/F			G/B							F/B		G/F	G/F				G/F		G/F	

Table 3.11 Genotypes generated for control samples using varying amounts of starting DNA template. The limit of detection appears to be sample dependent. SHM DNA could be fully genotyped down to 62.5pg DNA whereas Cambio™ Female control DNA showed dropout at 250pg.

A total of seven individual DNA samples at seven different DNA starting concentrations (n=49) were tested using the 21-SNP multiplex. From these results it was demonstrated that all samples provided a full, and correct, SNP profile at an optimal DNA amount of 1 ng and partial DNA profiles were obtained at a template level between 500 pg and 16 pg, lower levels were not tested. SGM+

amplification routinely gives a full profile above 100 pg starting DNA material (Gill *et. al.* 1997; Cotton *et. al.* 2000; Gill 2002) and LCN SGM+ is used to provide full or partial profiles at sub-optimal DNA concentrations <100 pg using LCN amplification conditions (Gill *et. al.* 2000b).

3.3.5 Testing artificially degraded DNA samples

Artificially degraded blood, saliva and semen samples (section 3.3.2) were re-amplified and analysed using the 21-SNP multiplex (Table 3.12).

Size of genome target (bp)		57	63	67	69	74	77	82	85	90	94	97	101	107	110	114	118	125	132	135	140	146
	Days in humidifier	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
Saliva	0	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	42	X/Y	T	F/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	62	X/Y	T	F/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	84	X/Y	T		A/T	A	G	A	C/G	A	C	F/C	C	T/A	T/A	A/T	C/F	C/A	T/C	C	C	T
	147	X/Y	T		A/T	A/F	G/F	A	C/G	A	C	F/C	C	T/A	T/A	F/T	C/F	C/A	T/C	C	C	T
	243	F/Y	T/F					F/A		A/F			C/F									
Semen	0	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	42	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	62	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	84	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	147	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	243	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
Blood	0	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	42	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	62	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	84	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	147	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	243	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T

Table 3.12 SNP profiles obtained from artificially degraded DNA samples. Grey boxes indicate complete locus dropout. F designations indicate single peaks falling below the homozygous threshold (*Ht*). Heterozygous genotypes are standardised as green peak base / blue peak base.

The percentage profile data for each sample was calculated, based on the number of alleles present. This data was compared to the results for the SNP 27-plex and the SGM+ results (Table 3.13).

The 21-SNP multiplex gave comparable results to SGM+ for the semen samples, showing a full profile at all time points. This indicated the multimix may be more optimised than the SNP 27-plex, which showed allele dropout at all time points, including the reference sample. The likelihood ratio (LR) for the semen samples was calculated to be 9 x 10^{-12} for SGM+ (i.e. it would be >9,000 million times more likely that this profile belonged to the suspect as opposed to another unrelated individual from the population), compared to 1.5 x 10^{-7} for the 21-SNP multiplex. In these instances it was suggested that SGM+ should be used in preference to SNPs, due to the higher match probabilities that can be gained. The LR values for SNP profiles varied when the percentage profile remained the same, due to different SNP loci being present in the resulting genotype, for example, SNP 27-plex data for saliva 42 days and 62 days (Table 3.13).

	Days in humidifier	SNP 27-plex			SGM+			21-SNP multiplex		
		No. of alleles	% profile	LR	No. of alleles	% profile	LR	No. of alleles	% profile	LR
Saliva	0	39	72	6.52E+07	21	95	9.01E+12	42	100	1.48E+07
	42	35	65	8.99E+05	11	50	6.76E+04	42	100	1.48E+07
	62	35	65	1.98E+06	12	55	1.25E+05	39	93	2.40E+06
	84	32	59	4.02E+04	11	50	6.76E+04	38	90	1.04E+06
	147	37	69	7.11E+06	4	18	1.01E+01	36	85	4.62E+05
	243	8	15	5.30	2	9	10	6	10	7.50
Semen	0	51	94	1.49E+09	22	100	9.01E+12	40	100	1.48E+07
	42	51	94	8.26E+08	22	100	9.01E+12	40	100	1.48E+07
	62	49	91	9.72E+07	22	100	9.01E+12	40	100	1.48E+07
	84	48	89	2.77E+07	22	100	9.01E+12	40	100	1.48E+07
	147	47	87	2.01E+07	22	100	9.01E+12	40	100	1.48E+07
	243	43	80	2.48E+06	21	95	4.60E+11	40	100	1.48E+07
Blood	0	48	89	2.32E+07	22	100	1.65E+11	42	100	1.37E+06
	42	49	91	2.78E+07	20	91	3.22E+09	42	100	1.37E+06
	62	47	87	1.67E+07	22	100	1.65E+11	42	100	1.37E+06
	84	45	83	6.62E+06	19	86	1.09E+09	42	100	1.37E+06
	147	44	81	6.19E+06	13	59	1.76E+07	42	100	1.37E+06
	243	30	56	1.33E+04	4	18	7.19	42	100	1.37E+06

Table 3.13 Percentage profile data and LR data for the SNP 27-plex, SGM+ 28 cycles and the 21-SNP multiplex. Data for the SNP 27-plex and SGM+ were obtained from earlier experiments on the artificially degraded samples (section 3.3.2).

The results for the artificially degraded saliva and blood samples were plotted on scattergraphs (Figure 3.4). The 21-SNP multiplex performed better than both the SNP 27-plex and SGM+ on these sample types. The artificially degraded blood samples gave a full profile at all time points, whereas SGM+ dropped to only an 18% profile after 243 days and the SNP 27-plex gave a 56% profile. This suggested that the smaller amplicon sizes used with the 21-SNP multiplex were allowing amplification of small fragments of DNA left in the blood samples after the longer degradation periods. Loci failing to amplify with SGM+ tended to be the higher molecular weight STRs (>125bp).

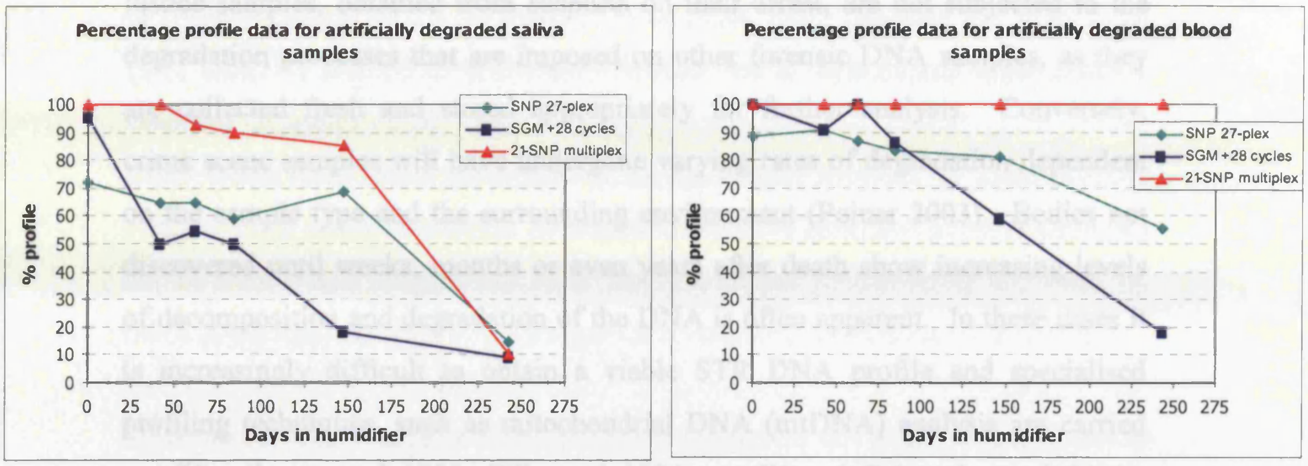


Figure 3.4 Scattergraphs of percentage profile data for artificially degraded saliva and blood samples. Graphsshow data obtained for the SNP 27-plex, SGM+ (28 cycles) and the 21-SNP multiplex.

All three profiling systems failed to successfully amplify the degraded saliva samples at the final time point of 243 days, with the maximum percentage profile gained for the SNP 27-plex (15%). The DNA in these samples would be subjected to more enzymatic activity than other sample types due to the increased number of enzymes present in saliva. The 21-SNP multiplex demonstrated better amplification of the saliva DNA at earlier time points, suggesting successful amplification of smaller DNA fragments still present in the samples.

3.4 Discussion

Forensic DNA samples are subject to varying rates of degradation. Criminal justice samples, obtained from suspects on their arrest, are not subjected to the degradation processes that are imposed on other forensic DNA samples, as they are collected fresh and stored appropriately for further analysis. Conversely, crime scene samples will have undergone varying rates of degradation dependent on the sample type and the surrounding environment (Poinar 2003). Bodies not discovered until weeks, months or even years after death show increasing levels of decomposition and degradation of the DNA is often apparent. In these cases it is increasingly difficult to obtain a viable STR DNA profile and specialised profiling techniques, such as mitochondrial DNA (mtDNA) analysis are carried out (Hagelberg *et. al.* 1991; Gill *et. al.* 1994; von Wurmb-Schwark *et. al.* 2003). Mitochondrial DNA persists for a longer time period in post-mortem samples as there is a greater number of mitochondria per cell, increasing the time it takes for degradation to occur (Butler and Levin 1998). Sequence analysis of mtDNA hypervariable regions may be undertaken, providing a profiling result where other methods fail (Hagelberg *et. al.* 1991; Holland *et. al.* 1993; Gill *et. al.* 1994; Holland and Parsons 1999; Budowle *et. al.* 2004a). Although this method partially overcomes the problem of identification, mtDNA is maternally inherited and its sequence is identical throughout the maternal lineage, meaning mothers and siblings and further progeny all share the same sequence. This factor reduces the discrimination power of mtDNA and decreases its usefulness as a tool for identification purposes (Butler and Levin 1998). Body fluid stains from crime scenes are also subject to DNA degradation, although they tend to have a lower rate of degradation as these sample types quickly dehydrate and restrict the oxidative DNA degradation process (Lindahl 1993).

The effect of DNA degradation in forensic DNA profiling is most apparent in mass disaster situations. Dependent on the type of disaster, victims will have been subjected to a wide range of extreme conditions including ultra-high temperatures and high levels of humidity, causing degradation of the DNA molecules. This makes it increasingly difficult to obtain a DNA profile that can be used for identification purposes. Examples of mass disaster situations include fires (Clayton *et. al.* 1995a), air crashes (Ballantyne 1997; Olaisen *et. al.* 1997; Leclair *et. al.* 2004), terrorist attacks (OrchidBiosciences 2002; Cash *et. al.* 2003; Holland *et. al.* 2003), tsunamis and earthquakes (Alonso *et. al.* 2005; Morgan and de Ville de Goyet 2005). In some of these cases, DNA profiling has proved the most effective method of body identification due to lack of any other forensic evidence (Clayton *et. al.* 1995a; OrchidBiosciences 2002; Leclair *et. al.* 2004). Other cases have relied more heavily on traditional methods of identification; victims of the Asian tsunami in December 2004 were identified predominantly by dental records and fingerprints with only 1% of the bodies being identified by DNA profiling (http://www.newscientist.com/channel/opinion/mg18725163.900).

DNA degradation begins with apoptosis (programmed cell death) and necrosis in post-mortem samples (Johnson and Ferris 2002). After this activity by enzymes such as micrococcal nuclease, DNase I and DNase II causes DNA fragmentation through single-stranded breaks and depurination of bases (Bär *et. al.* 1988; Lindahl 1993; Willerslev and Cooper 2005). As a consequence of this, current methods of DNA profiling using STR analysis may only provide a partial DNA profile resulting in a lower discriminating power than would otherwise be obtained (Whitaker *et. al.* 1995; Wiegand and Kleiber 2001; Tsukada *et. al.* 2002; Chung *et. al.* 2004). By defining the process of DNA degradation, new DNA profiling methods could be developed that take factors such as fragmentation into account, increasing the chance of amplifying the limited amount of DNA available.

In order to assess the ability of DNA profiling methods to amplify degraded DNA in the laboratory, artificially degraded DNA is required. By artificially degrading DNA using a robust method, sets of samples can be produced that are both quantitative and reproducible. A number of different methods have been used to artificially degrade DNA. Enzymes such as DNase I & II are routinely used to provide DNA in solution with varying fragment sizes (Wilcox and Smith 1976; Szopa and Rose 1986; Golenberg *et. al.* 1996; Wu *et. al.* 2000). DNase I favours purine-pyrimidine sequences (Staynov 2000) and DNase II is an enzyme found in lysozymes associated with cell apoptosis (Yasuda *et. al.* 1998). Although providing a range of DNA fragments in solution, this method of degrading DNA is not consistent with the degradation occurring *in situ* and so cannot be used as a direct comparison of the DNA found in such conditions. This is also true for boiled DNA samples, used in this study as a method of fragmenting DNA irrespective of the protection of the nucleosome. Some studies have used actual post-mortem samples in their work, such as animal tissue taken from a newly slaughtered animal (Johnson and Ferris 2002), ancient DNA samples from a variety of mummified or fossilised samples (Pääbo 1989) or post-mortem human samples (Akane *et. al.* 1993). Forensically directed research studies have used body fluid stains kept at room temperature for a number of years (Tsukada *et. al.* 2002; Butler *et. al.* 2003). Although viable for a small number of the forensic samples submitted for forensic DNA profiling, these stains are limited in the amount of degradation present as DNA has been shown to persist for many years in a dessicated form (Lindahl 1993; Willerslev and Cooper 2005). By introducing stains to a humid environment, the hydrolytic and oxidative processes of degradation are permitted to continue for prolonged periods of time (Lindahl 1993). For this study, artificially degraded samples were obtained from previous project work. Blood, semen and saliva samples had been spotted onto cotton squares and left in a humid environment at 37°C for a period of eight months.

Boiling DNA extracts allowed the DNA strands to be rapidly fragmented for use in this study. The DNA was extracted from cells prior to boiling, leaving it without nucleosome protection. The results showed the DNA became fragmented with increased periods of boiling, leaving the lower molecular weight amplicons relatively more amenable to amplification. The artificially degraded DNA samples showed a similar pattern of DNA fragmentation, with low molecular weight loci giving increased amplification efficiency after longer periods of degradation. Both methods are suggestive of DNA fragmenting with increased periods of degradation processes. The unprotected DNA in boiled samples would have undergone random shearing of the DNA molecule, leaving smaller and smaller lengths of DNA with time. Longer periods of boiling may have produced DNA fragments too small to be amplified with even the lowest molecular weight loci. The DNA in the artificially degraded samples may have been gradually fragmented as linker DNA joining the nucleosomes to each other was cleaved, leaving only the DNA fragments protected by each nucleosome. It is possible that after a longer period of boiling DNA amplification would not have been successful whereas the nucleosome would have continued to protect the DNA associated with it, causing the amplification efficiency to reach a plateau.

The work carried out in this study suggested that SNPs could have a number of advantages over STRs for degraded samples. Most sample types gave an increased percentage profile with SNPs and, although the discrimination power was lower than for STRs, this may be used as an adjunct to the SGM+ system. The SNP multiplex system was envisaged as an identification tool predominantly for use in circumstances where most DNA matter would be highly degraded, for example in mass disasters. In these circumstances SGM+ profiling could fail whereas SNPs may give a profile, even if only partial. Reference profiles of victims could be obtained from personal effects, rendering the discrimination power less relevant in these instances. The combination of a weak / partial SGM+ profile and a weak / partial SNP profile would also increase the discrimination power over a weak SGM+ profile alone.

Profiling of artificially degraded samples appeared to show a relationship between the size of the amplicon and the allele dropout seen. An increased fragment length above 186 bases was associated with a lower likelihood of amplification in samples that were artificially degraded. Protection of DNA by the nucleosome may be responsible for the small fragment sizes seen although other factors, such as the coiling of the DNA around the chromosome or the effects of secondary and tertiary protein structure, may have be involved. Five out of the ten STR amplicons used in SGM+ DNA profiling had lengths above 146 bases and these readily dropped out of the DNA profile in degraded samples.

The dilution series experiments indicated the 21-SNP multiplex was capable of amplifying DNA in low copy number conditions. A full SNP profile was obtained from sub-optimal (<500 pg) DNA template levels, and partial SNP profiles were seen at levels as low as 15 pg (the equivalent of approximately two cells). At low template levels, SNP interpretation would be subject to the same problems as STR profiling, i.e. there would be more chance of contamination being seen, stochastic variation could give heterozygous imbalance, allele dropout and preferentially amplification of loci. Interpretation criteria needed to be set for the 21-SNP multiplex, as outlined in chapter 4, using the dilution series data as a basis for low copy number genotypes.

The 21-SNP multiplex was designed to target smaller DNA fragments thought to be present when DNA becomes fragmented. The primary structure of nucleosomes confers protection onto 146 base pairs of the DNA strand, with the linker DNA joining two nucleosomes together being targeted for fragmentation first (Read *et. al.* 1985a; Read and Crane-Robinson 1985b). The results of the degradation experiments suggested that the 21-SNP multiplex was capable of amplification when traditional DNA profiling methods failed. Low copy number (LCN) SGM+ genotyping was not assessed as part of this study due to insufficient amounts of sample extract. In order to interpret LCN SGM+ profiles, two duplicate amplifications need to be performed and a consensus profile generated from the resulting genotypes (Gill 2001b). As part of the validation of the 21-SNP multiplex, casework samples profiled using LCN SGM+ were re-amplified and profiled for SNPs (chapter 7). In the artificially degraded samples analysed in this study, semen was shown to resist DNA degradation *in situ* probably as a result of the tight packaging of the DNA molecule within the sperm head. In these instances an increased LR could be gained by using SGM+ profiling, as STRs collectively give higher discrimination between non-related individuals within a population. Saliva and blood samples showed varying degrees of DNA degradation, with the 21-SNP multiplex more likely to successfully amplify after longer periods of degradation in both sample types.

4 The 21-SNP Multiplex Interpretation Criteria

4.1 Introduction

The increased use of DNA profiling in forensic casework led to a greater need for automated programs capable of accurately genotyping DNA samples that had been processed using STR DNA profiling systems (Perlin *et. al.* 1994; Perlin 2001). The SGM+ STR profiling system currently used in forensic casework in the UK is an optimised system that is both quantitative and well balanced within and between loci, allowing algorithms to be developed that can be used to automatically genotype profiles (Cotton *et. al.* 2000; Perlin 2001). Several STR automated genotyping systems for forensic use currently exist, and are used for different aspects of DNA profiling.

STRess™ (STR expert system suite), developed by The Forensic Science Service® in 1998 (Werrett *et. al.* 1998), was a program that *"accepts raw data [from Genotyper™ software], generates a file of allele designations and then compares this file to one generated by a human operator"*. At that time, DNA profiles analysed for the National DNA Database® were independently analysed by two separate operators before being compared by a third individual, to eliminate the possibility of operator differences. This analysis stage caused a bottleneck to the entire DNA profiling process and therefore automation was necessary. The optimised SGM+ system is a quantitative system and this allowed rule sets to be developed that could assess STR peak height and area data and accurately genotype them. For example, alleles had to be above a defined peak height to distinguish them from baseline noise and peak area ratios were calculated to assess the presence of a mixture (Werrett *et. al.* 1998).

The STRess™ program was complemented with an additional automated genotyping system known as TrueAllele™ (Perlin 2001). TrueAllele™ allowed peak data to be analysed and assessed for stutters, artefact peaks and preferential amplification, before generating genotype data for inputted samples. Both of these programs were developed to enable high throughput analysis of DNA profiles used for the National DNA Database® and as a consequence of this, neither were capable of analysing the more complex DNA mixtures or low copy number (LCN) profiles that required expert interpretation.

A recently developed program, *LoComatioN*, is *"a hypothesis driven expert system that enables LRs [likelihood ratios] for any number of different LCN scenarios to be evaluated"* (Gill *et. al.* 2005c). It remains necessary to manually genotype the data, but interpretation of resulting profiles is carried out using a set of algorithms allowing factors such as contamination and allele dropout to be included in the likelihood ratio calculation.

For SNP genotyping it was necessary to produce a system similar to those developed for STR DNA profiling. SNP genotyping programs for technologies such as microarrays and primer extension assays have been developed (chapter 2) but no program existed to analyse the data format generated by the SNP multiplex technique developed using the universal reporter primer assay (Hussain *et. al.* 2003; Dixon *et. al.* 2005a). A computer program for analysis of SNP data was developed to quantitatively analyse the peak data present, allowing accurate genotyping of samples independent of operator variability.

4.2 Materials & Methods

4.2.1 Celestial™ automated analysis program

In order for the SNP multiplex to be successfully implemented into a casework unit, the technique was tested against a number of set validation criteria. An important part of the validation was the ability to confidently assign genotypes to the sample data collected. This was carried out effectively by the use of an in-house program Celestial™, written in Visual Basic, to enable genotyping of the SNP 27-plex. The purpose of the validation was to define the interpretation criteria required for correct genotyping of the 21-SNP multiplex.

The program utilised the following data exported from Genotyper™ for each SNP locus for each sample: scan number, peak height (relative fluorescent units (rfu)), peak area (rfu) and size in base pairs. Each SNP locus was identified by Celestial™ according to its size in base pairs. The peak data were used to genotype SNP loci as homozygous or heterozygous and a SNP profile was generated for each sample analysed.

In forensic casework it is essential to keep error rates to a minimum. For this reason, the rules set for Celestial were more conservative than would be found in a non-caseworking environment.

4.2.2 Interpretation criteria

4.2.2.1 Heterozygote balance (Hb%)

In order to correctly interpret results from each SNP locus, three separate criteria were characterised (Figure 4.1). Firstly, the relationship of peak heights of each allele within a locus was established. This was given the term 'heterozygous balance' or *Hb%* (section 4.3.2). *Hb%* was calculated from analysis of a dilution series of control DNA samples (chapter 3) using the equation:

$$Hb(\%) = \left(\frac{\phi S}{\phi L}\right) x100 \qquad \textit{(Equation 4-1)}$$

[ϕS=smallest peak height (rfu); ϕL=largest peak height (rfu)]

Hb% for each SNP were calculated independently of each other as each SNP locus behaved differently within the multimix.

4.2.2.2 Homozygous thresholds (Ht_{max})

The homozygote threshold (Ht_{max}) of a SNP locus was defined as the maximum peak height of either allele A or allele B (ϕA or ϕB) when only one allele is present in a known heterozygous sample, plus 20% to allow for unobserved extreme variation, i.e.

$$Ht^{\phi A=0} = \phi B_{\max} + 0.2(\phi B_{\max})$$

(Equation 4-2)

$$\text{or } Ht^{\phi B=0} = \phi A_{\max} + 0.2(\phi A_{\max})$$

This observation can be seen when low levels of DNA are present and stochastic variation causes only one allele to be amplified. If a homozygous allele from an experimental sample fell below Ht_{max}, the locus was given an 'F' designation indicating that allele dropout may have occurred and the locus might be heterozygous. Allele dropout is occasionally seen with the 21-SNP multiplex in samples with optimal DNA amounts (0.5-1 ng) due to the sensitivity of the system preferentially amplifying alleles at some loci more than others.

4.2.2.3 Baseline threshold (Bt)

Lastly, the negative baseline (*Bt*) was set, according to observed allele drop-in peaks in negative control samples, to minimise the chance of genotyping false positives (section 4.3.4). Allele drop-in is a consequence of contaminant alleles and occurs predominantly as a consequence of the high sensitivity of the amplification method. Allele drop-in peaks are usually low level and can be distinguished from true allele peaks by their size in comparison to the rest of the profile.

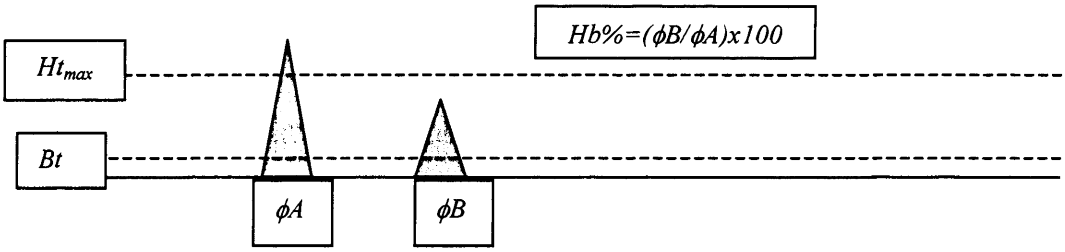


Figure 4.1 Diagrammatical representation of the interpretation criteria used in SNP analysis. *Ht_{max}* indicates the homozygous threshold, below which single peaks are given an 'F' designation to indicate possible allele dropout. *Bt* denotes the baseline threshold used to minimise the possibility of drop-in peaks being labelled as allelic peaks. *Hb%* characterises the heterozygous balance of the peak height of allele A (*φA*) compared to the peak height of allele B (*φB*). In this example *φA* is also the largest peak (*φL*) and *φB* is the smallest peak (*φS*).

4.2.3 Experimental procedures

To calculate interpretation rule sets, data were collected from the dilution series experiments carried out in chapter 3.

Samples were amplified with the 21-SNP multiplex using varying amounts of starting DNA template as follows; 0 pg, 16 pg, 32 pg, 62 pg, 125 pg, 250 pg, 500 pg, 1 ng, to give a range of data from optimal DNA starting template to LCN amounts (sub-125 pg).

Amplified products were then analysed using the AB 3100CE sequencer with two injection times of 12 and 20 seconds. Genotyper™ software was used to provide data in the necessary format - peak height and peak area data, allele size (bp) and genotype -for interpretation in Celestial™, in the form of *csv file.

4.3 Results

4.3.1 Celestial preliminary genotyping results

The preliminary rule sets for Celestial™ were relaxed to allow all data to be designated without ambiguity within Celestial™, i.e. *Hb%* was set to a base level threshold of 5% for all SNP loci, all Ht_{max} thresholds were set to 100rfu and the *Bt* was set to 50rfu, i.e. if *Hb%*>5% then the locus was recorded as a heterozygote, only single peaks with *Ht*<100rfu would be labelled with an 'F' designation and only peaks <50rfu were ignored from the interpretation as they fell below the baseline threshold. This allowed all data to appear without F designations and/or questioned heterozygotes (Table 4.1).

FileName	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
CAS reference	G	G/B	G/B	G/B	G/B	G	G	G/B	G	G/B	G/B	G	B	G	G	G/B	G	G	G	G	B
CAS 500pg	G	G/B	G/B	G/B		G	G	G/B	G	G/B	G/B	**G**	**B**	G	G	G/B	G	G	G	G	B
CAS 250pg	G	G/B	G/B	G/B	**G**	G	G	G/B	G	G/B	G/B	**G**	B	G	G	G/B	G	G	G	G	B
CAS 125pg	G			G/B		G	G		**G**	G/B	G/B			**G**	**G**	**B**		**G**	**G**	**G**	
CAS 62pg	G			G/B		G	G			G/B	G/B			**G**	**G**	**B**		**G**	**G**	**G**	
CAS 31pg	G					**G**				**B**	G/B			**G**	**G**			**G**		**G**	
CAS 16pg	G			G/B		**G**					G/B			**G**				G			B
DRJ reference	G/B	G	G/B	G/B	G/B	G	G	G	G	G	B	G/B	G	G/B	B	G/B	G	G	G	G/B	B
DRJ 500pg	G/B	G	G/B	G/B	G/B	G	G	G	G	G	B	G/B	G	G/B	B	G/B	G	G	G	G/B	B
DRJ 250pg	G/B	**G**	**G**	G/B		G	G	G	G	G	B	**B**	**G**	G/B	B	G/B	G	G	G	G/B	B
DRJ 125pg	**G**			G/B		G	G	**G**	**G**	G	B	**B**	**G**	G/B	B	**B**		G	**G**	**G**	B
DRJ 62pg	G/B		**G**	G/B		G	G	**G**	**G**	**G**	B	**B**	**G**	G/B	B			G		G/B	B
DRJ 31pg	**G**			G/B			G			**G**	**B**		**G**	G/B		**B**		**G**			
DRJ 16pg	**G**			G/B							**B**			**G**	B					**G**	
HER reference	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 500pg	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 250pg	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 125pg	G	**G**		G/B	**G**	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 62pg	G	**G**	**G**	G/B		G	G/B	G/B	**G**	G	B	**G**	**G**	**B**	G	G/B	G/B	G	G	G	B
HER 31pg	**G**			G/B	**G**	**G**	G/B	**G**	**G**	G	B	**G**		**B**	G	**B**	**B**	G		G	B
HER 16pg	**G**			**B**		**G**				G	B	**G**		**B**				G		**G**	B
SHM reference	G	G/B	B	G/B	G/B	G	G	G/B	G	G/B	G/B	G	G/B	G/B	G	G	G/B	G/B	G	G	B
SHM 500pg	G	G/B	**B**	G/B	**G**	G	G	G/B	G	G/B	G/B	G	G/B	G/B	G	G	G/B	G/B	G	G	B
SHM 250pg	G			G/B		G	G	**B**	G	G/B	G/B		**G**	G/B	G	G		G/B	**G**	G	B
SHM 125pg	**G**	**G**		G/B		G	G	G/B	G	G/B	G/B	G	G/B	G/B	G	G		G/B	G	G	B
SHM 62pg	G			G/B		G	G	**B**		G/B	**G**	**G**	**G**	**G**	G	G		G/B	**G**	G	B
SHM 31pg	**G**			G/B		**G**	G			G/B	**G**		**G**	G/B				G/B		**G**	
SHM 16pg	**G**			G/B						**G**	**B**			G/B				**B**		**G**	
ST reference	G	G/B	G/B	G/B	G/B	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 500pg	G	G/B	G/B	G/B	G/B	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 250pg	G	**G**	**G**	G/B	**G**	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 125pg	G	**G**		G/B	**G**	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 62pg	**G**			G/B		G/B	G	**G**	G	G	G/B	**G**	**G**	G	G	G/B	**G**	G	**G**	B	B
ST 31pg	**G**			G/B		**G**	G			G	**G**			**G**	G	**B**		**G**		**B**	
ST 16pg	**G**			**B**						**G**	**B**			**G**				**G**		**B**	
Cambio M reference	G/B	G/B	B	G	G	G	G/B	G/B	G	G/B	G/B	G	B	G	G	G	G	G	G/B	G	B
Cambio M 500pg	G/B	G/B	B	G	G	G	G/B	G/B	G	G/B	G/B	G	B	G	G	G	G	G	G/B	G	B
Cambio M 250pg	G/B	G/B	**B**	G	**G**	G	G/B	G/B	G	G/B	G/B	**G**	**B**	G	G	G	G	G	G/B	G	B
Cambio M 125pg	G/B	G/B	**B**	G	**G**	G	G/B	G/B	G	G/B	G/B	**G**	**B**	G	G	G	G	G	G/B	G	B
Cambio M 62pg	**G**			G	**G**	G			**G**	G/B	G/B		**B**	G	G	G		G	**B**	G	B
Cambio M 31pg	**G**			G		**G**				**B**	G/B			**G**	G	**G**		**G**		G	B
Cambio M 16pg	**G**										G/B			**G**	**G**			**G**	**B**	**G**	
Cambio F reference	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 500pg	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 250pg	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 125pg	G	G	G/B	G/B	**G**	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	**G**	G/B	B
Cambio F 62pg	**G**	**G**		G/B	**G**	G	G/B	**G**	**G**	G/B	B	**G**	**G**	G	G/B	G/B	G	G	**G**	G/B	B
Cambio F 31pg	**G**	**G**		G/B							B	**G**	**G**	G	**G**	**B**		G		G/B	B
Cambio F 16pg	**G**			G/B							**B**		**G**	**G**				**G**		**G**	

Table 4.1 Genotypes from a dilution series of each control sample. Celestial™ rule sets were set to allow all data to be captured within the program, without highlighting allele dropout or heterozygous imbalances. Results in BOLD type indicate heterozygotes / below threshold homozygotes.

4.3.2 Heterozygous balance (*Hb%*)

Data for all individual samples were collated for *Hb%* for all SNPs at each PCR template level. The data were tabulated and the lowest *Hb%* for each SNP at each PCR template level was noted, regardless of the individual (Table 4.2). At optimal DNA template levels (0.5-1.0ng) the lowest *Hb%* ($Hb\%_{min}$) exhibited was with Y3, at approximately 25%, for both the 12 and 20 second injection times from the same PCR amplification, i.e. the smaller peak was only 25% of the height of the larger peak (Table 4.2 **bold** type). The most balanced heterozygous SNPs at optimal PCR conditions were G and J2 at both the 12 and 20 second injection times with $Hb\%_{min}$>68%, comparable to existing STR multiplex systems (Gill *et. al.* 1997) (Table 4.2 **bold** type).

	12 second injection ($\phi S/\phi L$) x 100 (*Hb%*)				20 second injection ($\phi S/\phi L$) x 100 (*Hb%*)			
SNP locus	Sub 125pg	125pg	250pg	500pg-1ng	Sub 125pg	125pg	250pg	500pg-1ng
Amelo	#	#	23.5	31.0	#	#	25.9	35.7
D	25.0	39.2	48.0	47.8	#	41.3	55.2	49.7
U6	#	#	31.5	45.0	#	#	36.0	39.4
B6	#	#	23.6	44.2	#	#	23.6	45.1
N4	#	11.2	37.5	40.3	#	13.0	34.7	41.6
Y3	#	#	68.4	**25.1**	#	63.4	67.3	**24.4**
P5	34.3	#	55.1	54.1	32.3	#	34.6	47.8
A4	#	24.8	41.3	41.6	#	23.1	36.8	39.6
O6	16.2	50.0	57.8	40.2	15.6	52.3	61.1	41.3
Z2	25.7	29.7	36.3	38.6	25.2	29.8	37.9	39.9
K3	16.5	29.7	29.9	33.2	13.6	28.9	29.3	32.0
J2	#	#	22.6	**69.3**	#	#	24.2	**68.4**
Y6	27.4	#	50.2	35.1	30.0	#	51.2	35.9
P7	#	15.4	24.8	32.0	#	14.6	22.1	27.7
J8	#	#	40.7	34.5	#	#	40.9	58.7
X	#	#	41.7	56.5	#	#	39.8	62.0
F	32.7	#	46.1	36.9	33.0	#	52.9	38.4
G	31.6	#	69.5	**67.5**	35.0	#	69.3	**71.5**
L2	#	#	51.8	53.0	53.0	#	#	53.6
W3	#	#	13.3	52.7	#	18.3	42.1	53.9
H8	#	#	61.1	79.7	#	#	57.7	81.7

Table 4.2 *Hb%* collected from two runs of the AB 3100 instrument at 12 and 20 seconds. Heterozygote balance was exported from Celestial™ to Excel for collation and tabulation. # indicates loci with either allele dropout or total dropout, hence no heterozygous balance calculation.

As DNA template level decreased, *Hb%* decreased, indicating a larger imbalance between the two peak heights. This was due to stochastic variation at low levels, consistent with low copy number (LCN) SGM+ profiling. Using SGM+ an optimal DNA template would give *Hb%* > 0.6 but at LCN levels the distribution of *Hb%* can be almost random as a consequence of stochastic effects (Gill *et. al.* 2000b; Gill 2001a). This was an unavoidable consequence of low copy number DNA templates and special interpretation methods were therefore required for SNPs, as the multiplex had been designed for LCN and degraded templates. The most extreme $Hb\%_{min}$ were seen at 11.2% (12s) for N4 and 15.4% (12s) for P7 at a DNA template level of 125pg, closely followed by O6 (15.6% (12s) and 16.2% (20s)) and K3 (16.5% and 13.6% for 12s and 20s respectively) at the sub-125pg PCR template level. The lowest $Hb\%_{min}$, irrespective of DNA template level, was used for the rule sets in Celestial™ (appendix VII).

4.3.3 Homozygote thresholds (Ht_{max})

Ht_{max} for the known reference samples was estimated from the dilution series experiments. Data were tabulated, disregarding individual samples, to show Ht_{max} for each SNP allele for each instrument injection parameter (Table 4.3).

	12 second injection (Ht_{max})				20 second injection (Ht_{max})			
SNP locus	Sub 125pg	125pg	250pg	500-1000pg	Sub 125pg	125pg	250pg	500-1000pg
Amelo	403	#	#	#	560	#	#	527
D	615	#	#	#	859	#	#	#
U6	311	198	141	249	559	464	211	520
B6	328	207	#	#	450	439	#	#
N4	422	#	#	#	613	412	#	#
Y3	155	#	#	#	213	#	#	#
P5	424	#	#	#	617	#	#	#
A4	486	#	#	#	746	274	#	#
O6	152	#	#	#	615	#	#	#
Z2	380	#	#	#	795	#	372	#
K3	586	#	#	#	828	#	#	#
J2	170	#	#	#	#	449	#	#
Y6	454	#	#	#	526	#	#	#
P7	191	#	#	#	274	#	#	#
J8	160	#	#	**334**	#	372	#	**850**
X	390	191	#	#	546	334	#	221
F	**717**	#	#	#	**1068**	#	#	#
G	#	#	#	#	478	#	#	#
L2	230	#	#	#	329	#	#	#
W3	#	242	#	#	#	534	#	#
H8	134	#	#	#	#	377	#	#

Table 4.3 Observed homozygote peak heights (rfu) where allele dropout has occurred (Ht_{max}). Allele dropout was identified from known control sample heterozygotes and peaks heights for these collated within Excel. # indicates heterozygous loci giving no allele dropout.

At optimal DNA template amounts (0.5-1.0 ng), the maximum Ht_{max} (i.e. the maximum single peak height observed at a known heterozygous locus) was observed for J8 at both the 12-second injection time (334rfu) and the 20-second injection time (850rfu). At sub-125 pg template amounts the largest Ht_{max} was observed at locus *F*, at a height of 717rfu (12-sec) and 1068rfu (20-sec). This was at a template level below 125 pg and no allele dropout was observed for this SNP at the higher template concentrations.

Allele dropout was not observed at locus G at either optimal DNA amounts or the sub-125 pg level, for a 12 second injection time. Consequently an approximate theoretical dropout level for G was estimated from the observed $Hb\%_{min}$ and the negative baseline (*Bt*) (section 4.3.4), using the equation:

$$Ht(theoretical) = \frac{(Bt-1)}{(Hb\%_{\min}/100)} \qquad \textit{(Equation 4-3)}$$

This equation allowed the theoretical maximum peak height (Ht_{max}) to be calculated based on the observed *Hb%* by comparing the minimum peak height of the largest peak that would cause the smaller peak height to drop below *Bt* (Figure 4.2). For example, if *Bt* was set to 100rfu, the smallest peak height would need to be <100rfu to 'drop out' of the profile. If the $Hb\%_{min}$ was calculated to 50%, both values could be substituted into the equation to give an estimated value for *Ht* (Figure 4.2).

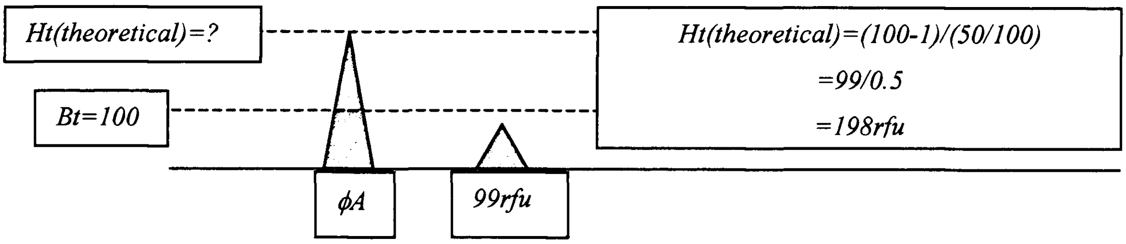


Figure 4.2 Example of the theoretical calculation for *Ht* based on the value of $Hb\%_{min}$ and *Bt*. In this example $Hb\%_{min}$ is set to 50% and *Bt* is 100rfu.

To allow for unobserved extremes (potential outlier data), Ht_{max} was adjusted upwards by 20% within the Celestial™ rule set, in order to be conservative (appendix VII). Again these guidelines were built upon principles already established for STRs (Gill *et. al.* 1997; Gill *et. al.* 2000b). With STRs, Ht_{max} is set to 150 rfu (peak height), signifying potential allele dropout across all multiplexed loci, whereas the SNP multiplex used a different guideline *per locus* programmed into Celestial™.

4.3.4 Negative control thresholds (*Bt*)

LCN is characterised by allele dropout and drop-in (laboratory contamination by single alleles measured by reference to negative controls) (Gill *et. al.* 2000b). A 96-well microtitre plate was prepared for SNP amplification using water controls as negatives instead of DNA samples. The plate was processed through the system and any drop-in peaks were identified, for both a 12 second injection time and a 20 second injection time (Table 4.4). For a 12 second injection time the largest drop-in peak seen was at D (blue) at 81rfu peak height. For 20 seconds, the largest peak was 150rfu at G (green). The baseline level *Bt* was set according to the greatest drop-in peak seen plus an arbitrary ~25% to capture unobserved outliers. Consequently the thresholds were set at 100rfu and 200rfu for 12 seconds and 20 seconds respectively, and these were programmed into Celestial™ (appendix VII).

	12 second injection (peak height rfu)		20 second injection (peak height rfu)	
SNP locus	Green peak	Blue peak	Green peak	Blue peak
Amelo		53 (1)	76 (1)	77 (1)
D	58 (1)	**81** (1)	118 (6)	130 (3)
U6				
B6	57 (1)			73 (1)
N4			92 (2)	56 (1)
Y3				
P5			91 (1)	75 (1)
A4		51 (1)	79 (2)	72 (2)
06	56 (1)			
Z2			133 (2)	
K3	52 (1)		76 (1)	
J2				
Y6			66 (1)	
P7				
J8			60 (1)	
X				
F			119 (1)	
G			**150** (2)	
L2			87 (2)	
W3				
H8	56 (1)			

Table 4.4 Peak height data for allele drop-in peaks seen on a 96-well negative (deionised water) control plate. Brackets indicate the number of peaks seen. Data is for both a 12 second and 20 second injection time.

4.3.5 Analysis of dilution series data

Once the rule sets for heterozygous balance (*Hb%*), homozygote thresholds (Ht_{max}) and the negative baseline (*Bt*) had been calculated (as described in sections 4.3.2-4.3.4) and programmed into Celestial™, data from the dilution series experiments were re-analysed to ensure all incidences of allele dropout were highlighted by the program (Table 4.5).

FileName	Lane	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
CAS reference	A01	G	G/B	G/B	G/B	G/B	G	G	G/B	G	G/B	G/B	G	B	G	G	G/B	G	G	G	G	B
CAS 500pg	A02	G/F	G/B	G/B	G/B		G	G	G/B	G	G/B	G/B	G/F	F/B	G	G	G/B	G	G	G	G	B
CAS 250pg	A03	G	G/B	G/B	G/B	G/F	G	G	G/B	G	G/B	G/B	G/F	B	G	G	G/B	G	G	G	G	B
CAS 125pg	A04	G/F			G/B		G	G		G/F	G/B	G/B			G/F	G/F	F/B		G/F	G/F	G/F	
CAS 62pg	A05	G/F			G/B		G	G			G/B	G/B			G/F	G/F	F/B		G/F	G/F	G/F	
CAS 31pg	A06	G/F					G/F				F/B	G/B			G/F	G/F			G/F		G/F	
CAS 16pg	A07	G/F			G/B		G/F					G/B			G/F				G			B
DRJ reference	B01	G/B	G	G/B	G/B	G/B	G	G	G	G	G	B	G/B	G	G/B	B	G/B	G	G	G	G/B	B
DRJ 500pg	B02	G/B	G	G/B	G/B	G/B	G	G	G	G	G	B	G/B	G	G/B	B	G/B	G	G	G	G/B	B
DRJ 250pg	B03	G/B	G/F	G/F	G/B		G	G	G	G	G	B	F/B	G/F	G/B	B	G/B	G	G	G	G/B	B
DRJ 125pg	B04	G/F			G/B		G	G	G/F	G/F	G	B	F/B	G/F	G/B	B	F/B		G	G/F	G/F	B
DRJ 62pg	B05	G/B		G/F	G/B		G	G	G/F	G/F	G/F	B	F/B	G/F	G/B	B			G		G/B	B
DRJ 31pg	B06	G/F			G/B			G			G/F	F/B		G/F	G/B		F/B		G/F			
DRJ 16pg	B07	G/F			G/B							F/B			G/F	B					G/F	
HER reference	C01	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 500pg	C02	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 250pg	C03	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 125pg	C04	G	G/F		G/B	G/F	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 62pg	C05	G	G/F	G/F	G/B		G	G/B	G/B	G/F	G	B	G/F	G/F	F/B	G	G/B	G/B	G	G	G	B
HER 31pg	C06	G/F			G/B	G/F	G/F	G/B	G/F	G/F	G	B	G/F		F/B	G	F/B	F/B	G		G	B
HER 16pg	C07	G/F			F/B		G/F				G	B	G/F		F/B				G		G/F	B
SHM reference	D01	G	G/B	B	G/B	G/B	G	G	G/B	G	G/B	G/B	G	G/B	G/B	G	G	G/B	G/B	G	G	B
SHM 500pg	D02	G	G/B	F/B	G/B	G/F	G	G	G/B	G	G/B	G/B	G	G/B	G/B	G	G	G/B	G/B	G	G	B
SHM 250pg	D03	G			G/B		G	G	F/B	G	G/B	G/B		G/F	G/B	G	G		G/B	G/F	G	B
SHM 125pg	D04	G/F	G/F		G/B		G	G	G/B	G	G/B	G/B	G/F	G/B	G/B	G	G		G/B	G	G	B
SHM 62pg	D05	G			G/B		G	G	F/B		G/B	G/F	G/F	G/F	G/F	G	G		G/B	G/F	G	B
SHM 31pg	D06	G/F			G/B		G/F	G			G/B	G/F		G/F	G/B				G/B		G/F	
SHM 16pg	D07	G/F			G/B						G/F	F/B			G/B				F/B		G/F	
ST reference	E01	G	G/B	G/B	G/B	G/B	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 500pg	E02	G	G/B	G/B	G/B	G/B	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 250pg	E03	G	G/F	G/F	G/B	G/F	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 125pg	E04	G	G/F		G/B	G/F	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 62pg	E05	G/F			G/B		G/B	G	G/F	G	G	G/B	G/F	G/F	G	G	G/B	G/F	G	G/F	B	B
ST 31pg	E06	G/F			G/B		G/F	G			G	G/F			G/F	G	F/B		G/F		F/B	
ST 16pg	E07	G/F			F/B						G/F	F/B			G/F				G/F		F/B	
Cambio M reference	F01	G/B	G/B	B	G	G	G	G/B	G/B	G	G/B	G/B	G	B	G	G	G	G	G	G/B	G	B
Cambio M 500pg	F02	G/B	G/B	B	G	G	G	G/B	G/B	G	G/B	G/B	G	B	G	G	G	G	G	G/B	G	B
Cambio M 250pg	F03	G/B	G/B	F/B	G	G/F	G	G/B	G/B	G	G/B	G/B	G/F	F/B	G	G	G	G	G	G/B	G	B
Cambio M 125pg	F04	G/B	G/B	F/B	G	G/F	G	G/B	G/B	G	G/B	G/B	G/F	F/B	G	G	G	G	G	G/B	G	B
Cambio M 62pg	F05	G/F			G	G/F	G			G/F	G/B	G/B		F/B	G	G	G		G	F/B	G	B
Cambio M 31pg	F06	G/F			G		G/F				F/B	G/B			G/F	G	G/F		G/F		G	B
Cambio M 16pg	F07	G/F										G/B			G/F	G/F			G/F	F/B	G/F	
Cambio F reference	G01	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 500pg	G02	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 250pg	G03	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 125pg	G04	G	G	G/B	G/B	G/F	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G/F	G/B	B
Cambio F 62pg	G05	G/F	G/F		G/B	G/F	G	G/B	G/F	G/F	G/B	B	G/F	G/F	G	G/B	G/B	G	G	G/F	G/B	B
Cambio F 31pg	G06	G/F	G/F		G/B							B	G/F	G/F	G	G/F	F/B		G		G/B	B
Cambio F 16pg	G07	G/F			G/B							F/B		G/F	G/F				G/F		G/F	

Table 4.5 Dilution series genotype data generated using the validated rule-sets for homozygote thresholds and heterozygous balance.

All heterozygous loci were genotyped correctly by Celestial™ with no questioned heterozygotes observed. Single peaks falling below Ht_{max} were given an 'F' designation. Some homozygous loci became designated with an 'F', indicating possible allele dropout; these were mostly seen at the sub-optimal template levels for all samples.

4.4 Discussion

Identifying the interpretation criteria was as a crucial part of the validation of the 21-SNP multiplex. Due to factors affecting the amplification of DNA templates at low levels, it was necessary to devise a set of criteria that could readily highlight any anomalies in data collected. Allele dropout is a common feature of LCN templates, with one allele being preferentially amplified over the other due to the low levels of DNA present (Whitaker *et. al.* 2001; Gill 2001b). It was important to construct a system capable of analysing data and automatically generating genotypes based on a standard rule set.

By preparing a dilution series of control samples, the system was tested against parameters where dropout was known to occur. The low levels of DNA present would 'force' allele dropout and allow it to be characterised. By amplifying low template levels of DNA, background levels of allele 'drop in' could also be assessed and a baseline threshold was set above which peaks could be genotyped with greater accuracy.

Heterozygous balance (*Hb%*) was shown to be variable between all SNP loci used in the multiplex. Due to the large number of primers used, some preferential amplification of loci was already known to occur, even at optimal template levels. The URP-ARMS method of amplification was developed to minimise the amount of variation seen between loci by standardising the melting temperature of the primers used in the second phase of amplification (Hussain *et. al.* 2003). The use of three Universal tails, one for each forward primer and one reverse primer, allowed each SNP amplicon to be tagged with either forward Universal plus the one reverse Universal tail. The 3' forward Universal tails were targeted for amplification in phase three of the PCR process, allowing each product containing a tail to be fluorescently labelled. Sequence variation caused some primers to be more efficient than others. Although not completely balanced, the 21-SNP multiplex could still be characterised by examining each locus individually and using an interpretation rule for each one. Unlike automated STR genotyping systems, this program used locus-specific rule sets and characterised each peak according to the rules laid out for that particular SNP. STR programs interpret peak characteristics based on criteria relevant to the whole data set (Werrett *et. al.* 1998; Perlin 2001).

Allele dropout leading to false positive homozygotes was defined from the dilution series of control samples. The amount of dropout seen was, again, variable between the different SNP loci with some showing dropout with a large single peak and others showing no dropout, even at sub-optimal DNA template levels. By characterising each SNP locus individually, the program was able to accurately determine genotypes for samples showing variable template levels of DNA.

Celestial™ was shown to genotype all control samples correctly, even at sub-optimal levels. By using an automated program, genotyping of SNP data became independent of operator variation. This allowed all data generated during the validation to be analysed using the same set of interpretation criteria, regardless of operator and allowed confidence in the genotyping method.

5 The 21-SNP Multiplex Population Studies

5.1 Introduction

Population genetics is the application of statistical analysis of allele frequencies and allele frequency spectra to populations of organisms. It includes the study of genetic variation and attempts to understand the processes involved in evolutionary and adaptive changes within species through time. Genetic variation is a natural phenomenon modulated by mutation, population size, genetic drift and selection, passed through the population over time through biological, demographic and historical processes (Chakravarti 1999). Variation becomes divergent in different sub-populations through genetic drift, natural selection, demographic history and gene flow.

The advent of the Human Genome Project and subsequent availability of large amounts of sequence data, further highlighted the amount of variability between individuals at certain genetic sites. SNPs have been found to occur approximately once in every 1kb of DNA (Chakravarti 1999; Stoneking 2001) and have been identified for use in many different areas of population genetics. *"Most human variation influenced by genes can be traced to SNPs, especially in medically important traits indicating disease susceptibility"* (Stoneking 2001). If SNPs are not directly responsible for disease susceptibility they can still be used in gene mapping to identify such traits (Riley *et. al.* 2000; Schork *et. al.* 2000; Shastry 2002; Clark 2003; Schmith *et. al.* 2003; Kuno *et. al.* 2004; Powell *et. al.* 2004). SNPs can also be used to provide patterns of molecular genetic variation which can subsequently be used to reconstruct the evolutionary history of human populations (Collins 2000; Collins *et. al.* 2001; Reich *et. al.* 2001; Smith *et. al.* 2001; Stoneking 2001; Kaessmann *et. al.* 2002).

Most identified SNPs have been made available within the public domain via websites such as the International HapMap Consortium (http://www.hapmap.org) and the SNP Consortium (http://snp.cshl.org). At an early stage in human forensic population studies SNPs should be characterised and assessed for Hardy-Weinberg equilibrium (HWE). This allows loci to be assessed for their use in forensic identification as the frequency of a particular genotype can be calculated from the frequencies of the alleles present. This is done by screening a number of individuals from a population and analysing the allele frequencies of selected SNPs within this population. From the observed allele frequencies, the expected genotype frequencies can be calculated. If the observed genotype frequencies are close to the expected frequencies then the population is said to be in HWE for that locus (Norton and Neel 1965; Hosking *et. al.* 2004; Butler 2005b). All SNPs selected for publication on public domain websites undergo testing with different population samples, including European, African and Asian databases (Reich *et. al.* 2003; Hinds *et. al.* 2005). Selection of appropriate SNPs for forensic identification purposes requires loci to be in Hardy-Weinberg equilibrium and to have allele frequencies that vary within different populations, preferably with frequencies between 0.2 and 0.8 (Delahunty *et. al.* 1996; Chakraborty *et. al.* 1999; Gill 2001a; Petkovski *et. al.* 2003; Inagaki *et. al.* 2004; Lee *et. al.* 2005; Dixon *et. al.* 2005a).

SNPs must also be tested for independence between the various loci selected. Independence indicates that recombination events occur freely and randomly between loci and there are no factors influencing the inheritance of one allele with another or increasing the likelihood of their co-occurrence within individuals (Weir 1996; Butler 2005b). If loci demonstrate independence from each other the product rule can be used for match probability and likelihood ratio calculations, increasing the usefulness of the loci under investigation (section 1.3). Independence testing can be carried out using computer programs available on the internet, such as GDA (Genetic Data Analysis) (Lewis and Zaykin 2001), GENEPOP (http://wbiomed.curtin.edu.au/genepop/index.html; Raymond and Rousset 1995), and TFPGA (Tools for Population Genetic Analyses) (http://www.marksgeneticsoftware.net/). Each program can perform a variety of genetic tests such as HWE, linkage equilibrium testing, Exact tests and tests for F-statistics (Butler 2005b).

Typical human sub-populations are predominantly defined by geographical separation (e.g. islands) or by clines (where the extremities of a population are separated by considerable distance). Cosmopolitan human populations are not defined by geographical boundaries due to the ability to travel worldwide. Consequently, the boundaries that maintain sub-populations are defined largely by constraints to mating preferences imposed mainly by tradition, religion, geopolitical boundaries and language. Typically, the white Caucasian population is virtually unconstrained by marriage traditions whereas in contrast, some recent immigrant groups established in the UK in the 1950s exhibit strong consanguineous mating preferences. For example, differentiation between eight Indian Hindu castes has been estimated to reflect high levels of inbreeding within the founder population from the Indian sub-continent (Bittles 2001). The British Asian population is sub-structured in terms of caste and within castes there are extended family networks, especially within Pakistani Moslem societies. The population is not constrained geographically, since marriages are relatively common between extended family members of the local immigrant group and the original Indian sub-continental population. This means that the concept of the sub-population is not straightforward since simple identifiable subdivisions cannot be identified. There is considerable overlap, making population sub-structure difficult to identify (Overall *et. al.* 2003). Furthermore, the fact that census information divides people into broad categories, e.g. White Caucasian; Pakistani; Bangladeshi and other Asian gives us no clues about the complex ethnic diversity within these groups, hence any attempt to define the sizes of sub-populations within the UK, or any other post-industrial society would be nothing more than guess-work.

Due to the presence of sub-structuring within the population, it becomes necessary to apply a correction factor to the allele frequency data, in order to allow an assessment of match probabilities and likelihood ratios, given that alleles may be identical by descent (IBD). The inheritance of alleles is not completely random as most parents share some common ancestry. In highly inbred populations this will be indicated by a decrease in the number of expected heterozygotes for a locus and a subsequent increase in the number of observed homozygotes. For STR databases this population substructure can be adjusted for by using Weir & Cockerham's theta (θ) calculation (Weir and Cockerham 1984; Nichols and Balding 1991; Balding and Nichols 1994; Balding *et. al.* 1996; Weir 1996; Evett and Weir 1998; Curran *et. al.* 2003; Gill *et. al.* 2003; Buckleton *et. al.* 2005; Butler 2005b). As outlined by the National Research Council Committee on DNA Forensic Science recommendations (NRCII), the value for θ is an empirically determined measure of population substructure and is set to a value of 0.01 for general populations and 0.03 for smaller, isolated populations (NRCII 1996). Calculations are carried out using θ to give a corrected allele frequency value for each locus analysed.

As well as identification involving non-related unknown individuals, SNPs can be used for paternity testing, for determination of parentage; and kinship cases, where relatives are available for genetic comparison with remains from, for example, mass disaster situations or unidentified bodies. Traditionally STRs have been used for such cases with great success (Jamieson 1994; Whitaker *et. al.* 1995; Clayton *et. al.* 1995a; Clayton *et. al.* 1995b; Cash *et. al.* 2003; Holland *et. al.* 2003; Leclair *et. al.* 2004; Alonso *et. al.* 2005; Budowle *et. al.* 2005), however the availability of a SNP multiplex for such analyses could prove useful in situations where the quality and quantity of DNA is limited (Amorim and Pereira 2005).

In cases of disputed paternity it is possible to calculate a probability of an alleged father being the biological parent of a questioned child. Calculations assume the DNA profile of the mother and child are known and the DNA profile of the alleged father is compared to these. Calculations can be carried out using two methods: probability of parentage (the *paternity index* PI) and paternity exclusion (the *exclusion probability* P_E).

PI is calculated using a likelihood ratio:

$$LR = \frac{\Pr(E|H_p)}{\Pr(E|H_d)}$$

It is based on the following hypotheses:

H_p : *the alleged father is the father of the child*
H_d : *another unknown individual is the father of the child.*

The likelihood ratio compares the strength of the evidence of the two propositions. The exclusion probability is calculated from allele frequencies within a population and does not depend on the genotypes in any particular case.

It is calculated from the combined frequencies of all the genotypes that would be excluded if the pedigree relationships were true assuming Hardy-Weinberg equilibrium (Weir 1996; Jamieson and Taylor 1997; Evett and Weir 1998; Ayres 2005; Butler 2005b).

5.2 Materials & Methods

5.2.1 DNA extraction and quantification

DNA was extracted from a variety of samples (Table 5.1) using Qiagen™ QiaAmp Mini-Kits (Cat. No. 51306). Samples had been stored frozen at -20°C and were thawed at room temperature prior to DNA extraction. The manufacturer's protocol for each sample type was used to obtain up to 2ng/µL DNA suspended in 1 x TE Buffer (100mM Tris, 1mM EDTA disodium). Samples were quantified using PicoGreen (Ahn *et. al.* 1996) and/or a UV spectrophotometer (Biochrom Ltd, UK), according to the manufacturers' protocols.

Sample reference	Sample type	Number of samples
Population database – White Caucasians	Buccal swab	201
Population database – Afro-Caribbeans	Buccal swab	71
Population database – Indian Sub-continent	Buccal swab	86
Kuwaiti family samples	Liquid blood	104

Table 5.1 Sample types used for SNP multiplex validation experiments. All samples were extracted using Qiagen™ extraction kits to give quant values of up to 2ng/µL.

5.2.2 SNP multiplex amplification

The SNP multimix for each amplification reaction consisted of oligonucleotide primers (synthesised by IBA, Germany) at varying concentrations (primer sequences are listed in appendix IV), 0.4 µg bovine serum albumin (Boehringer Mannheim, Germany), 225µM dNTPs (dATP, dCTP, dTTP, dGTP; Boehringer Mannheim, Germany), 1 x PCR Buffer II containing 1.5mM $MgCl_2$ (Applied Biosystems™, UK) and 5 units AmpliTaq Gold® (Applied Biosystems™, UK). DNA was added up to a maximum amount of 1 ng per reaction.

DNA extracts were amplified in a total reaction volume of 25 µL in 0.2 mL tubes, without mineral oil, on a thermal cycler (Applied Biosystems GeneAmp PCR system 9600) using the parameters set out in appendix V.

5.2.3 Detection of PCR products using capillary electrophoresis

1.1 µL of each PCR product and 10 µL GS-HD400 ROX size standard (Applied Biosystems, UK Part no. 402985):HI-DI Formamide (Applied Biosystems) [ratio 1:37] was added to each well in a 96-well micro-titre plate. Samples were run on a capillary electrophoresis (CE) sequencer (ABI model 3100) using Collection software v1.1 (ABI) according to the manufacturer's protocol, using a 12 second injection time.

5.2.4 Analysis and interpretation of results

Sample data from the 3100CE instrument was analysed using ABI Prism™ Genescan™ Analysis v3.7.1 and ABI Prism™ Genotyper™ software v3.7 NT. SNP data extracted from Genotyper™ (peak height, peak area, scan number, size in bases) were transformed into *.csv format and analysed by Celestial™ (chapter 4).

5.2.5 Statistical analyses

Chi-squared analysis and likelihood ratio calculations were carried out by generating the appropriate formulae within Microsoft® Excel. The following software programs were used for analysis of the population data:

5.2.5.1 Genetic Data Analysis (GDA) software

GDA software was downloaded from the following Internet site - http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php. The software was used for calculation of HWE and linkage disequilibrium using Exact tests.

5.2.5.2 CERVUS software

CERVUS software was downloaded from the following Internet site - http://helios.bto.ed.ac.uk/evolgen/cervus/cervus.html. The program was designed for large-scale parentage analysis. For this study it was used to assess the validity of paternity testing using SNP loci. Genotype data in text file format was used to analyse allele frequencies, run appropriate simulations and carry out likelihood-based parentage analysis.

5.3 SNP Allele Frequencies

Genotype data was generated for 201 White Caucasian, 71 British Afro-Caribbean and 86 Indian sub-continent DNA samples. These data were used to assess the viability of the SNPs used in the multiplex for forensic purposes. It was essential that all SNPs followed Hardy-Weinberg equilibrium (HWE), to allow the data to be used in the calculation of likelihood ratios. HWE was initially tested using a traditional Goodness-of-fit test, based on Chi-squared (χ^2) analysis of the expected and observed genotypes (Table 5.2) (section 1.8).

Population size	**201**	
		Frequency
Allele A	308	0.76617
Allele B	94	0.23383
No of alleles	402	
	p_a^2	0.58702
	$2p_ap_b$	0.35831
	p_b^2	0.05468
Expected	Hom A	117.99005
	Het AB	72.01990
	Hom B	10.99005
Observed	Hom A	**119**
	Het AB	**70**
	Hom B	**12**
Chi-squared	Hom A	0.00864
	Het AB	0.05665
	Hom B	0.09281
X^2 total		0.15811
95% confidence		3.841
1 degree freedom		
Conforms to HWE?		**TRUE**
Match Probability		0.47596
Likelihood ratio		2.10101

Table 5.2 An example of a Goodness-of-Fit test for Hardy-Weinberg equilibrium.

The allele frequencies of each SNP were tabulated (Table 5.3) and HWE was re-calculated and assessed by Exact tests, using GDA software (section 1.8.2) (Table 5.4).

Likelihood ratios were calculated using the formula $LR = \frac{\Pr(E|H_p)}{\Pr(E|H_d)}$. Where:

H_p : the profile came from the suspect

H_d : the profile came from an unknown unrelated individual. (Buckleton *et. al.* 2005; Butler 2005b)

		White Caucasian		British Afro-Caribbean		Indian Sub-Continent	
SNP locus	Allele 1 (green) / Allele 2 (blue)	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
D	T / C	0.52	0.48	0.27	0.73	0.49	0.51
U6	A / T	0.37	0.63	0.10	0.90	0.32	0.68
B6	A / T	0.64	0.36	0.77	0.23	0.57	0.43
N4	A / T	0.57	0.43	0.51	0.49	0.53	0.47
Y3	G / C	0.92	0.08	0.96	0.04	0.94	0.06
P5	T / A	0.72	0.28	0.59	0.41	0.80	0.20
A4	C / G	0.71	0.29	0.51	0.49	0.66	0.34
06	A / T	0.75	0.25	0.82	0.18	0.77	0.23
Z2	C / T	0.56	0.44	0.45	0.55	0.45	0.55
K3	G / C	0.31	0.69	0.25	0.75	0.39	0.61
J2	C / T	0.92	0.08	0.94	0.06	0.94	0.06
Y6	T / A	0.63	0.37	0.57	0.43	0.53	0.47
P7	T / A	0.62	0.38	0.73	0.27	0.79	0.21
J8	A / T	0.77	0.23	0.86	0.14	0.72	0.28
X	C / A	0.79	0.21	0.89	0.11	0.78	0.22
F	C / A	0.78	0.22	0.85	0.15	0.80	0.20
G	T / C	0.75	0.25	0.64	0.36	0.59	0.41
L2	C / T	0.79	0.21	0.94	0.06	0.90	0.10
W3	C / G	0.77	0.23	0.87	0.13	0.77	0.23
H8	A / T	0.11	0.89	0.10	0.90	0.15	0.85
Multiplex likelihood ratio (LR)		4,460,764		364,761		3,173,898	

Table 5.3 Allele frequencies for each of the 20 SNP loci used in the multiplex for each ethnic group studied and overall likelihood ratios for each group.

The allele frequencies for each ethnic group exhibited divergence from each other (Figure 5.1). The British Afro-Caribbean ethnic group demonstrated the most deviation from the other two ethnic groups tested.

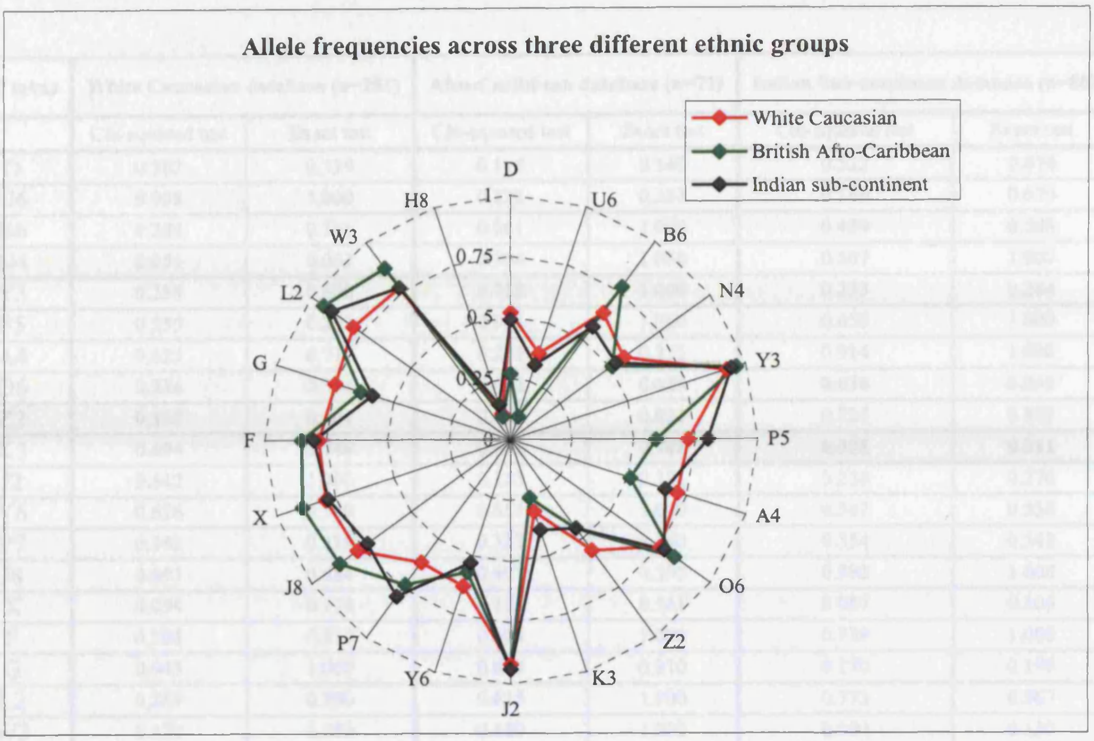


Figure 5.1 Radar graph showing the spread of allele frequencies for each ethnic group for each of the 20 SNP loci used in the multiplex.

Using both statistical tests, HWE demonstrated no significant deviation from expectation (p>0.05) for all 20 SNPs in the white Caucasian population, 19 out of 20 SNPs for the Afro-Caribbean population (locus O6, $p < 0.03$) and 18 out of 20 SNPs for the Indian sub-continent population (locus K3, $p < 0.02$; locus O6, $p = 0.036$) (Table 5.4). Using Exact tests, locus O6 in the Indian sub-continent population was not significantly different ($p = 0.059$).

SNP locus	White Caucasian database (n=201)		Afro-Caribbean database (n=71)		Indian Sub-continent database (n=86)	
	Chi-squared test	Exact test	Chi-squared test	Exact test	Chi-squared test	Exact test
D	0.282	0.339	0.115	0.140	0.522	0.674
U6	0.908	1.000	0.222	0.288	0.166	0.070
B6	0.284	0.361	0.681	1.000	0.489	0.505
N4	0.051	0.062	0.904	1.000	0.807	1.000
Y3	0.236	0.600	0.758	1.000	0.233	0.284
P5	0.255	0.272	0.994	1.000	0.655	1.000
A4	0.625	0.717	0.285	0.353	0.914	1.000
O6	0.286	0.354	**0.022**	**0.029**	**0.036**	**0.059**
Z2	0.330	0.401	0.782	0.821	0.725	0.838
K3	0.694	0.744	0.661	0.748	**0.008**	**0.011**
J2	0.842	1.000	0.153	0.231	0.238	0.276
Y6	0.626	0.749	0.855	1.000	0.347	0.538
P7	0.342	0.328	0.327	0.360	0.354	0.343
J8	0.691	0.684	0.497	0.595	0.992	1.000
X	0.094	0.114	0.135	0.161	0.057	0.105
F	0.205	0.217	0.603	1.000	0.729	1.000
G	0.943	1.000	0.664	0.810	0.170	0.190
L2	0.269	0.390	0.615	1.000	0.773	0.567
W3	0.172	0.152	0.880	1.000	0.092	0.120
H8	0.341	0.290	0.070	0.109	0.952	1.000

Table 5.4 Hardy-Weinberg equilibrium probability values for each SNP within the three main ethnic code populations, calculated using Goodness-of-fit Chi-squared analysis and Exact tests. (n=number of individuals sampled)

The use of multiple loci within the SNP multiplex meant that simultaneously applying 60 significance tests (p>0.05) would naturally result in 5% of them giving p<0.05 (Weir 1996). To make a distinction between "comparison-wise" and "experiment-wise" significance levels, a Bonferroni correction was used (Tarone 1990; Bland and Altman 1995; Weir 1996; Tanner *et. al.* 1997; Evett and Weir 1998) which gave a revised significance test level of p=0.01. Re-examination of the data indicated that only locus K3 (Indian sub-continent) gave a p<0.01.

On close inspection, the raw data for K3 (data not shown) had an excess of heterozygotes within the population set suggesting the deviation was probably sampling error (Pudovkin *et. al.* 1996), rather than a genetic or biochemical abnormality such as primer binding site mutation or population sub-structuring effect – both of which would increase the levels of homozygosity (Devlin *et. al.* 1990; Steinberger *et. al.* 1993).

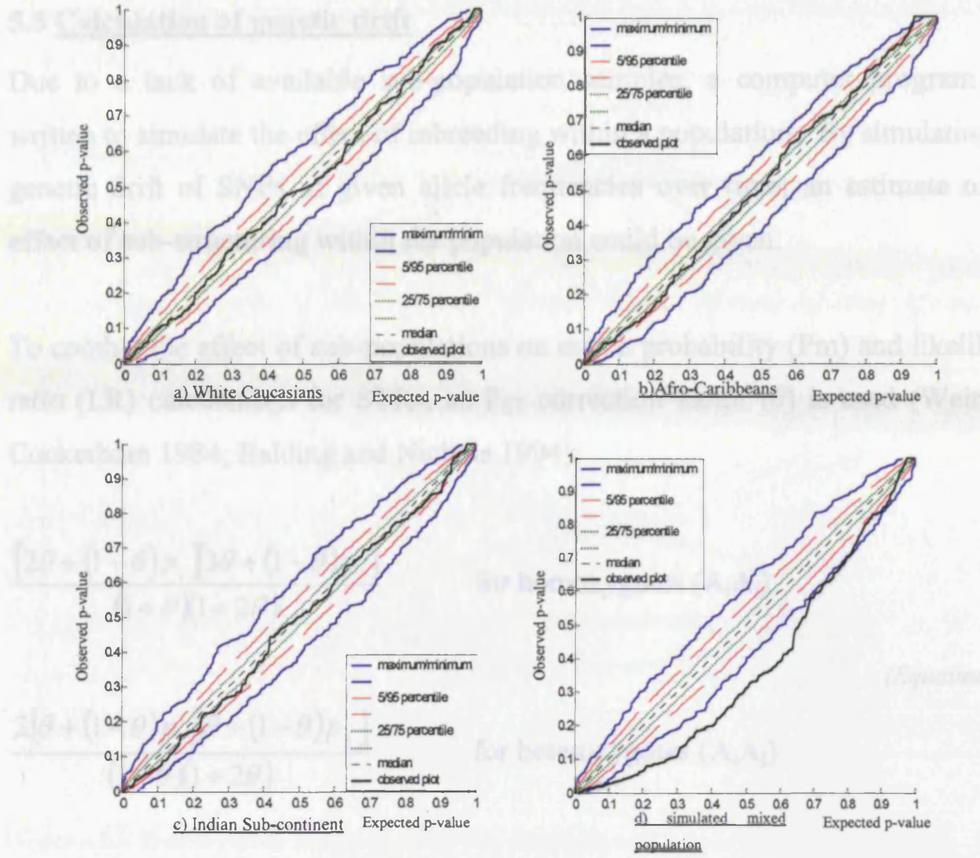
5.4 Linkage Disequilibrium

Exact tests for linkage disequilibrium were carried out on the population data using Genetic Data Analysis (GDA) software (Weir 1996; Lewis and Zaykin 2001) to detect associations between alleles at different loci (section 1.8.2).

Linkage disequilibrium was calculated by looking at the frequencies of the alleles at all the loci under investigation, using genotypic data (Weir 1996) to assess the Chi-squared statistic. Exact tests were performed by comparing the observed two-locus genotypic counts with the values expected under various hypotheses (Zaykin *et. al.* 1995). This gave the significance of the association as a probability value (p-value) that was used to assess the linkage disequilibrium seen between the two loci under investigation, using the assumption of 95% (0.05) significance.

To create probability (P-P) plots for visualisation of the generated data, a random number matrix was simulated using statistical programming software, MatLab®6 (The MathWorks, Inc.). The matrix was generated by firstly selecting 210 random numbers (the same number of associations observed for each locus-locus comparison) a thousand different times. The data was then sorted within each group of 210 and across each set of 1000. A matrix was plotted using the median set of values, plus the maximum & minimum and the 5th, 25th, 75th and 95th percentiles, generating bins within which the experimental data should lie, given a randomly mating population.

Probability data (p-values), calculated from each locus-locus association for each of the three populations tested, ranged from 0.0 – 1.0, with little deviation from the 95% confidence level imposed (Figure 5.2). P-values were plotted against the random number matrix as a probability (P-P) plot (Curran *et. al.* 2003; Buckleton *et. al.* 2005) (Figure 5.2a-c). All data fitted within the random number bins for each ethnic group indicating that the SNP loci were behaving as would be expected within a randomly-mating population with little or no linkage disequilibrium (Zaykin *et. al.* 1995; Collins 2000; Ayres and Balding 2001; Collins *et. al.* 2001).


a) White Caucasians

b)Afro-Caribbeans

c) Indian Sub-continent

d) simulated mixed population

Figure 5.2 Exact test P-P plots for all three population databases and an artificially mixed population. Single ethnic group plots (a-c). The artificially mixed population (d). x values = expected p-values; y values = observed values.

To demonstrate the effectiveness of the test, a random sample generator[4] was used to artificially create a population of 100 individuals from the White Caucasian and Indian sub-continent SNP genotype data. Fifty individuals from each of the two ethnic groups were randomly selected (without replacement) from the population database and amalgamated to produce a new population. Allele data was analysed using GDA software (Weir 1996; Lewis and Zaykin 2001) generating *p*-values deviating from expected values, as would be expected in a sub-structured population (Figure 5.2d). Separately, the two populations used to make up the artificial population were shown to be in Hardy-Weinberg equilibrium but when combined, the data did not conform. This was because the artificial population was effectively sub-structured resulting in a demonstrable increased homozygosity, known as the Wahlund effect (Wahlund 1928).

[4] A random sample generator was written using Visual Basic for Applications in Microsoft Excel.

5.5 Calculation of genetic drift

Due to a lack of available sub-population samples, a computer program was written to simulate the effect of inbreeding within a population. By simulating the genetic drift of SNPs of given allele frequencies over time; an estimate of the effect of sub-structuring within the population could be given.

To combat the effect of sub-populations on match probability (Pm) and likelihood ratio (LR) calculations for STRs, an F_{ST} correction factor (θ) is used (Weir and Cockerham 1984; Balding and Nichols 1994):

$$\frac{[2\theta + (1-\theta)p_i][3\theta + (1-\theta)p_i]}{(1+\theta)(1+2\theta)} \quad \text{for homozygotes } (A_iA_i)$$

(Equation 5-1)

$$\frac{2[\theta + (1-\theta)p_i][\theta + (1-\theta)p_j]}{(1+\theta)(1+2\theta)} \quad \text{for heterozygotes } (A_iA_j)$$

Where θ = 0.01 or 0.03 depending on the sub-population the sample has been derived from; and p = the allele frequency of i or j within the population.

Curran *et al.* (2003) simulated the effect of population sub-division and subsequent LR calculations using the product rule calculation compared to a simulated 'true' product rule calculation (Curran *et. al.* 2003). The method devised by Curran *et al.* was used as a basis for the computer simulations designed to assess the effect of population sub-division in biallelic SNPs. Simulated data could be used to ascertain whether the Balding-Nichols correction factor for STRs could also be applied to SNPs. Genotypes for one thousand sub-populations were generated from an ancestral population and were simulated to randomly breed amongst themselves for a set number of generations. The number of generations varied depending on the size of the sub-population and the value given for θ. All sub-populations were then converged to produce a new population from which the effects of genetic drift could be measured (Figure 5.3).

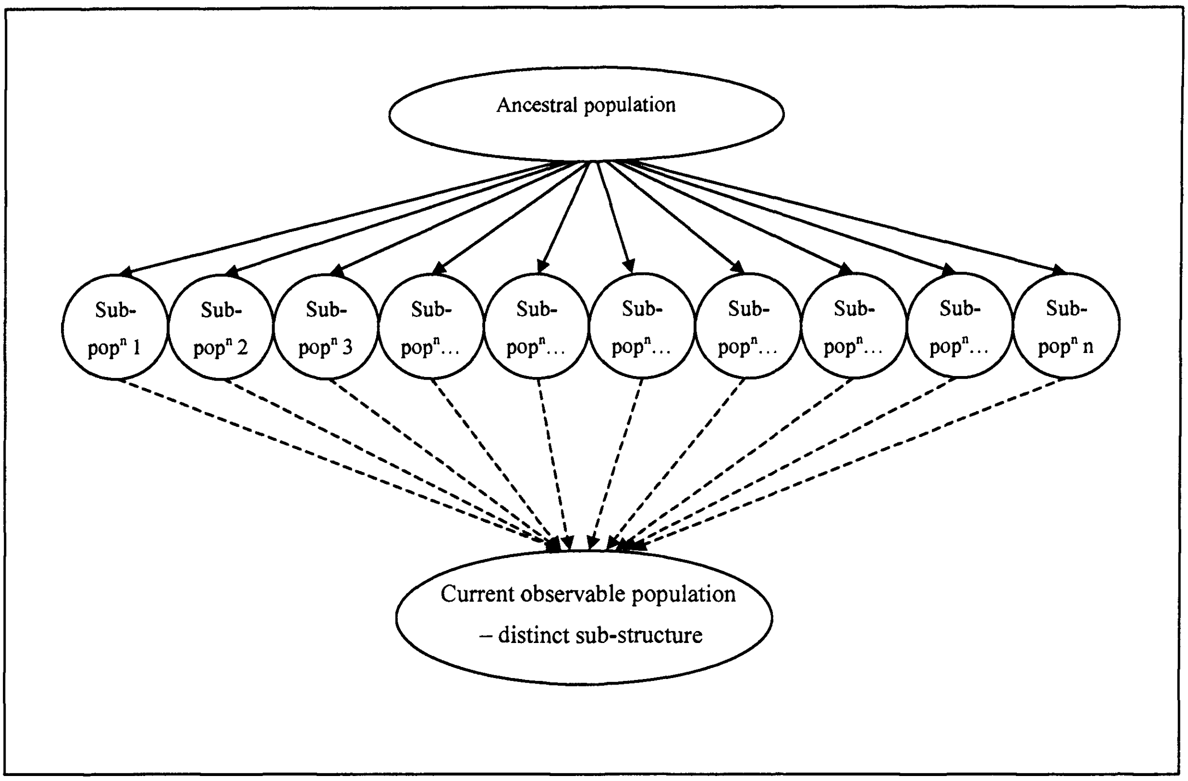


Figure 5.3 Evolutionary model used in the simulation of a sub-divided population. ***Taken from J. Curran et. al. (2003) "What is the magnitude of the subpopulation effect?" Foren. Sci. Int. 135(1):1-8.***

One thousand sub-populations of size n (where n = 200 or 1000) were created using a random number generator[5]. The genotypes for each SNP locus were randomly generated from an 'ancestral' gene pool, based on the mean allele frequencies of the twenty SNP loci used in the 21-SNP multiplex system (Table 5.3). The allele frequencies were calculated from the white Caucasian database, giving eleven frequencies in total rounded to the nearest 0.1, for computational ease. These frequencies formed the basis of the ancestral population (Figure 5.3). To achieve a desired level of drift within the separate sub-populations, random breeding was simulated (within each sub-population, with no migration or mutation) for a fixed number of generations (t), dependent on the size of the sub-population (N_S) and θ ($\theta = 0.01$ or 0.03), where:

[5] All simulations were carried out using MatLab®6 (MathWorks, Inc.)

$$t = \frac{\ln(1-\theta)}{\ln\left(1-\frac{1}{2N_S}\right)}$$ *(Equation 5-2 taken from Curran et. al. (2003))*

One thousand individuals were then chosen randomly across all sub-populations to generate a new allele frequency database. For each individual within the database, two likelihood ratios were calculated: the first was a 'gold standard' estimate based on the allele frequencies of the sub-population from which the sample was chosen; the second estimate was derived from the new allele frequency values of the total combined population, adjusted for θ using the Balding and Nichols (BN) correction (Balding and Nichols 1994).

Simulations were generated for sub-populations of 200 and 1000 individuals using values of 0.01 and 0.03 for θ. Figure 5.4 illustrates the results obtained for a simulation with a sub-population size of 200 individuals using a θ value of 0.03.

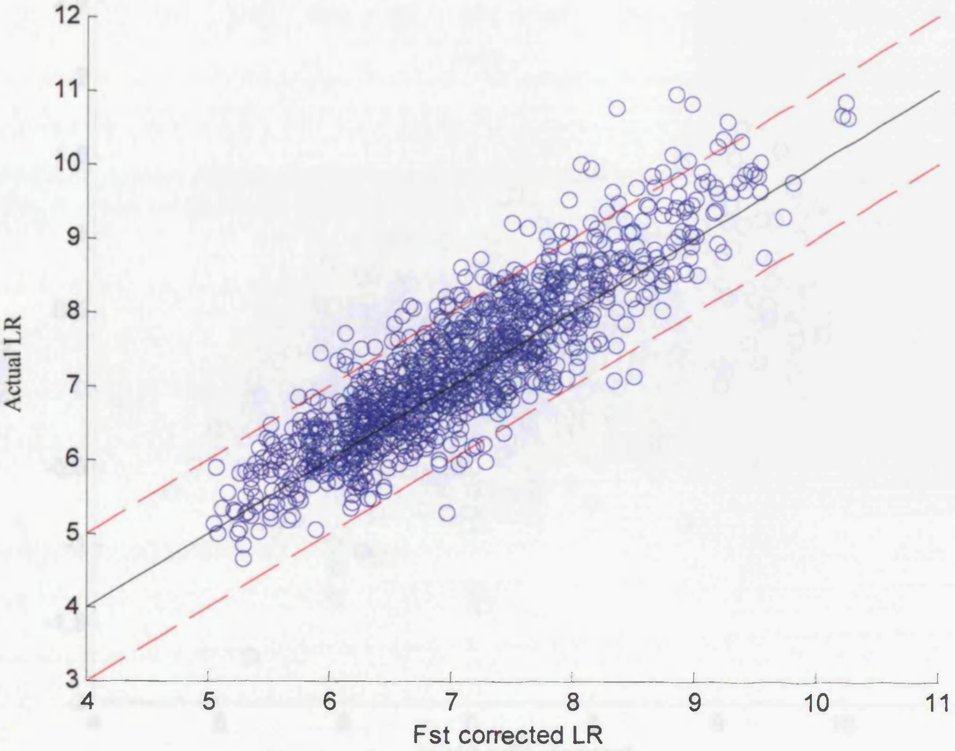


Figure 5.4 Plot of BN corrected LR vs. gold standard LR, θ =0.03, sample size =200. Samples below the solid black line are non-conservative and those below the dashed line are non-conservative by an order of magnitude or more.

For all simulations, application of the BN correction was generally conservative. For the example given, only nine individual samples out of 1000 deviated from the 'gold standard' LR by more than one order of magnitude (Figure 5.4). None of the results were greater than two orders of magnitude, for any of the simulated populations generated.

As well as directly comparing the gold standard LR and the BN corrected LR, $\log_{10}$ ratios of the two values were calculated for each individual sample (d_{obs}), using the following equation:

$$d_{obs} = \log_{10}\left(\frac{LR_{gold}}{LR_{F_{ST}}}\right) \qquad \textit{(Equation 5-3)}$$

If the $LR_{gold} < LR_{fst}$ then d_{obs} was negative and vice-versa. These results were plotted on a graph using the $\log_{10}$ gold standard LR compared to the calculated d_{obs} ratio (Figure 5.5).

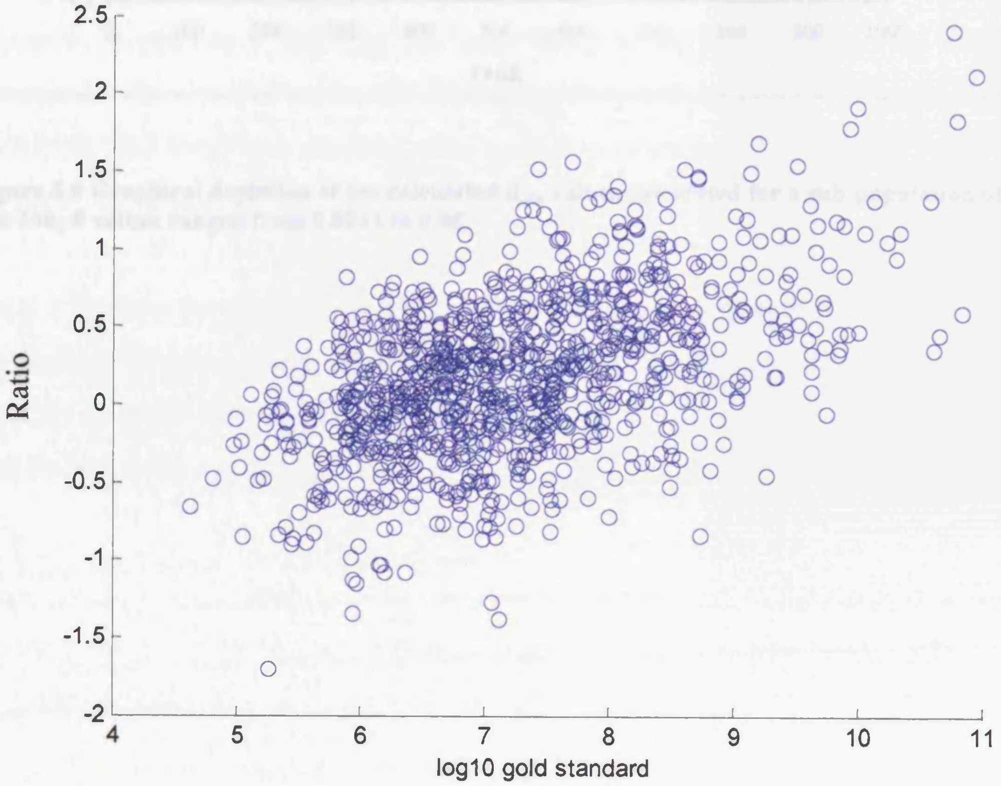


Figure 5.5 Plot of $\log_{10}$ gold standard vs. the calculated ratio d_{obs} where $\theta = 0.03$, sample size = 200.

The data (d_{obs}) were also ranked in ascending order to illustrate the proportion of data that were typically under or over-estimated relative to the gold standard LR (Figure 5.6). Values of θ from 0.05 down to the lowest possible value, dependent on population size, were used to create a set of simulations with varying amounts of genetic drift within the sub-populations.

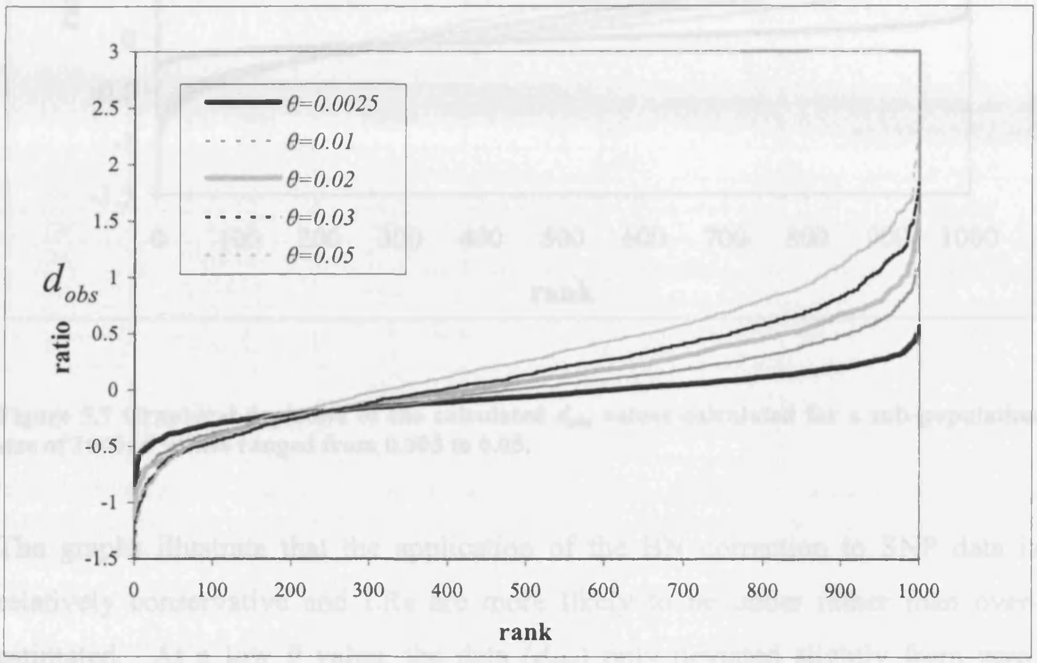


Figure 5.6 Graphical depiction of the calculated d_{obs} values calculated for a sub-population of size 200; θ values ranged from 0.0025 to 0.05.

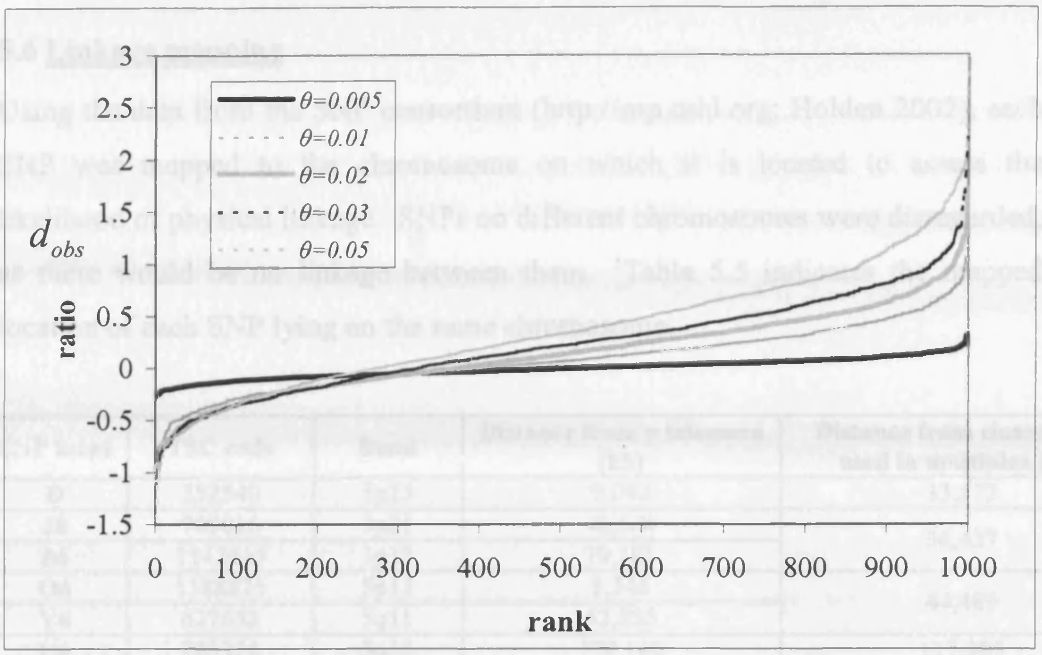


Figure 5.7 Graphical depiction of the calculated d_{obs} values calculated for a sub-population size of 1000; θ values ranged from 0.005 to 0.05.

The graphs illustrate that the application of the BN correction to SNP data is relatively conservative and LRs are more likely to be under rather than over-estimated. At a low θ value, the data (d_{obs}) only deviated slightly from zero, indicating that there is a minimal effect of sub-population drift and it is readily corrected using the BN correction.

From a forensic perspective, the aim of DNA profiling interpretation is to report a conservative figure. These simulations confirm that the BN correction method is unlikely to under-estimate the true LR value by more than an order of magnitude, and consequently appears to be a robust estimator for SNP LR calculations.

5.6 Linkage mapping

Using the data from the SNP consortium (http://snp.cshl.org; Holden 2002), each SNP was mapped to the chromosome on which it is located to assess the likelihood of physical linkage. SNPs on different chromosomes were disregarded, as there would be no linkage between them. Table 5.5 indicates the mapped location of each SNP lying on the same chromosome.

SNP locus	TSC code	Band	Distance from p telomere (kb)	Distance from closest SNP used in multiplex (kb)
D	252540	3p25	9,092	33,572
J8	709016	3p21	42,664	36,437
B6	1342445	3p13	79,101	
O6	1588825	5p15	8,346	44,489
Y6	627632	5q11	52,835	
U6	746324	5q35	170,140	117,305
P7	897904	6p23	14,070	153,973
Z2	86795	6q27	168,043	
X	31988	8p23	238	91,431
A4	421768	8q21	91,669	

Table 5.5 SNP loci that lie on the same chromosome. SNPs were selected based on a maximised distance away from other SNPs on the same chromosome to negate the effects of physical linkage.

On chromosomes 6 and 8, one SNP lay on the short arm (P7 = 6p23; X = 8p23) and one on the long arm (Z2 = 6q27; A4 = 8q21). Linkage disequilibrium can typically extend up to a few megabases (Collins *et. al.* 2001) and is frequently used for disease mapping studies (Terwilliger and Weiss 1998; Clark 2003; Kuno *et. al.* 2004). The shortest distance between any two SNPs in this study was more than 33Mb (D and J8 on chromosome 3). This was sufficient distance to ensure that multiple chromosomal recombination events would result in linkage equilibrium between any pair of loci (Petes 2001). The SNPs used in another multiplex system for parentage analysis (Orchid Biosciences Inc.) are often less than half this distance (http://www.cstl.nist.gov/biotech/strbase/SNP.htm). Consequently, the assumption of independence was reasonable with regard to physical linkage.

5.7 Paternity Testing

The DNA from blood samples obtained from members of thirteen Kuwaiti families was extracted and amplified using the 21-SNP multiplex. The samples had been obtained from the Wetherby Casework Unit where paternity testing had already been performed in relation to immigration regulations. Full SNP genotyping results are shown in appendix VIII.

Exclusion probabilities (P_E) and the probability of parentage (PI) were calculated for each DNA profile analysed from each child within a family. Following STR analysis, the maternal parentage for each child was known and analysis of the SNP genotypes was employed to test the paternity within each family. Statistical calculations for P_E and PI were carried out using the computer program CERVUS (http://helios.bto.ed.ac.uk/evolgen/cervus/cervus.html; Marshall *et. al.* 1998; Slate *et. al.* 2000).

CERVUS was designed for large-scale parentage analysis. Using genotype data in text file format, the program analysed the allele frequencies of each SNP locus before carrying out likelihood-based parentage analysis. Each sample was analysed by assessing the genotype of the known parent (in the Kuwaiti samples this was always the mother) against the genotype of the child. The likelihood of the alleged father being the true father was then assessed against all possible allele combinations. This value was represented as a paternity index (PI) (Table 5.6). The PI value is interpreted as the likelihood of observing the paternal component of the putative offspring's DNA profile in the alleged father compared to a random man from the general population, i.e. "*it is X (PI value) times more likely that the alleged father is the true biological father of the child compared to a random, non-related male*".

The exclusion probability (P_E) for each child was also calculated. This value represents the probability of a random man in the general population being excluded as the father of the child (Buckleton *et. al.* 2005; Butler 2005b). It is based on the allele frequencies of the SNP loci, rather than the genotypes of the individuals involved.

In each case, the number of loci compared was noted (Table 5.6). Some DNA samples failed to give a full profile and only results for SNP loci present in both samples were compared. The PI values obtained varied depending on the allele frequencies of the genotypes. A PI value of over one hundred is an internationally accepted minimum at which paternity can be verified using STR markers (http://www.dnasupport.co.uk; Panke *et. al.* 2001; Butler 2005b). This value was used as a basis for the assessment of the use of the 21-SNP multiplex for paternity testing. The value for P_E was used as a comparison against other studies investigating the use of both SNPs and STRs for paternity testing. The determination of exclusion probabilities can be done without having the DNA profile of the alleged father, as P_E is calculated from the allelic frequency within the population and can be conditioned depending on the genotypes of the child and mother (Chakraborty *et. al.* 1999).

Offspring (O) ID	O loci typed	Candidate parent (CP) ID	CP loci typed	O-CP loci compared	O-CP loci mismatching	Exclusion probability (P_E)	Paternity Index (PI)
CHILD1A	21	FATHER1	21	21	0	0.997	44134
CHILD1B	21	FATHER1	21	21	0	0.998	15304
CHILD1C	21	FATHER1	21	21	0	0.984	46
CHILD1D	21	FATHER1	21	21	0	0.996	6153
CHILD1E	21	FATHER1	21	21	0	0.967	46
CHILD2A	21	FATHER2	21	21	0	0.931	27
CHILD2B	21	FATHER2	21	21	0	0.987	6871
CHILD2C	21	FATHER2	21	21	0	0.944	82
CHILD2E	21	FATHER2	21	21	0	0.972	650
CHILD2F	20	FATHER2	21	20	0	0.977	9514
CHILD3A	21	FATHER3	21	21	0	0.994	719123
CHILD3B	21	FATHER3	21	21	0	0.995	853473
CHILD3C	21	FATHER3	21	21	0	0.998	13052467
CHILD3D	21	FATHER3	21	21	0	0.997	2633158
CHILD3E	21	FATHER3	21	21	0	0.983	10873
CHILD4A	21	FATHER4	20	20	0	0.975	299
CHILD4B	21	FATHER4	20	20	0	0.999	4009747
CHILD4C	21	FATHER4	20	20	0	0.993	18893
CHILD4D	21	FATHER4	20	20	0	0.975	396
CHILD4E	21	FATHER4	20	20	0	0.998	339598
CHILD4F	21	FATHER4	20	20	0	0.991	7274
CHILD5A	21	FATHER5	21	21	0	0.992	84240
CHILD5B	21	FATHER5	21	21	0	0.993	182546
CHILD5C	21	FATHER5	21	21	0	0.998	9732812
CHILD5D	21	FATHER5	21	21	0	0.994	1312157
CHILD5E	20	FATHER5	21	20	0	0.997	16059442
CHIILD5F	21	FATHER5	21	21	0	0.996	683173
CHILD6A	21	FATHER6	21	21	0	0.981	396846
CHILD6B	20	FATHER6	21	20	0	0.951	19558
CHILD6C	21	FATHER6	21	21	0	0.978	392509
CHILD6D	21	FATHER6	21	21	0	0.962	41205
CHILD6E	21	FATHER6	21	21	0	0.958	67196
CHILD6F	21	FATHER6	21	21	0	0.929	5693
CHILD6G	**21**	**FATHER6**	**21**	**21**	**3**	0.992	0
CHILD7A	21	FATHER7	21	21	0	0.833	2
CHILD7B	21	FATHER7	21	21	0	0.999	96966
CHILD7C	21	FATHER7	21	21	0	0.920	17
CHILD7D	21	FATHER7	21	21	0	0.929	27
CHILD7E	21	FATHER7	21	21	0	0.990	5270
CHILD7F	21	FATHER7	21	21	0	0.988	482

Offspring (O) ID	O loci typed	Candidate parent (CP) ID	CP loci typed	O-CP loci compared	O-CP loci mismatching	Exclusion probability (P_E)	Paternity Index (PI)
CHILD8A	**21**	**FATHER8**	**20**	**20**	**4**	0.990	0
CHILD8B	21	FATHER8	20	20	0	0.994	4048344
CHILD8C	21	FATHER8	20	20	0	0.997	352730
CHILD8D	**21**	**FATHER8**	**20**	**20**	**3**	0.970	0
CHILD8E	**21**	**FATHER8**	**20**	**20**	**2**	0.962	0
CHILD9A	21	FATHER9	18	18	0	0.953	25610
CHILD9B	21	FATHER9	18	18	0	0.937	46439
CHILD9C	21	FATHER9	18	18	0	0.966	215344
CHILD9D	21	FATHER9	18	18	0	0.973	199073
CHILD9E	21	FATHER9	18	18	0	0.968	479765
CHILD10A	21	FATHER10	20	20	0	0.994	333166
CHILD10B	21	FATHER10	20	20	0	0.998	20361388
CHILD10C	21	FATHER10	20	20	0	0.994	454165
CHILD10D	21	FATHER10	20	20	0	0.997	1202324
CHILD10E	21	FATHER10	20	20	0	0.932	1237
CHILD10F	21	FATHER10	20	20	0	0.998	3563158
CHILD10G	21	FATHER10	20	20	0	0.999	40788973
CHILD11A	21	FATHER11	20	20	0	0.997	808731
CHILD11B	21	FATHER11	20	20	0	0.994	232885
CHILD11C	21	FATHER11	20	20	0	0.981	10095
CHILD11D	21	FATHER11	20	20	0	0.977	8127
CHILD11E	21	FATHER11	20	20	0	0.940	2030
CHILD11F	21	FATHER11	20	20	0	0.922	291
CHILD11G	20	FATHER11	20	19	0	0.997	131563
CHILD12A	21	FATHER12	20	20	0	0.968	4915
CHILD12B	21	FATHER12	20	20	0	0.952	2659
CHILD12C	21	FATHER12	20	20	0	0.974	5550
CHILD12D	21	FATHER12	20	20	0	0.968	16006
CHILD12E	21	FATHER12	20	20	0	0.921	324
CHILD12F	21	FATHER12	20	20	0	0.892	137
CHILD12G	21	FATHER12	20	20	0	0.859	39
CHILD12H	21	FATHER12	20	20	0	0.958	2070
CHILD13A	21	FATHER13	21	21	0	0.983	68375
CHILD13B	21	FATHER13	21	21	0	0.996	3621034
CHILD13C	21	FATHER13	21	21	0	0.982	39088
CHILD13D	21	FATHER13	21	21	0	0.937	1192
CHILD13E	21	FATHER13	21	21	0	0.996	7997532

Table 5.6 Exclusion probability (P_E) and paternity index (PI) values for Kuwaiti family samples. Tables indicate how many loci for each individual were genotyped and cross-compared for parentage analysis. Samples in BOLD indicate excluded fathers. See appendix VIII for full genotype data.

The P_E and PI values were ranked and displayed graphically, to assess the range of values observed for each statistical test. P_E values ranged from 0.833 – 0.999 for the 77 relationships tested, with a mean value of 0.972 (standard deviation = 0.032) (Figure 5.8).

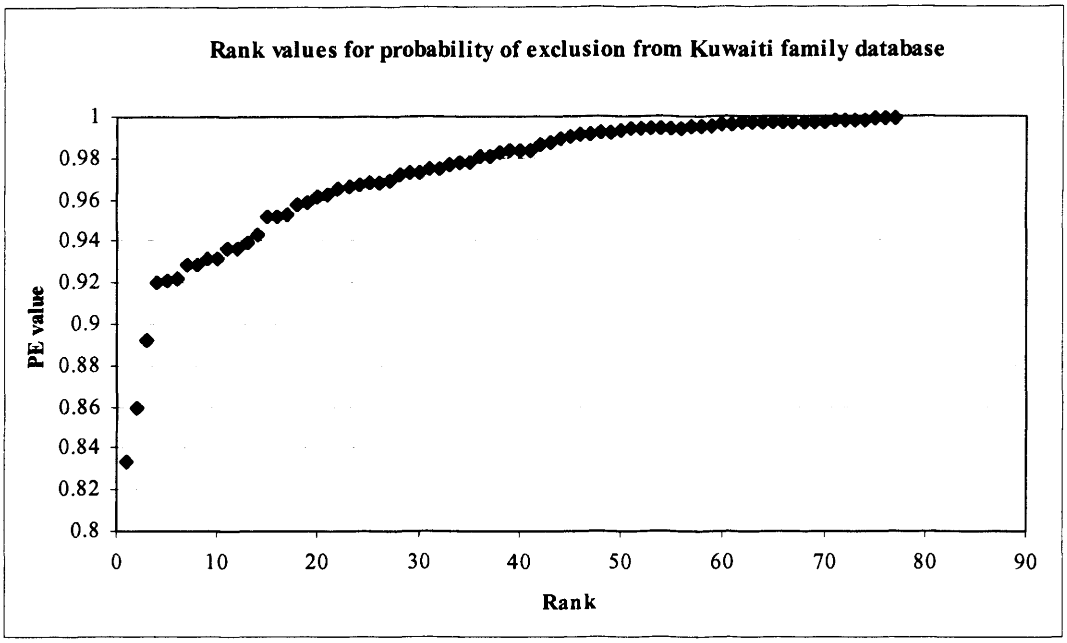


Figure 5.8 A scattergraph of the values observed for the probability of exclusion (P_E) within the Kuwaiti family database.

Chakraborty *et. al.* (1999) assessed the use of SNPs for paternity analysis. Computational data indicated that a core set of approximately 40 SNP loci, with an allele frequency of 0.4-0.5, would be required to give a P_E of 0.999 when both mother and child data were available. Petkovski *et. al.* (2005) reported paternity testing of a set of 36 autosomal SNPs with an average P_E of 0.9999. This set has subsequently been increased to 51 loci, giving a P_E of 0.99999999 (Petkovski *et. al.* 2005). A 39-SNP multiplex system was shown to give a P_E of 0.9999995 when tested for a known mother-child relationship, however data was only available for one case study (Inagaki *et. al.* 2004). An assessment of 24 SNP loci for paternity analysis in Koreans gave an average P_E of 0.989 (Lee *et. al.* 2005).

In comparison, STR multiplex kits available on the commercial market have a P_E value of more than 0.9999, making them much more discriminating than the 21-

SNP multiplex used in this study. SGM Plus™ (Cotton *et. al.* 2000; Steinlechner *et. al.* 2001; Soltyszewski *et. al.* 2005), Identifiler™ (http://docs.appliedbiosystems.com/pebiodocs/04323291.pdf) and Powerplex®-16 (Okamoto *et. al.* 2003; Greenspoon *et. al.* 2004; Konjhodžić *et. al.* 2004) have P_E values significantly higher than 0.9999, due to the increased number of STR loci used in each kit.

PI values ranged from $1x10^{-12}$ – $4.1x10^{7}$ (Figure 5.9), with 79% of the non-excluded data having a value >1000 and 89% higher than 100. The negative PI values were calculated in families where the man was excluded from being the biological father by more than two mismatched loci. True exclusion assesses each locus individually and allows a person to be excluded if one loci mismatches. In practice, mutation can occur leading to a mismatch at one or two loci, and this is compensated for in statistical calculations (Chakraborty *et. al.* 1999; Amorim and Pereira 2005). In STRs, two loci must mismatch in order for a man to be excluded from being the alleged father (http://dna-view.com/mudisc.htm; Ayres 2005).

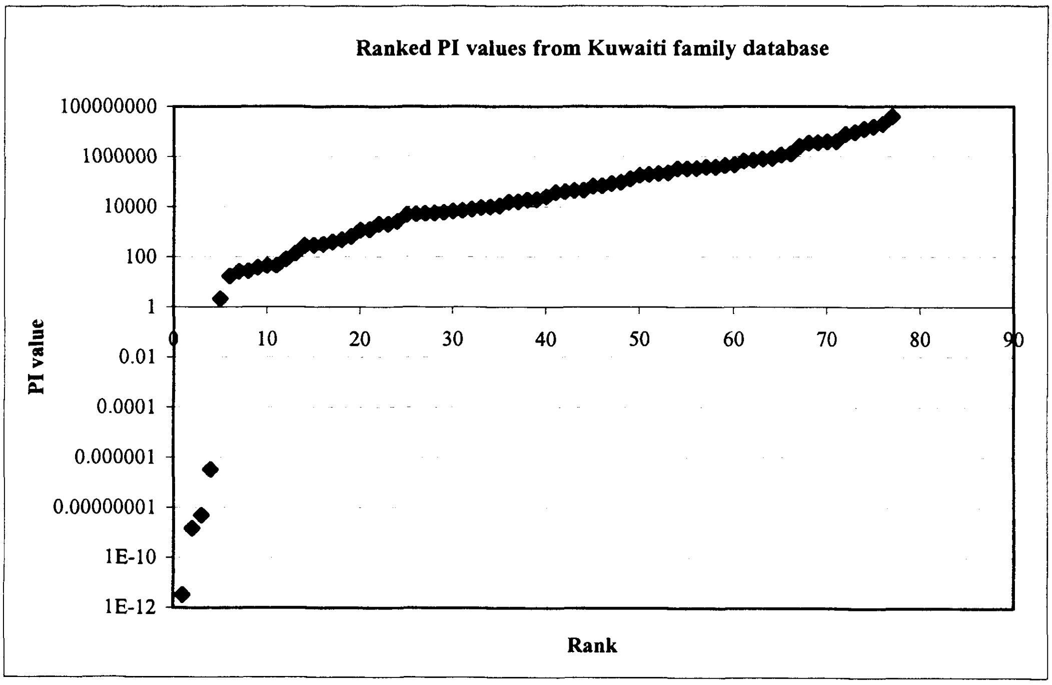


Figure 5.9 A scattergraph of values observed for the paternity index (PI), calculated for each child-alleged father relationship in the Kuwaiti family samples.

PI values of less than zero were tabulated as zero as the probability of the alleged father being the true father became insignificant due to exclusion at more than one locus (Table 5.1). A PI of zero was evident in two different families. Child 6G in family 6 had three mismatched loci when compared to the candidate father (father 6), leading to a PI value of zero. STR profiling and further family investigation indicated that this man was unrelated to child 6G (Figure 5.10). In family 8, three offspring had loci mismatching with the alleged father (father 8), giving PI values of zero (Table 5.6). Child 8A had five loci with no match to the alleged father, child 8D had four mismatches and child 8E had three mismatching loci. Again, STR profiling and family investigations found this man to be unrelated to the three offspring (Figure 5.11).

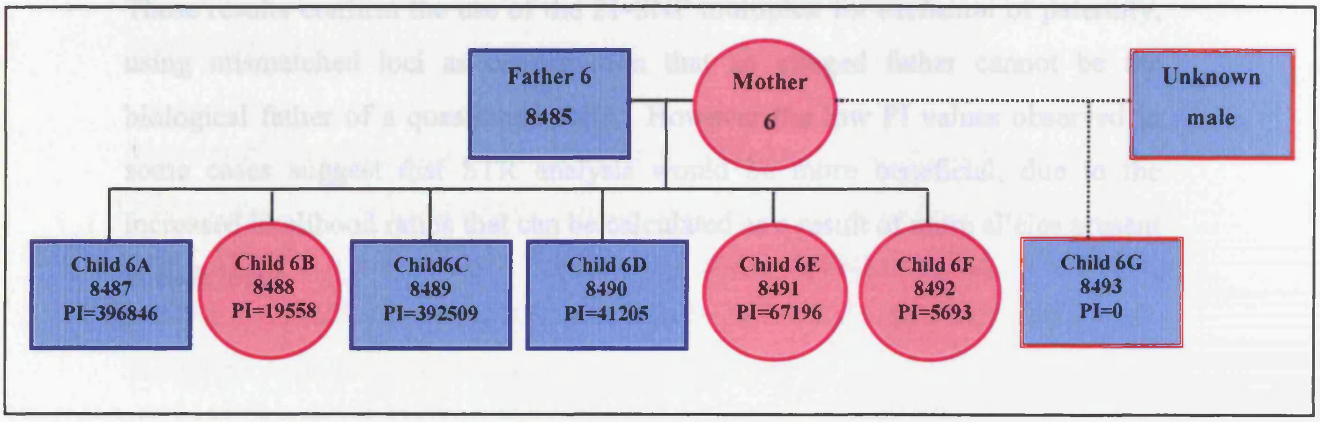


Figure 5.10 Family tree for family 6 depicting the relationships between the seven offspring genotyped and the parents.

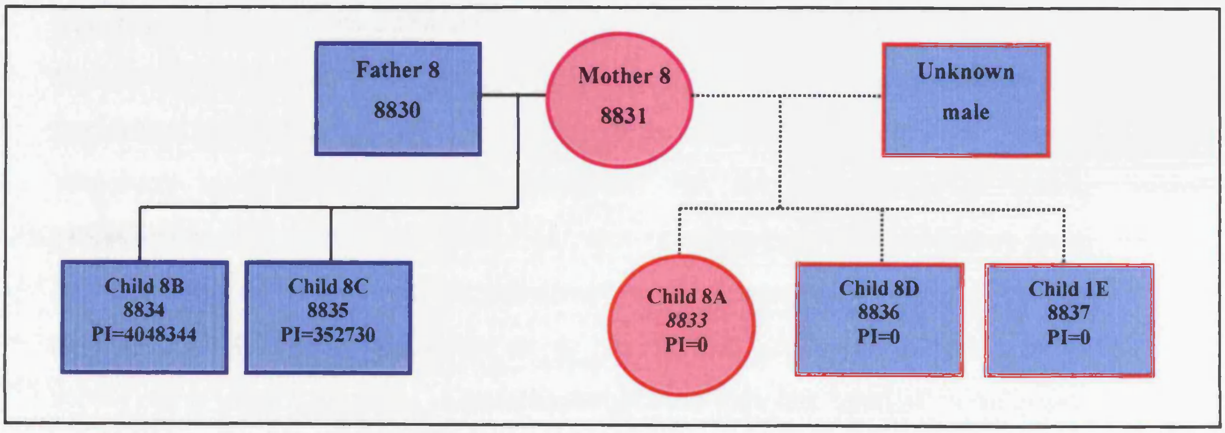


Figure 5.11 Family tree for family 8 depicting the relationships between the five offspring and the parents.

All offspring in families 3, 4, 5, 8, 9, 10, 11 and 13 had PI values >100, supporting the case for paternity against the alleged father. The values ranged from 137 to >40,000,000, dependent on the genotypes of the offspring, the known mother and the alleged father. The samples with the highest PI values were shown to have rare alleles at certain SNP loci, greatly increasing the significance of the result (see appendix VIII for full SNP genotypes). Some PI values were less than the threshold value of 100, although paternity was confirmed using STR analysis. In these cases, all loci matched the alleged father but the allelic frequencies, once the mother's alleles had been removed, were too high to give a significant result compared to an unrelated man from the general population. This is most likely due to the biallelic nature of the SNPs tested.

These results confirm the use of the 21-SNP multiplex for *exclusion* of paternity, using mismatched loci as confirmation that an alleged father cannot be the biological father of a questioned child. However the low PI values observed in some cases suggest that STR analysis would be more beneficial, due to the increased likelihood ratios that can be calculated as a result of more alleles present at each locus.

5.8 Discussion

It is important to fully recognise the attributes of the DNA profiling system in use for forensic identification. Factors such as sub-structuring of populations, as well as sampling error and linkage distances can affect the strength of the evidence obtained. The population studies carried out on the 21-SNP multiplex incorporated an analysis of the main types of irregularities, including linkage disequilibrium, Hardy-Weinberg proportions and a calculation of F_{ST} to allow correction factors to be used when calculating match probabilities. All SNP loci demonstrated Hardy-Weinberg equilibrium and no linkage disequilibrium between SNPs (including those on the same chromosome). The minimum distance between SNPs on the same chromosome was over 33Mb, sufficient distance to ensure that multiple recombination events would result in linkage equilibrium between any pair of loci (Petes 2001).

Many studies on the population genetics of STR loci have been carried out as a consequence of these loci being routinely used in forensic casework across the world (Devlin *et. al.* 1990; Gill *et. al.* 1991; Nichols and Balding 1991; Steinberger *et. al.* 1993; Balding and Nichols 1994; Budowle 1995; Deka *et. al.* 1995; Gill and Evett 1995a; Balding *et. al.* 1996; Evett *et. al.* 1997; Foreman *et. al.* 1998; Foreman and Lambert 2000; Foreman and Evett 2001; Ayres *et. al.* 2002a; Curran *et. al.* 2003; Overall *et. al.* 2003). The National Institute of Standards and Technology (NIST) currently holds references for over 750 STR population studies on its STRbase™ website (Ruitberg *et. al.* 2001). The criteria for selection of STR loci can be applied to the selection of SNP loci as traditional population genetics analyses holds for both types of DNA polymorphism. Hardy-Weinberg equilibrium (HWE) is essential for the calculation of match probabilities and subsequent likelihood ratio calculations. Any deviation from HWE is suggestive of population sub-structure and / or sampling errors (Evett *et. al.* 1996; Weir 1996; Buckleton *et. al.* 2005) and is likely to give a non-conservative likelihood ratio. A sample size greater than one hundred is sufficient to provide enough genotype information to project the frequency of alleles in a larger population (http://www.promega.com/geneticidproc/ussymp8proc/13.html; Chakraborty 1992). In this study less than 100 individuals were available for the

African-Caribbean and Indian sub-continent databases, meaning the true allele frequencies may deviate slightly from those calculated here.

The allele frequencies at a particular locus are not fixed within the population and change over time as a consequence of mutation and genetic drift, however in any particular population, allele frequencies should maintain HWE. Admixing between different populations can cause deviation from HWE and leads to phenomena such as the Wahlund effect (Wahlund 1928), where excess homozygosity is observed within a population due to the presence of sub-populations with different allele frequencies. In reality, the presence of sub-structuring within a population is minimal and has a much less severe affect. In spite of this, it is important to include an estimate of sub-population bias in all calculations for forensic purposes (Balding and Nichols 1994). As members of the same sub-population are more likely to share common alleles, i.e. alleles identical by descent, the allele frequencies within that sub-population will give a lower likelihood ratio than would be found in the general population (Budowle 1995; Foreman *et. al.* 1998; Foreman and Lambert 2000). For calculation of DNA profiles present on the UK National DNA Database®, three broad allele frequency databases are used: Caucasian, African-Caribbean and Indian sub-continent and the BN correction (Equation 5-1) is applied to all LR calculations. The value for θ is routinely set to 0.01 for Caucasians and 0.03 for all other ethnic groups, based on analyses of STR population databases in the 1990s and the recommendations of the National Research Council in 1994 (Budowle 1995; Gill and Evett 1995a; Balding *et. al.* 1996; NRCII 1996; Foreman *et. al.* 1998). Both of these estimates are highly conservative and represent extreme values of inbreeding and/or genetic drift for all populations.

A real population of forensic interest will contain several sub-populations that will not be completely separate, and may exist as several levels of geographical and social structure. There are a large number of studies which report estimated values for θ for different populations and sub-populations (Bowcock *et. al.* 1991; Kidd *et. al.* 1991; Morris *et. al.* 1991; Lin *et. al.* 1994; Weir 1994; Foreman and Lambert 2000; Zarrabeitia *et. al.* 2003). Values are highly variable dependent on the population under consideration, but all are significantly lower than 0.03, with

Caucasian populations having $\theta < 0.01$ in most instances. The populations found to have the highest values of θ are the same cultures with the highest levels of consanguinity. Globally, the most common form of consanguineous union is between first cousins, in which spouses share 1/8 of their genes inherited from a common ancestor, making their progeny homozygous at 1/16 of all loci (Bittles 1998). The level of consanguineous unions is directly related to the religious, ethnic and / or tribal traditions of a population. For example, in Christianity, the Orthodox churches prohibit consanguineous marriage, the Roman Catholic church currently requires Diocesan permission for marriages between first cousins, and the Protestant denominations permit marriages up to and including first cousin unions (Bittles 1998). Within the Islamic and Buddhist communities, first cousin marriages are permitted, but are forbidden by the Sikh religion. The increasing diversity of religious factions within Western countries, due to immigration and across-country marriages, is leading to an increasing number of individuals sharing a common ancestor. Migrant communities permanently gaining residence in Western countries demonstrate an increased level of within-community marriage, further sub-dividing the population. It is due to these sub-populations that allele frequencies for whole populations need to be corrected for, to allow for higher levels of inbreeding within some sub-populations (Zhivotovsky *et. al.* 2001).

Due to a lack of available sub-population data, simulations were carried out using $\theta = 0.01$ and 0.03, to assess the effect of inbreeding on SNP allele frequencies, over a number of generations. The method was derived from simulations for STR loci carried out by Curran *et. al.* (2003). The resulting genotype frequencies were then used to calculate LR values for each sub-population and these values were compared to LR values for the whole population, calculated using the BN correction for F_{ST} (Balding and Nichols 1994). The BN correction was found to be conservative compared to the simulated sub-populations, suggesting the BN correction was sufficient for SNP LR calculations.

The Exact Test data for linkage disequilibrium (LD) (Weir 1996; Lewis and Zaykin 2001) indicated that there was no deviation from what would be expected within a randomly-mating population, for all three populations tested –white

Caucasian, British afro-Caribbean and Indian sub-continent. LD occurs when alleles at two loci occur together in gametes more frequently than expected given the known allele frequencies and recombination fraction between the two loci. Evidence for linkage disequilibrium can be helpful in mapping disease genes since it suggests that the two loci may be very close to one another in the genome. The Wahlund effect was demonstrated on an artificially created population based on white Caucasian and Indian sub-continent data. The Exact Test p-values deviated significantly from that expected from the random number matrix and indicated the variation expected if the populations under study were effectively sub-structured. This simulation also demonstrated the ability of the 21-SNP multiplex to effectively illustrate the presence of sub-structuring within a population. All SNPs were shown to be in linkage equilibrium, i.e. all loci are independently inherited. As a consequence of this, all loci can be multiplied together using the product rule for match probability, and subsequent LR, calculations. LD studies using SNP loci are increasingly used for disease mapping in the human genome (Terwilliger and Weiss 1998; Kruglyak 1999; Collins 2000; Collins *et. al.* 2001; Ennis *et. al.* 2001; Saunders *et. al.* 2001; Halldorsson *et. al.* 2004; Kuno *et. al.* 2004). LD can also be used as an indicator of population admixture, as demonstrated by the artificially-created population in this study (Lin *et. al.* 1994; Zaykin *et. al.* 1995; Smith *et. al.* 2001; Gu and Rao 2003).

The independent inheritance of alleles is also important when it comes to paternity testing using SNP loci. It is essential that each locus is independently inherited in order to maximise the evidential value of the test. The paternity index (PI) is the most commonly used form of statistical evidence in the case of paternity analysis (Butler 2005b). PI is calculated using a likelihood ratio examining the chance of seeing the genotypes of each locus in a random unrelated individual compared to the suspected father. PI values for thirteen different families were calculated from SNP genotype data using the computer program CERVUS (http://helios.bto.ed.ac.uk/evolgen/cervus/cervus.html; Marshall *et. al.* 1998; Slate *et. al.* 2000). 89% of the PI values were higher than the accepted minimum value of 100 and two men were excluded as biological fathers of four offspring, using the observation of over two mismatched loci as a guideline.

SNPs have been investigated as a potential alternative to STRs in paternity analysis. SNPs have a lower mutation rate compared to STRs, due to their biallelic nature, and the chance of positively excluding a potential male through mutation at some loci is decreased (Amorim and Pereira 2005; Ayres 2005). However STR calculations have been derived which incorporate the chance of a mutational event having occurred, thus allowing a more stringent approach to paternity analysis using STR loci (Dawid *et. al.* 2001; Ayres 2002b).

Many studies on paternity analysis using SNPs employ an assessment of the probability of exclusion (P_E). This approach is known as the 'frequentist approach' as it considers the probability of the evidence under one hypothesis (Buckleton *et. al.* 2005). In the case of paternity analysis, the frequentist approach uses the value of P_E as a method of excluding a non-related random man from the general population, however it makes no inference to the data supplied from the alleged father. From the data generated for the Kuwaiti families, the PI value was found to be more informative than the P_E value. For child 6G and 8A (Table 5.6) a P_E value >0.99 was observed yet the PI values for both offspring was less than zero, due to mismatched loci. This suggests that P_E is only useful if the alleged father is a potential parent as it gives the probability of a random person being excluded as a potential parent.

Computer simulations assessing the utility of SNPs for paternity analysis suggest that SNPs with an allele frequency of 0.5 are the most discriminating and a set of 40-50 loci would be capable of giving a PI and P_E value similar to STRs (Chakraborty *et. al.* 1999; Amorim and Pereira 2005; Ayres 2005). The 21-SNP multiplex assessed in this study incorporated loci with allele frequencies between 0.1 and 0.9 (Table 5.3). This led to decreased values for PI except in families where loci with low allele frequencies were present. In cases with rare genotypes the PI was increased to greater than one million. The 21-SNP multiplex was capable of excluding an alleged father as the biological father by assessing the number of mismatched loci present, however some PI values fell below the accepted minimum of 100. This suggests that this multiplex does not contain sufficient loci to be as discriminating as current STR methods of parentage analysis.

6 European Collaborative Degradation Study

6.1 Introduction

Chapters 3, 4 and 5 outlined the development and validation of a SNP multiplex system for use as an alternative forensic DNA profiling method (Dixon *et. al.* 2005a). The discrimination power of the 21-SNP multiplex (approximately one in five million) is lower than that gained if a full profile were obtained from STRs (approximately one in a thousand million), therefore its use is limited to situations where routine DNA profiling is not possible.

A discussion held by the joint European Network of Forensic Science Institutes (ENFSI), European DNA Profiling Group (EDNAP) and Scientific Working Group on DNA Analysis Methods (SWGDAM) made recommendations on the development of DNA profiling for degraded samples within the forensic community (Gill *et. al.* 2004a). Existing Short Tandem Repeat (STR) systems used in European national DNA databases (NDNADBs) include seven core STR loci recommended by ENFSI. The core loci[6] are included in commercially available multiplexes (Cotton *et. al.* 2000; Collins *et. al.* 2004; Greenspoon *et. al.* 2004). However, all current markers are relatively high in molecular weight (between 150 – 450 bp) (Gill 2002).

It has been demonstrated that smaller amplicons are much more likely to be amplified in samples containing degraded DNA (Hellmann *et. al.* 2001; Wiegand and Kleiber 2001; Krenke *et. al.* 2002; Ohtaki *et. al.* 2002; Butler *et. al.* 2003; Chung *et. al.* 2004; Schumm *et. al.* 2004; Coble and Butler 2005; Butler 2005b). There are two kinds of markers that can bring the size of the amplicon substantially below 150 bp: 'mini-STRs' that have short flanking regions to the tandem repeat sequence and SNPs.

A small number of validated SNP assays are used in casework and these include mini-sequencing assays for mitochondrial DNA (mtDNA) (Tully *et. al.* 1996; Coble *et. al.* 2004; Just *et. al.* 2004; Vallone *et. al.* 2004), Y chromosome (Sanchez *et. al.* 2005), a red hair marker assay (Grimes *et. al.* 2001) and autosomal multiplexes (Dixon *et. al.* 2005a).

[6] Core European STR loci - THO1, VWA, D21S11, FGA, D3S1358, D8S1179, D18S51.

To achieve the ultimate lower limit of small amplicons, SNPs are preferable, but the downside is that a panel of 45-50 loci would be needed to achieve match probabilities comparable with existing STR multiplexes (Chakraborty *et. al.* 1999; Gill 2001a). Furthermore, the larger the multiplex, the more difficult it is to reliably and to reproducibly construct (Budowle 2004b); loss of amplification efficiency may ensue, effectively defeating the object of the exercise. To circumvent this problem, several SNP multiplexes of a dozen loci each can be used in concurrent multi-tube reactions, however the sample size needs to be sufficient to allow this option (Bell *et. al.* 2002; Inagaki *et. al.* 2004). Large amounts of DNA from, e.g. bones, can be analysed in this way, but the study of many small forensic stains is precluded, as the amount of DNA extract available is limited. In addition, the binary nature of SNPs means that their statistical characteristics are not amenable to the interpretation of complex samples such as mixtures. A robust, highly quantitative SNP assay would be required to allow determination of mixtures using an interpretation strategy based on heterozygous balance and homozygous thresholds (Gill 2001a).

Mini-STR multiplex systems have been developed as an alternative method of DNA profiling using traditional STR genetic markers (Butler *et. al.* 2003; Chung *et. al.* 2004; Drabek *et. al.* 2004). Primers are designed closer to the STR locus, sometimes overlapping the repeat region. This approach has the benefit of shortening the length of the amplicon whilst maintaining the higher discrimination powers seen with STRs as opposed to SNPs. The resulting profiles are also consistent with STR profiles currently used in national DNA databases, meaning a direct comparison can be made. Problems can be encountered due to the polymorphic nature of STRs, making the primer binding sites more prone to primer-binding site variants. This can increase the number of null alleles seen and can lead to a higher number of imbalanced peaks within a profile.

It was agreed that markers such as SNPs have the potential to usefully complement existing STR systems, but there was no published data comparing the performance of STRs against SNPs at the time. Furthermore, there was no comparative data published on the performance of other DNA profiling techniques, such as low copy number (LCN) DNA profiling using SGM+ or mini-STR multiplex systems, when looking at degraded DNA samples.

I set up a collaborative study to assess the different DNA profiling techniques available throughout Europe and the United States for their usefulness in genotyping artificially degraded samples. The study was designed to assess the extraction method employed, the current DNA profiling technique used and the effectiveness of new profiling techniques (SNPs and mini-STRs). Experimental work and sample genotyping was carried out by each individual laboratory and results were sent to myself electronically for data analysis and interpretation (Dixon *et. al.* 2005b).

6.2 Materials & Methods

6.2.1 Production of degraded DNA samples

A new set of artificially degraded samples was created, to supply enough samples for each laboratory involved in the collaboration (Pulker 2004). Liquid blood and saliva samples were collected from two unrelated, white Caucasian volunteers (one male, one female), whose SNP loci had been fully sequenced as part of the 21-SNP multiplex validation. For artificial degradation, samples were set up in 0.2 ml PCR tubes containing a 4mm^2 cotton square saturated with 25 μL sterile deionised water. 5 μL of whole blood or 10 μL of saliva was added directly to each cotton square.

Samples were placed in an incubator at 37°C, to accelerate enzymatic degradation. The individual tubes allowed each sample to be contained within a "micro-environment" and prevented any loss of material by leeching and, because the tubes were manufactured for PCR, there would be no loss of moisture by evaporation. As the cotton remained saturated the environment immediately associated with the sample maintained 100% humidity. The stains were sampled at specific time intervals and then frozen at -20°C to suspend the degradation process; 44 samples of each type were frozen at each time interval to provide a store for the collaborative study.

6.2.2 Exercise design

Each laboratory was supplied with one 'Foren-SNPs' multiplex kit[7] (The Forensic Science Service®, UK), one mini-SGM miniplex kit and one NC01 mini-STR kit (National Institute of Standards and Technology (NIST) laboratory, US), as well as sets of artificially degraded DNA stains as follows:

Reference 1 (3 or 4 stains for each time point):

Blood samples – 0,2,8,16 weeks degradation
Saliva samples – 0,2,8,12 weeks degradation

[7] Trademark name given to the 21-SNP multiplex system outlined in chapters 4-6.

Reference 2 (3 or 4 stains for each time point):

Blood samples – 0,2,8,16 weeks degradation
Saliva samples – 0,2,8,12 weeks degradation

Each laboratory was asked to perform the following procedures:

- Extract the DNA stains using standard laboratory protocols;
- Pool each extract for each individual at each time point (Figure 6.1);
- Perform standard DNA profiling techniques on all samples (standard being defined as the DNA profiling technique routinely used for analysis of samples in the laboratory carrying out the work);
- Amplify the samples using the SNP multiplex kit provided (each lab was provided with one Foren-SNPs™ kit comprising primer mix, reaction mix and Amplitaq Gold®);
- Amplify the samples using the mini-STR kits provided (each kit contained primer mix and protocol; labs provided their own Amplitaq Gold® and PCR buffer);
- Record all results on the spreadsheets provided, to allow easy collation of all data.

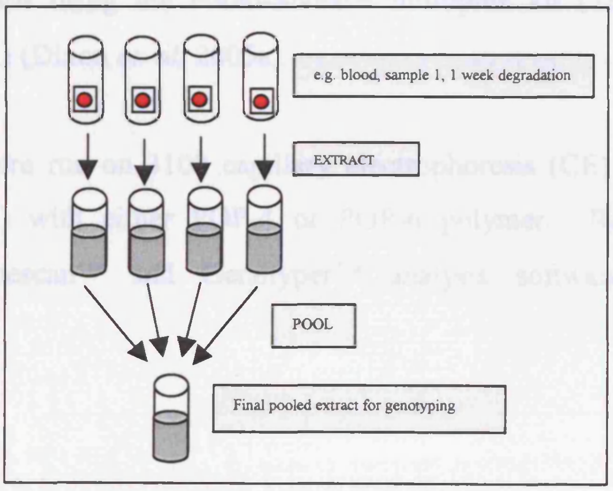

e.g. blood, sample 1, 1 week degradation
EXTRACT
POOL
Final pooled extract for genotyping

Figure 6.1 Preparation of artificially degraded DNA extracts. Separate extractions from the same degradation period were pooled together to minimise the likelihood of stochastic variation from the cotton stains.

6.2.3 Experimental protocols

DNA extractions were carried out using either QIAamp mini-kits (Cat. No. 51306) or QIAshredder (Cat. No. 79656) supplied by Qiagen™ (Scherczinger *et. al.* 1997; Sinclair and McKechnie 2000), Chelex-100 (Walsh *et. al.* 1991), or by phenol chloroform methods (Dimo-Simonin and Brandt-Casadevall 1996).

Samples were quantified using Picogreen (Ahn *et. al.* 1996), Quantifiler™ Human DNA Quantification kit (Cat. No. 4343895) or Slot-blot methodology (Waye *et. al.* 1991). One laboratory performed quantification using a real-time quantitative PCR assay with a fluorogenic Taqman probe, targeting the human Alu repetitive sequence, with PCR primers adopted from Nicklas and Buel (Nicklas and Buel 2003).

Each laboratory used STR multiplex kits according to the manufacturer's protocol. The following STR kits were used in the study: Either AMP*Fl*STR® SGM Plus™ (Applied Biosystems) (Cotton *et. al.* 2000), AMP*Fl*STR® Identifiler (Applied Biosystems) (Collins *et. al.* 2004) or Powerplex®16 system (Promega) (Greenspoon *et. al.* 2004); plus mini-SGM and miniNC01 (National Institute of Standards and Technology (NIST), US) (Butler *et. al.* 2003). SNP analysis was carried out by all labs using the Foren-SNPs™ multiplex kit (The Forensic Science Service®, UK) (Dixon *et. al.* 2005a).

All PCR products were run on 3100 capillary electrophoresis (CE) sequencers (Applied Biosystems) with either POP-4 or POP-6 polymer. Results were analysed using Genescan™ and Genotyper™ analysis software (Applied Biosystems).

6.2.4 Data analysis

Each laboratory was given an identifier number and genotyping results for each DNA profiling system for each laboratory were collated on Microsoft® Excel spreadsheets.

Genotypes were analysed as percentages – e.g. for SGM+ a full genotype comprised 22 alleles; thus a profile with 11 alleles was 50% of a full profile. Converting into percentages allowed direct comparisons between the different multiplex systems. Data were analysed with Minitab™ Release 14 software using analysis of variance (ANOVA), box-whisker plots and median polish analysis (Tukey 1977).

Median polish analysis was carried out in order to standardise the data, allowing data sets from all laboratories to be compared regardless of variability in laboratory techniques, operator differences and sampling limitations (Tukey 1977).

Identifiler® and Powerplex®-16 were omitted from the final results analysis, except for the inter-laboratory comparison, because only one laboratory used each multiplex. Low copy number (LCN) SGM+ results were also disregarded from intra-laboratory analyses, because only two laboratories submitted data.

6.3 Results

Results were received from nine laboratories from six European countries and the United States. All laboratories provided data from one standard STR multiplex kit plus the Foren-SNPs™ kit and mini-STR kits (Figure 6.2).

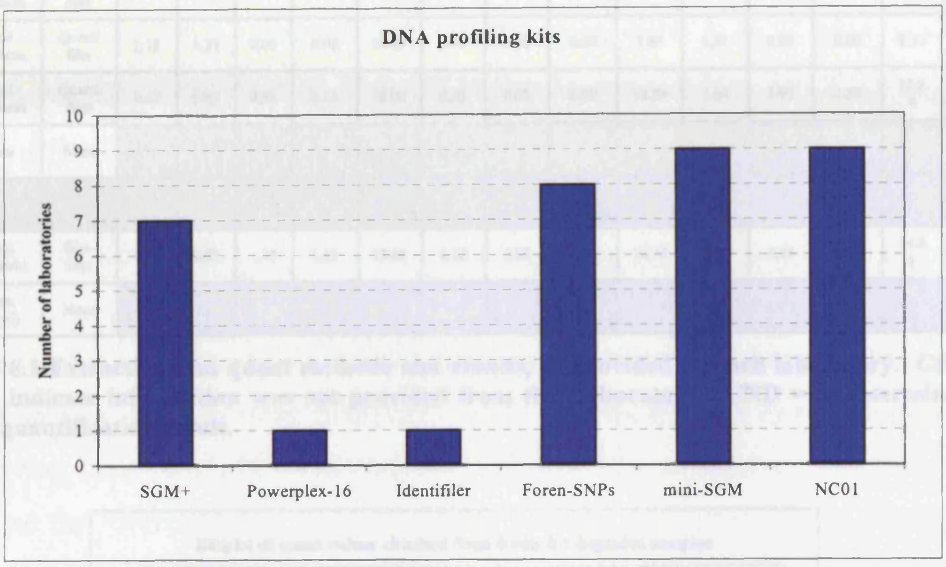


Figure 6.2 DNA profiling kits used for the collaborative study. NB. One lab did not submit data for the Foren-SNPs™ kit.

6.3.1 Extraction methods

Details of extraction techniques and corresponding quant values were submitted from six laboratories (Table 6.1). Quant values ranged from 0 ng/µL for heavily degraded samples to 33 ng/µL for a reference sample stain, with the range of values varying greatly from laboratory to laboratory, especially for the reference samples (Figure 6.3).

			Quant values (ng/µL)															
			Ref 1 blood				Ref 1 saliva				Ref 2 blood				Ref 2 saliva			
Lab ID	Extraction protocol	Quant	0wk	2wk	8wk	16wk	0wk	2wk	8wk	12wk	0wk	2wk	8wk	16wk	0wk	2wk	8wk	12wk
1	Qiagen (manual)	Picogreen	1.91	0.22	0.22	0.03	1.03	0.07	0.01	0.01	2.13	0.23	0.23	0.02	0.36	0.03	0.03	0.01
2	Qiagen (robot)	Quantifiler	0.63	0.01	0.00	0.00	0.82	0.00	0.00	0.00	0.72	0.01	0.01	0.00	0.36	0.00	0.00	0.00
3	Phenol chloroform	Quantifiler	2.31	0.22	0.06	0.02	6.59	0.00	0.00	0.00	5.16	0.92	0.25	0.46	8.95	0.06	0.01	0.03
4	Phenol chloroform	Quantifiler	2.18	1.29	0.00	0.00	UND	0.00	0.00	0.00	3.65	2.35	0.96	0.00	8.31	0.00	0.00	0.00
5	Phenol chloroform	Quantifiler	9.67	0.93	0.53	0.12	19.00	0.10	0.03	0.03	10.29	1.64	1.97	1.58	15.84	0.06	0.15	0.06
6	Chelex	None																
7																		
8	Phenol chloroform	Slot-blot	11.79	0.57	1.00	1.68	33.46	0.12	0.03	0.05	10.15	2.33	4.13	0.80	14.88	0.36	0.23	0.05
9	Qiagen (manual)	None																

Table 6.1 Extraction and quant methods and results, as provided by each laboratory. Grey boxes indicate information was not provided from that laboratory. UND = undetermined DNA quantification result.

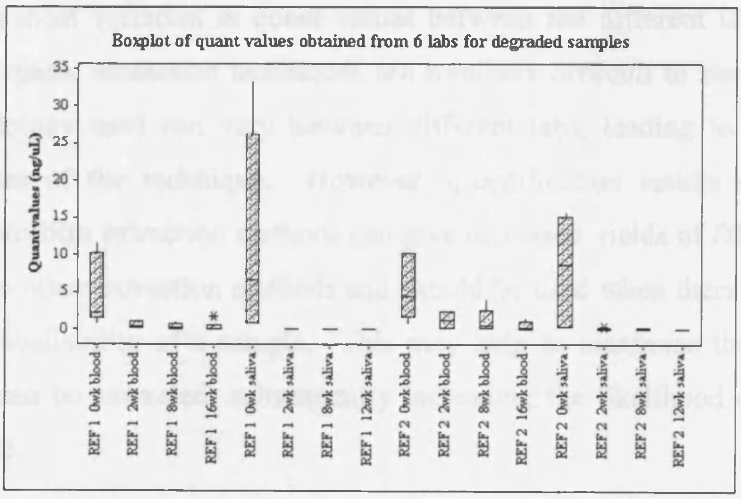


Figure 6.3 Box and whisker plot showing the range of quant values received for each reference individual for each sample type. Calculations are based on data submitted from six out of the nine participating laboratories.

The range of values seen for the degraded samples (i.e. samples ≥2 wks incubation) was smaller for saliva samples compared to blood samples. The inter-quartile (IQ) range for degraded saliva samples varied from 0.03 ng/µL to 0.17 ng/µL, compared to 0.5 ng/µL to 2.3 ng/µL for blood samples. All results gained showed a DNA concentration of <2.5 ng/µL.

In comparison, undegraded control (time zero) reference samples showed considerable variation in the amount of DNA recovered between laboratories. More DNA was recovered with phenol-chloroform compared to Qiagen™ but the variation was much greater in the former (IQ range = 27 ng/µL and 1.4 ng/µL respectively) (Figure 6.3). The method of quantification may have affected the DNA quantification values gained. Both laboratories using phenol chloroform extraction followed by Quantifiler™ quantification (labs 3 & 4) gave similar values, whereas quantification with qPCR (lab 5) and slot-blot (lab 8) produced much greater values (Table 6.1). However, all phenol chloroform values (for control samples) were greater than those gained with Qiagen™, regardless of the quantification method.

The variation seen between different extraction techniques was less marked as the DNA samples became more degraded (Figure 6.3), most likely as a consequence of having little or no DNA available to extract. Phenol chloroform extraction showed the most variation in quant values between the different labs (data not shown). Organic extraction techniques are routinely difficult to standardise and the methodology used can vary between different labs, leading to variation in success rates of the technique. However, quantification results suggest that phenol chloroform extraction methods can give increased yields of DNA template compared to other extraction methods and should be used when there is known to be limited availability of a sample. This may help to maximise the amount of DNA that can be extracted, subsequently increasing the likelihood of gaining a DNA profile.

6.3.2 Analysis of Variance (ANOVA) calculations

Sample data was run through Minitab™ Release 14 software to give ANOVA calculations (Table 6.2). Assuming a 95% confidence level, there was no significant difference found between the percentage profiles gained using the different multiplex kits (p=0.061), suggesting each kit gave comparable results for each sample and sample type. A significant difference was seen between results gained from different labs (p<0.001); from different individuals (p=0.006); different sample types – blood and saliva (p<0.001); and from the different degradation times. There was also a difference between the percentage profiles gained for the different individuals when looking at each sample type (p=0.044), i.e. there was no relationship between the results obtained for each blood sample or each saliva sample, independent of reference individual.

Analysis of variance (ANOVA) tests	**DF**	**SS**	**F**	**P**
Multiplex	3	11705	2.49	0.061
lab ID	7	96328	8.78	**0.000**
ref ID	1	11829	7.55	**0.006**
sample type	1	177422	118.4	**0.000**
degradation time	3	280931	59.78	**0.000**
Multiplex * lab ID	21	20122	0.61	0.909
Multiplex * ref ID	3	553	0.12	0.950
Multiplex * sample type	3	2293	0.51	0.676
Multiplex * degradation time	9	2949	0.21	0.993
lab ID * ref ID	7	5841	0.53	0.809
lab ID * sample type	7	15431	1.47	0.176
ref ID * sample type	1	6126	4.09	**0.044**
lab ID * degradation time	21	47521	1.44	0.098
ref ID * degradation time	3	2867	0.61	0.609
Multiplex * lab ID * ref ID	21	3545	0.11	1.000
Multiplex * lab ID * degradation time	63	20937	0.21	1.000
Multiplex * ref ID * degradation time	9	2765	0.2	0.994
lab ID * ref ID * degradation time	21	30254	0.92	0.566
Multiplex * lab ID * ref * degradation time	63	13408	0.14	1.000
Multiplex * lab ID * sample type	21	8114	0.26	1.000
Multiplex * ref ID * sample type	3	262	0.06	0.981
lab ID * ref ID * sample type	7	14042	1.34	0.231
Multiplex * lab ID * ref ID * sample type	21	3571	0.11	1.000

Table 6.2 ANOVA results for percentage profile data for each laboratory for each sample type using each multiplex kit.

There was no significance found in the results gained for the different multiplexes compared to any other factor. This suggested each multiplex could be used independently by any lab and it would be other factors that affected the result, i.e. sample type, individual sample or degree of degradation, as opposed to the multiplex used. There was also no intra-laboratory effect seen, i.e. if a lab performed well with one multiplex then it would also perform well with another, and vice versa.

There was no correlation found between the results gained for matching sample types for different individuals, i.e. the two saliva samples and two blood samples degraded at different rates to each other. More work would need to be carried out on different individuals to assess the degradation rates more fully.

6.3.3 Box and whisker plot analysis

ANOVA had indicated a significant difference between the results gained for each laboratory (section 6.3.2). Percentage profiles were calculated for each sample from each laboratory and the intra-laboratory variation was compared using box and whisker plots (Figure 6.4-6.7). Variation between labs would indicate the need for further statistical testing to allow a direct comparison of samples from the whole data set, otherwise data would appear skewed.

Variation was seen between the different labs for both individuals and sample types. Reference samples and the most highly degraded samples gave the least amount of variation between the data sets. Reference DNA samples generally had higher quant values, allowing an optimal amount of DNA (~1 ng) to be added to each amplification reaction. The addition of optimal DNA to each multiplex reaction would decrease the variability seen.

The most consistent multiplex across all laboratories was the mini-STR NC01 kit (Figure 6.7), with both reference blood samples and reference 2 saliva sample giving 100% profiles across all nine laboratories. This multiplex consisted of only three STR loci, D10, D14 and D22, not found in other commercially available STR kits.

It is important to take into account the relative number of loci present in each kit when assessing the results obtained. SGM+ contained 10 STR loci, plus Amelogenin; Powerplex-16 and Identifiler™ contained 15 STR loci, plus Amelogenin; Foren-SNPs™ contained 20 SNP loci plus Amelogenin; mini-SGM contained five STR loci plus Amelogenin and NC01 contained three STR loci. It is well-documented that an increase in the number of loci (and therefore an increase in the number of primer pairs used) within a multiplex makes amplification much less consistent as primers can interact with each other (Shuber *et. al.* 1995; Butler 2005a). As well as this, some primer pairs may be more efficient than others, giving an imbalance in the resulting profiles.

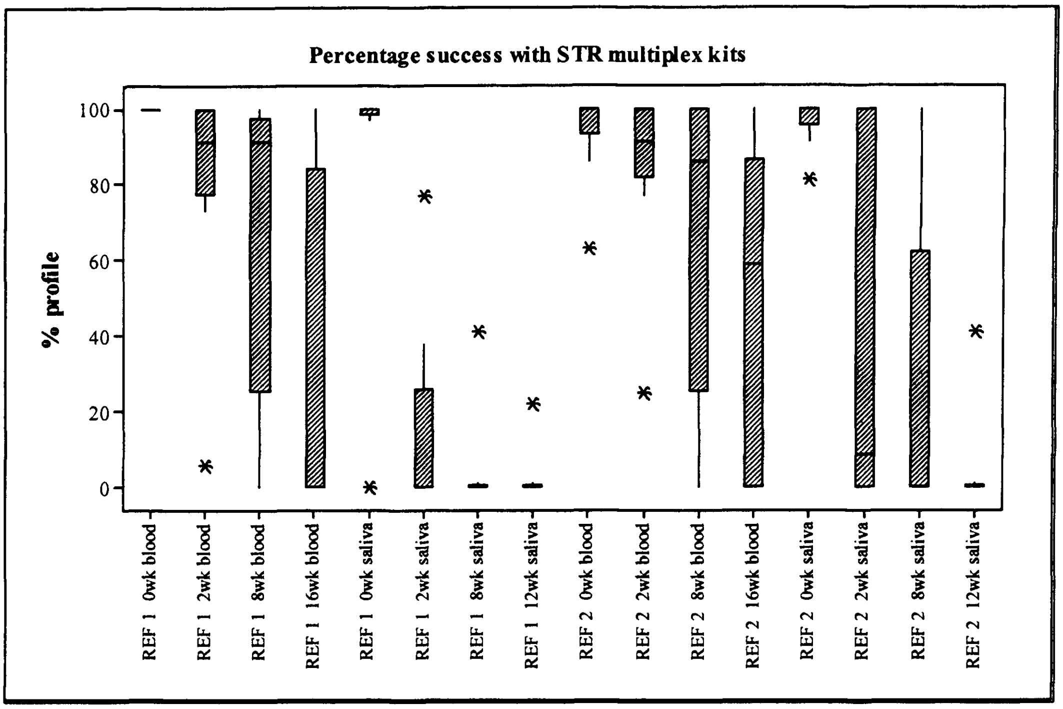


Figure 6.4 Box and whisker plot showing the variation in successful amplification per sample between the participating laboratories, using standard STR multiplex DNA profiling kits

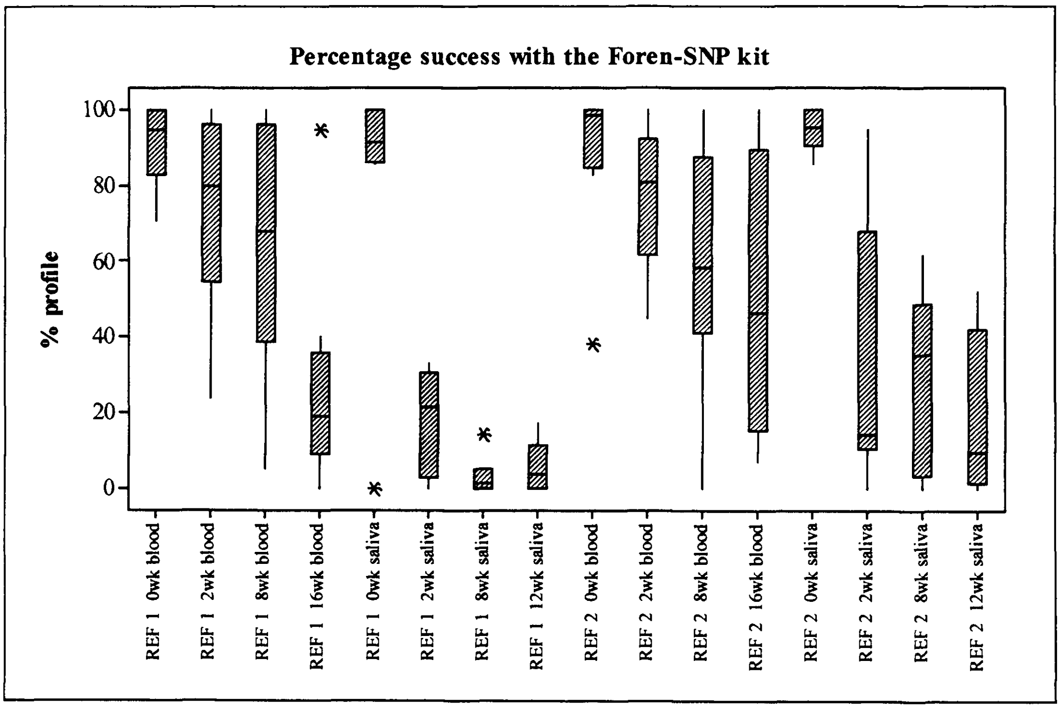


Figure 6.5 Box and whisker plot showing the variation in successful amplification per sample between the participating laboratories, using the Foren-SNP™ multiplex DNA profiling kit

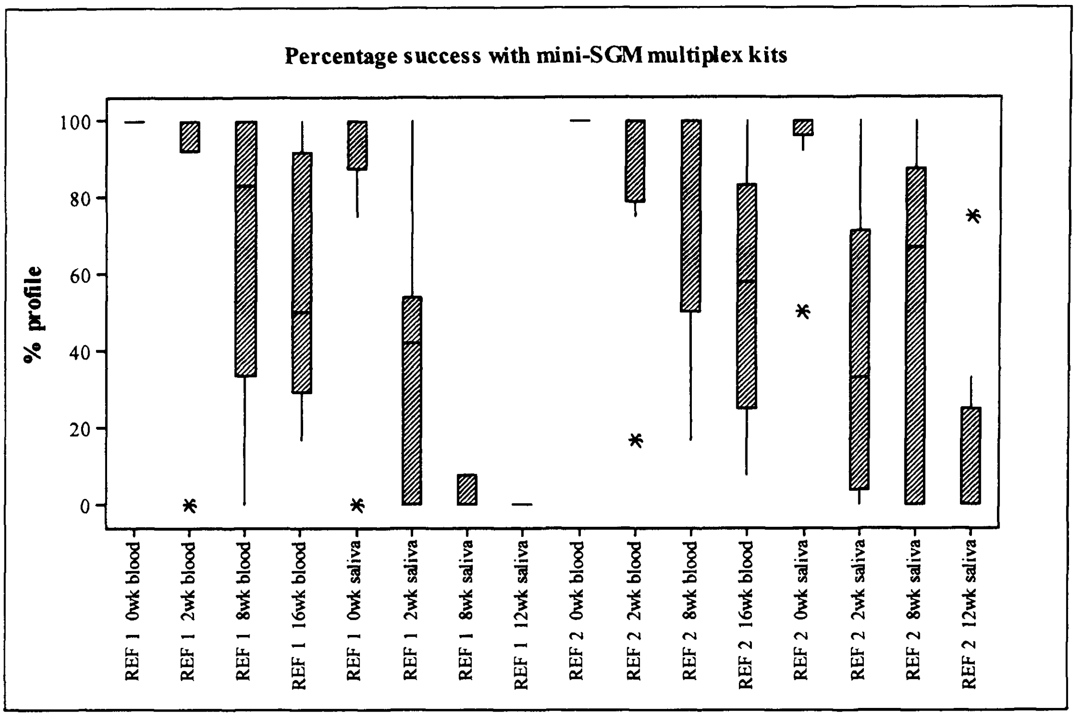


Figure 6.6 Box and whisker plot showing the variation in successful amplification per sample between the participating laboratories, using the mini-SGM DNA profiling kit

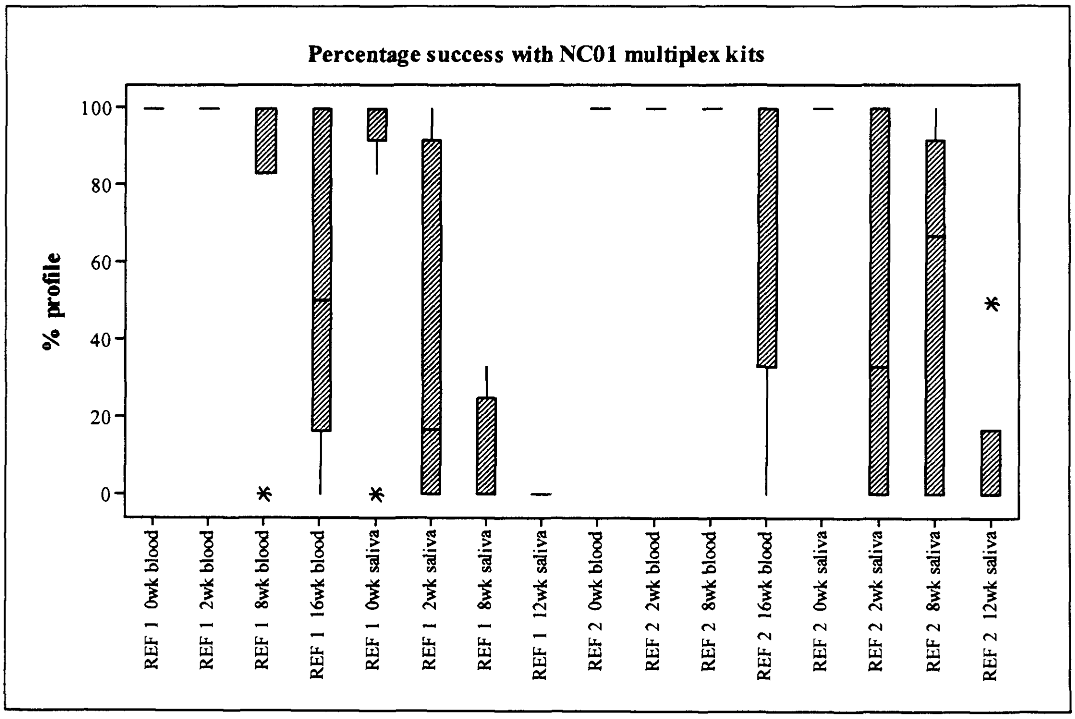


Figure 6.7 Box and whisker plot showing the variation in successful amplification per sample between the participating laboratories, using the NC01 mini-STR DNA profiling kit

6.3.4 Sample – sample variation

Due to variations between the different laboratories, results needed to be standardised before all data could be used independently of lab effect. This was carried out using Median polish analysis (Tukey 1977). Median polish analysis used the residuals of the data to allow a normalised data set to be used for analysis (Table 6.3), by transforming the data into residuals, analysis of variance became applicable.

Reference ID	Weeks degradation	Median polish values across laboratories (% profiles)				
		SGM+	Foren-SNPs	Mini-SGM	NC01	LCN SGM+
Ref 1 blood	0	100	92	100	100	100
	2	82	80	100	100	100
	8	80	67	83	100	100
	16	2	19	56	50	91
Ref 1 saliva	0	100	93	100	100	100
	2	2	19	42	17	100
	8	0	4	0	0	45
	12	0	9	0	0	18
Ref2 blood	0	100	94	100	100	100
	2	86	84	92	100	100
	8	82	66	85	100	82
	16	59	57	58	100	73
Ref2 saliva	0	100	95	100	100	100
	2	9	19	33	33	100
	8	0	28	67	67	100
	12	0	19	0	0	91

Table 6.3 Percentage profiles obtained for each sample using data from all labs analysed by Median polish calculations.

The data for each sample type and individual is shown graphically (Figure 6.8). As indicated by ANOVA tests (section 6.3.2), there was a significant difference in the profiles gained for each sample type (p = <0.001). This would be expected due to the different physiological characteristics of saliva and blood. DNA is much more prone to degradation in saliva due to the presence of bacteria and enzymes that can readily break down the DNA molecules.

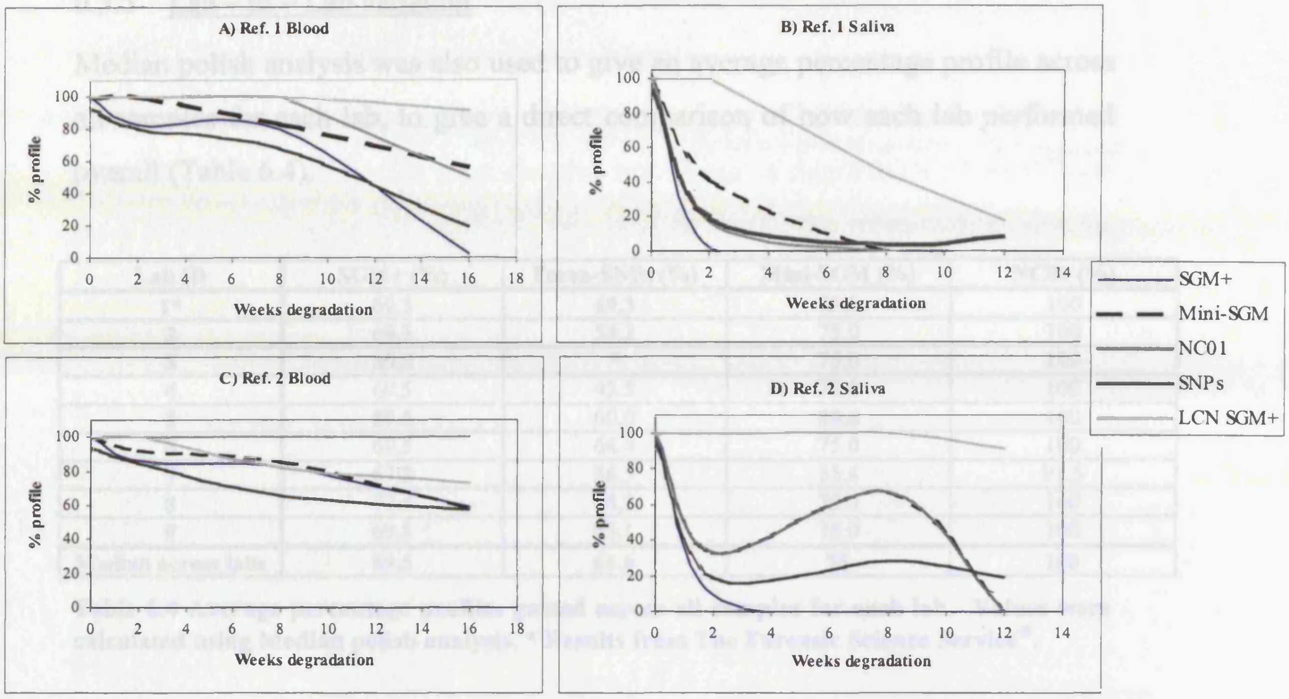


Figure 6.8 Percentage profiles obtained across all labs for all samples and sample types. A) reference 1 blood B) reference 1 saliva C) reference 2 blood D) reference 2 saliva. Values were calculated using median polish analysis to standardise the data obtained from all laboratories.

The four degradation series demonstrated different levels of degradation over the incubation period. The least degradation was seen in the reference 2 blood sample which still gave >50% profile, using all four profiling techniques, after 16 weeks degradation (Table 6.3). Reference 1 saliva showed the highest level of degradation, with the percentage profile obtained for each system (excluding LCN SGM+) dropping significantly after 2 weeks. LCN SGM+ continued to give a full profile until 8 weeks degradation.

All multiplexes showed similar degradation patterns with a particular reference and sample type but this pattern was not replicated between samples (Figure 6.8). LCN SGM+ worked significantly better in three out of four samples, compared to any other profiling method. The SGM+ profiling technique appeared to be the least efficient method, with Foren-SNPs™ working less efficiently than the two mini-STR systems.

6.3.5 Lab – to – Lab variation

Median polish analysis was also used to give an average percentage profile across all samples for each lab, to give a direct comparison of how each lab performed overall (Table 6.4).

Lab ID	SGM+ (%)	Foren-SNPs (%)	Mini-SGM (%)	NC01 (%)
1*	69.5	**69.3**	75.0	100
2	69.5	58.2	75.0	100
3	69.5	*	75.0	100
4	69.5	43.5	75.0	100
5	**89.5**	60.0	**89.6**	100
6	69.5	64.4	75.0	100
7	67.2	56.5	35.4	91.5
8	77.4	68.3	75.0	100
9	69.5	63.1	75.0	100
Median across labs	**69.5**	**61.6**	**75**	**100**

Table 6.4 Average percentage profiles gained across all samples for each lab. Values were calculated using Median polish analysis. * Results from The Forensic Science Service®.

The NC01 mini-STR multiplex kit performed the best overall, giving a median value of 100%. SNPs showed the most variation between the different labs (Figure 6.9) whereas the mini-STR multiplexes showed the greatest consistency.

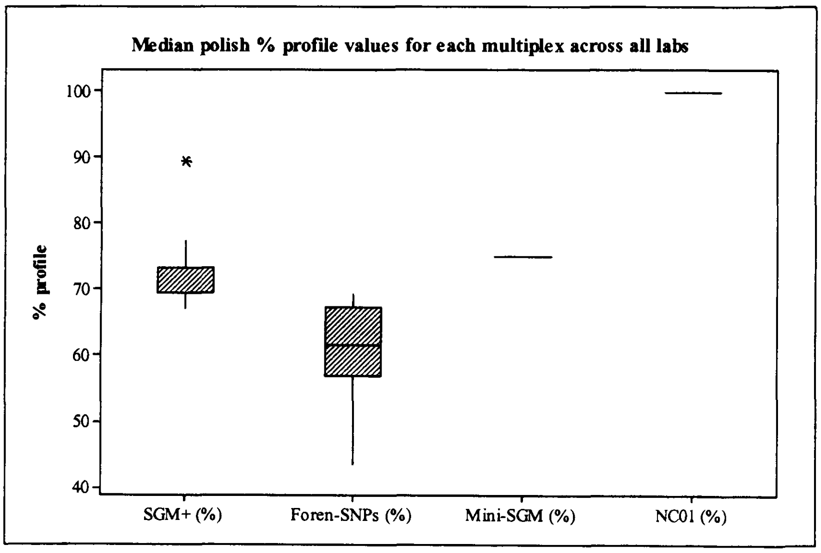


Figure 6.9 Box and whisker plot showing the variation seen in median percentage profiles across labs. Values were calculated using Median polish analysis.

6.3.6 Degradation patterns (allele dropout vs. amplicon size)

6.3.6.1 Total allele dropout in reference samples

Allele dropout was measured for each reference sample. Initial studies looked at total dropout for each reference sample, regardless of degradation time (Figure 6.10 & 6.11). Regression analysis was used to assess the relationship between amplicon size and the proportion of dropout seen.

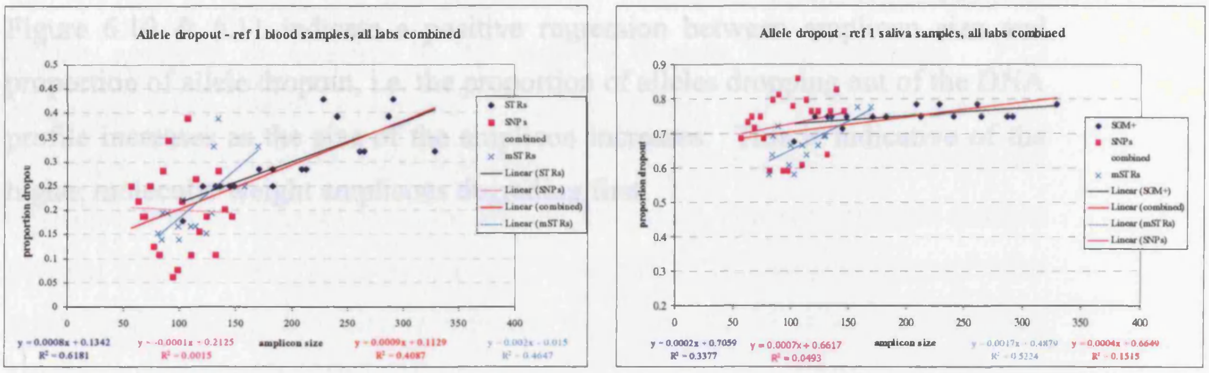


Figure 6.10 Allele dropout compared to amplicon size for reference 1 degraded blood and saliva samples. Proportion of allele dropout was calculated across the degradation set, regardless of incubation time.

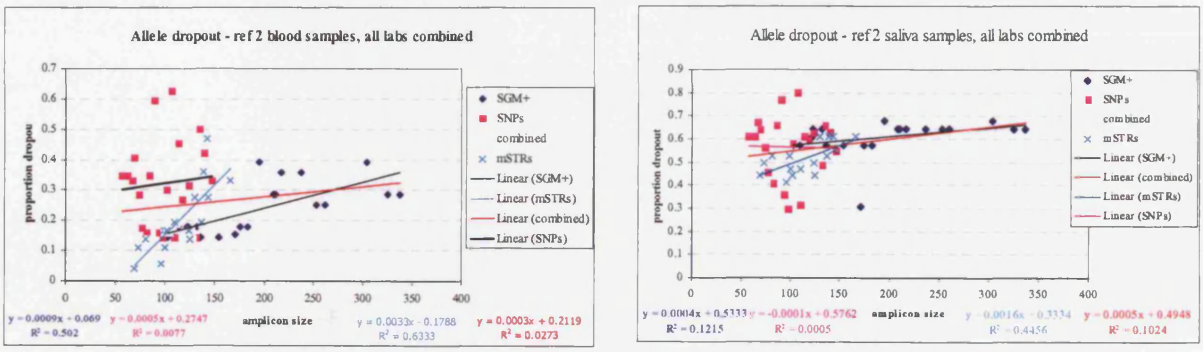


Figure 6.11 Allele dropout compared to amplicon size for reference 2 degraded blood and saliva samples. Proportion of allele dropout was calculated across the degradation set, regardless of incubation time.

Regression analysis displayed no relationship between the proportion of allele dropout and amplicon size for any of the samples used, when looking at SNPs (R-squared = 0.0005-0.0493). STRs and mini-STRs were evaluated both separately and as a combined set. Initial studies using computer simulations (data not shown) suggested a linear relationship between degradation and amplicon size, but these were carried out regardless of the protection factor conferred by the nucleosome. The R-squared values for STRs varied between 0.1215 and 0.6181 compared to 0.4087 – 0.6333 for mini-STRs.

For each sample, all observed dropout for each multiplex was combined together to observe whether all data followed the same regression. This data is indicated by the red regression line in figures 6.10 & 6.11. The data became skewed due to the SNP allele dropout data, which showed a random array of scatter points.

Figure 6.10 & 6.11 indicate a positive regression between amplicon size and proportion of allele dropout, i.e. the proportion of alleles dropping out of the DNA profile increases as the size of the amplicon increases. This is indicative of the higher molecular weight amplicons degrading first.

6.3.6.2 Total allele dropout across degradation periods

Allele dropout was measured for each sample at each stage of degradation. The regression scatters seen for reference 1 blood for each multiplex are shown (Figure 6.12), data for other samples followed a similar pattern. All multiplexes showed increasing dropout seen with degradation times with the exception of the SNP data which appeared to be random and independent of amplicon size and was the only multiplex that showed allele dropout in control samples (i.e. samples that had not been subject to any degradation time). The slope of the regressions for SGM+ appeared to decrease as the samples became more degraded. This suggested more alleles at the low molecular weight end of the profile were starting to drop out as the sample became more highly degraded, decreasing the significance of the regression. The mini-STR systems appeared to show random dropout with the less degraded samples. A positive regression was observed after eight weeks degradation and this began to plateau at 16 weeks degradation, similar to the SGM+ results.

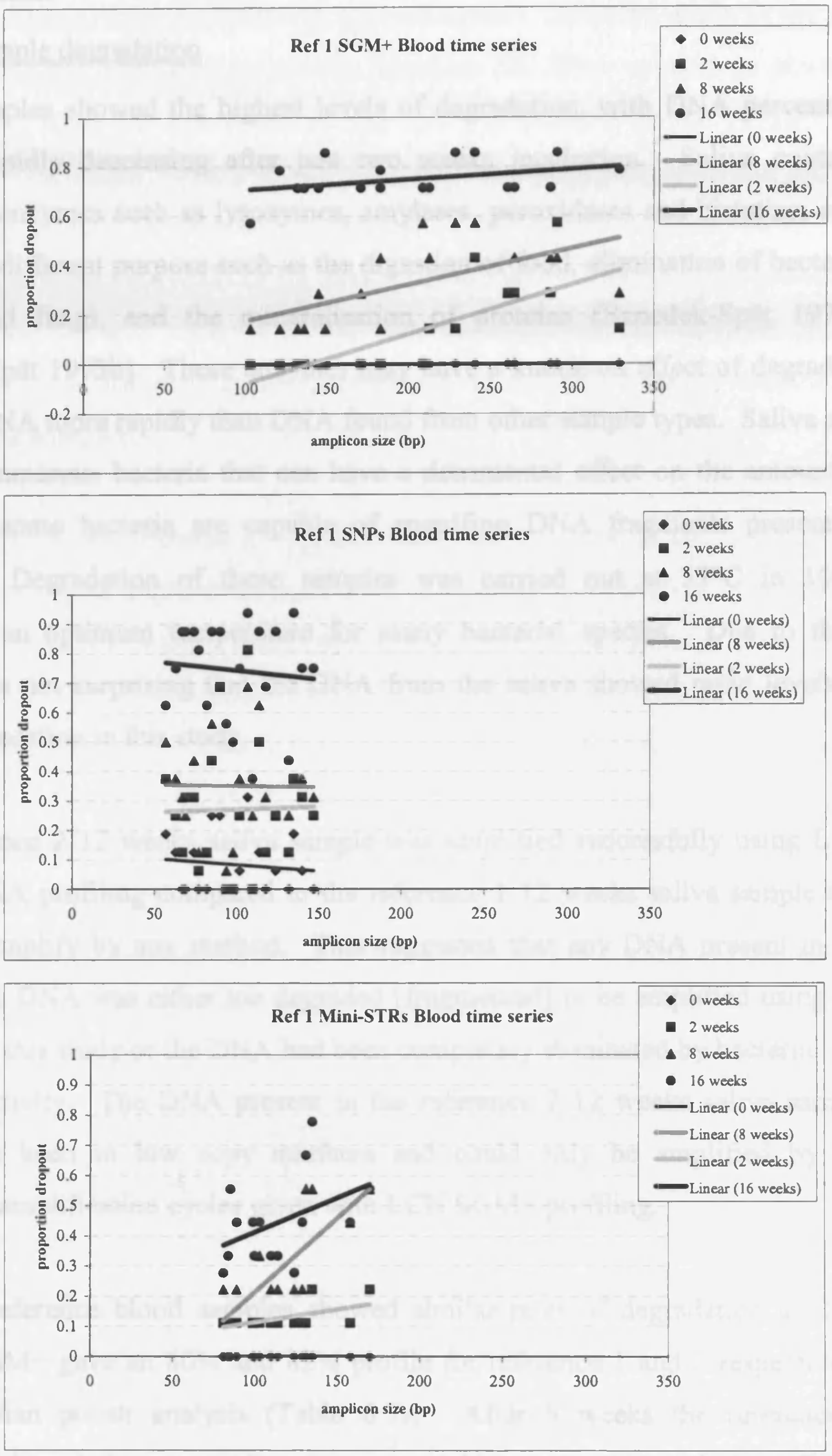


Figure 6.12 Degradation time series plots for each multiplex. Graphs indicate the proportion of allele dropout seen compared to amplicon size for reference 1 blood samples. A) SGM+ profiles B) SNP multiplex profiles C) mini-STR profiles. NB. Mini-STR multiplexes (mini-SGM & NC01) were combined for the mini-STR analysis.

6.4 Discussion

6.4.1 Sample degradation

Saliva samples showed the highest levels of degradation, with DNA percentage profiles rapidly decreasing after just two weeks incubation. Saliva contains numerous enzymes such as lysozymes, amylases, peroxidases and histatins; each used for a different purpose such as the digestion of food, elimination of bacteria, viruses and fungi, and the mineralisation of proteins (Benedek-Spät 1973a; Benedek-Spät 1973b). These enzymes may have a knock-on effect of degrading salivary DNA more rapidly than DNA found from other sample types. Saliva also contains numerous bacteria that can have a detrimental effect on the amount of DNA, as some bacteria are capable of engulfing DNA fragments present in solution. Degradation of these samples was carried out at 37°C in 100% humidity, an optimum temperature for many bacterial species. Due to these factors it is not surprising that the DNA from the saliva showed rapid levels of DNA degradation in this study.

The reference 2 12 weeks saliva sample was amplified successfully using LCN SGM+ DNA profiling compared to the reference 1 12 weeks saliva sample that failed to amplify by any method. This suggested that any DNA present in the reference 1 DNA was either too degraded [fragmented] to be amplified using the systems in this study or the DNA had been completely eliminated by bacterial and enzyme activity. The DNA present in the reference 2 12 weeks saliva sample must have been in low copy numbers and could only be amplified by the additional amplification cycles given with LCN SGM+ profiling.

The two reference blood samples showed similar rates of degradation up to 8 weeks (SGM+ gave an 80% and 82% profile for reference 1 and 2 respectively, using median polish analysis (Table 6.3)). After 8 weeks the reference 1 bloodstain degraded more rapidly, giving a variable success rate with the different systems after 16 weeks (Figure 6.8A). The reference 2 bloodstain showed the least degradation out of all four samples (Figure 6.8C).

Blood is made up of three main cell types – red blood cells (erythrocytes), white blood cells (leukocytes) and platelets (thrombocytes). Both the erythrocytes and thrombocytes are anucleate in nature, therefore the DNA present in blood is derived only from the leukocytes or free DNA in solution (Poste 1973). The number of leukocytes present in an individual varies greatly, with the normal range lying somewhere between 4,500 and 11,000 per cubic millimetre. The number of cells can decrease during resting periods and increase during periods of exercise (Nieman and Pedersen 1999) and, as leukocytes are involved in the immune response, the number also increases if an individual has an infection or suffers from allergies.

The number of cells present in the reference samples used in this study could explain the variable degradation rates seen. The reference 2 individual may have had a higher white blood cell count, meaning more cells were present in the stains to begin with. The DNA degradation rate may have been the same for both sets of stains, accounting for the similar results seen in the samples up to 8 weeks degradation. After this point, the number of cells present in reference 1 would have decreased to a low copy number level, decreasing the success rate of all DNA profiling methods except for LCN SGM+ (Figure 6.8A). The number of cells present in the reference 2 stains allowed standard DNA profiling techniques to still attain >50% success rates after 16 weeks degradation.

6.4.2 Low copy number amplification

Using PCR simulations, it has been shown that a minimum of 25 molecules are required to give a full SGM+ DNA profile using 28-cycle PCR (P Gill, per comms). This number can be decreased to just one molecule by increasing the number of amplification cycles from 28 to 34, i.e. performing low copy number amplification parameters.

Further work simulating degradation of DNA fragments has shown that once DNA starts to degrade, the number of molecules present decreases below the 25 molecules required for standard SGM+ profiling (Figure 6.13). These simulations help understand why LCN SGM+ is more successful than any other method that uses 28 cycle PCR. Both mini-STR kits were amplified using 32 cycle PCR, according to protocol, allowing both of these DNA profiling methods to work routinely better than SGM+.

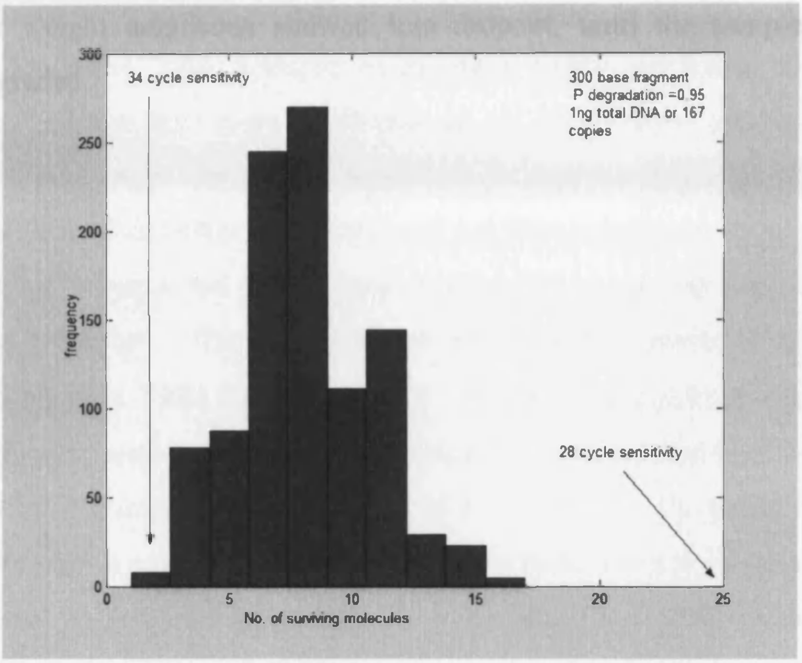


Figure 6.13 Graphical representation of the number of molecules seen when simulating the degradation of DNA.

The decrease in percentage profile seen with LCN SGM+ profiling after longer periods of degradation indicate that the DNA in these instances had become fragmented and the higher molecular weight STR loci failed to amplify.

6.4.3 Size of amplicons

All samples showed a pattern of proportion dropout similar to that shown for each multiplex (Figure 6.12). The SNP loci showed a random dropout pattern, indicating that success rates were independent of the amplicon length. The SNP multiplex was designed with all PCR products below 146bp in length, to allow amplification of degraded DNA. Dropout is most likely to have occurred as a consequence of the multimix not being optimised, due to the number of loci amplified in one reaction (Dixon *et. al.* 2005a), as opposed to the size of the amplicons.

Both mini-SGM and NC01 STR loci were analysed collectively as 'mini-STRs', with a maximum amplicon size of 170bp. The SGM+ and mini-STR dropout results showed a similar pattern whereby the higher molecular weight amplicons were the first to drop out of the profile, giving a positive regression. The lower molecular weight amplicons showed less dropout, until the samples became highly degraded.

6.5 Conclusion

A previous EDNAP study using DNA degraded by sonication and DNase I (Schneider *et. al.* 2004), and other studies using degraded body fluid stains (Wiegand and Kleiber 2001; Krenke *et. al.* 2002; Ohtaki *et. al.* 2002; Butler *et. al.* 2003; Chung *et. al.* 2004; Schumm *et. al.* 2004; Coble and Butler 2005; Butler 2005b) and telogen hair roots (Hellmann *et. al.* 2001), have demonstrated the efficacy of low molecular weight amplicons to analyse degraded DNA. The experiment described in this paper followed a different design to those previously described, as it simulated a time-course series of stains degraded by *in situ* enzymatic processes. This was achieved by incubating material spotted with saliva and blood in 100% humidity at 37°C. Under these conditions, degradation was greatly accelerated compared to the dried-state process and total degradation was achieved within a short time-period of 12-16 weeks. By taking samples at regular intervals, a complete time-course was produced and a point reached which corresponded to the time where little or no amplifiable DNA remained. We showed that saliva degraded faster than blood, but this is not surprising as this body fluid contains enzymes such as lysozymes, amylases, peroxidases and histatins, as well as numerous bacteria, which contribute nucleases, e.g. Micrococcus *sp.* contribute micrococcal nuclease. Micrococcal nuclease is a non-specific endonuclease, that cuts adjacent to any base, with the rate of cleavage reported to be 30 times greater at the 5' side of A or T rather than G or C (fortunately most STR sequences tend to be GC-rich). Mammalian cells contain two additional DNases that cleave non-specifically; DNase I, which slightly favours purine-pyrimidine sequences (Staynov 2000) and DNase II, an enzyme found in lysozomes associated with cell apoptosis (Yasuda *et. al.* 1998).

Median polish analysis was carried out in order to standardise the data, allowing data sets from all laboratories to be compared regardless of variability in laboratory techniques, operator differences and sampling limitations (Tukey 1977). Transformed data was analysed to investigate degradation rates, allele dropout and performance of the four assays used in this study. The artificially degraded samples gave similar results across all laboratories, showing the method produced samples with consistent levels of degradation across all sets.

The mini-STR assays tested gave the best results overall, when compared with standard SGM+ profiling and the Foren-SNPs™ kit (Dixon *et. al.* 2005b). Low copy number (LCN) DNA profiling proved to be the most successful method of amplification, although this technique was only carried out by three laboratories; one using Powerplex®16 and two using SGM+. LCN profiling only differs from standard DNA profiling by the number of cycles used for PCR amplification (Whitaker *et. al.* 2001). By increasing the number from 28 cycles to 34 cycles, the chance of amplifying the few molecules present in the DNA extract is improved. The mini-STR assays tested in this study used 32 cycles in PCR amplification, making the method more consistent with LCN profiling (Butler *et. al.* 2003; Drabek *et. al.* 2004; Coble and Butler 2005). As well as increased cycles for amplification, the reduced amplicon size targeted with mini-STRs allowed the more degraded (and therefore more fragmented) DNA samples to be amplified with greater success. The mini-STR assays were also the most robust in this study as the number of loci targeted was lower than the other DNA profiling methods tested. NC01, giving the highest percentage profiles overall, only contained three STR loci and therefore would generally have been easier to optimise than the Foren-SNP™ multiplex containing 21 loci.

The Foren-SNP™ kit performed poorest out of the four assays tested in this study. This particular kit was used as it was the only fully-validated forensic SNP multiplex available (Dixon *et. al.* 2005a). Other SNP multiplexes have been developed, but lack the quantitative and qualitative properties for forensic use (Kwok 2001; Inagaki *et. al.* 2004; Budowle 2004b). SNP assays based on primer extension biochemistry, such as GenomeLab™ SNPStream® (Beckman Coulter) and SNaPshot™ multiplex system (Applied Biosystems™), are capable of genotyping thousands of SNPs in a single run but require an increased volume of either initial DNA template or PCR product, both of which are limited in crime scene samples. They also have the disadvantage of being multi-stage procedures, with sample tubes needing to be opened at various stages within the process. The Foren-SNPs™ kit allowed amplification of all 21 loci in a single tube reaction, which were then analysed on an electrophoresis instrument. A more highly optimised SNP multiplex system could give better results on degraded samples as SNP loci do benefit from being single base sites allowing targeting of much smaller amplicons (Chakraborty *et. al.* 1999; Gill *et. al.* 2000a; Gill 2001a). The ability to obtain a result using SNPs would be beneficial with discrete forensic sample types, especially if the sample failed to give a profile using standard STR DNA profiling, however the biallelic nature of SNPs makes it difficult to interpret mixtures and a perfectly balanced assay would be required to make this feasible (Gill 2001a). For these reasons, and to be consistent with current national DNA databases, it would be preferable to use STRs for forensic identification as they are more amenable to mixture interpretation and a high discrimination power can be gained from fewer loci (Gill *et. al.* 2004a).

Degraded samples continue to be the most problematic for current forensic profiling methods, in part because while it is ideal to maximize the amount of information gleaned from the extracted DNA, it is imperative that any system be robust for forensic application. The observed degradation pattern of high molecular weight loci failing to amplify with increased levels of degradation has been an enduring feature of this study, providing the incentive to produce a system targeting the low molecular weight loci most likely to remain in higher copy number in degraded samples. It is therefore proposed that any further research into DNA profiling focuses on reducing the size of STR amplicons, such that they can be more successfully amplified in degraded samples, as well as supporting amplification under low copy number conditions (Dixon *et. al.* 2005b; Gill *et. al.* 2006).

7 Casework Samples

7.1 Introduction

The first criminal case using DNA profiling for identification occurred in the UK (http://www.forensic.gov.uk/forensic_t/inside/news/list_casefiles.php?case=1). In 1983, a 15 year old schoolgirl, Lynda Mann, was found raped and murdered. A semen sample taken from the body was found to belong to a person with type A blood group and an enzyme profile matching 10% of the male population. With no other forensic evidence available at the time, the murder enquiry was suspended. Three years later another 15 year old, Dawn Ashworth, was found strangled and sexually assaulted in the same area. Semen samples again showed a suspect with type A blood and the same enzyme profile as the murderer of Lynda Mann. In 1985, Jeffreys published a method of identification using DNA 'fingerprints' (Jeffreys *et. al.* 1985a; Jeffreys *et. al.* 1985b). Gill *et. al.* further developed this work to allow DNA profiling of material for forensic use (Gill *et. al.* 1985b). DNA profiling proved conclusively that the same person had killed both girls and that the suspect who had admitted both of the murders could not have been the murderer.
For the first time in criminal history "*this suspect became the first person in the world to be exonerated of murder through the use of DNA profiling*" (http://www.forensic.gov.uk/forensic_t/inside/news/list_casefiles.php?case=1).
The first DNA 'mass screen' was then carried out, to profile men in the local area in the hope of finding the murderer. In 1988 Colin Pitchfork was sentenced to life imprisonment after confessing to the murders of both girls.

Since these early cases of DNA being used as evidence, the technology and sensitivity of the systems used for DNA profiling has rapidly evolved. The current SGM+ system used for the National DNA Database® has a discrimination power of one in 1000 million for unrelated individuals (Cotton *et. al.* 2000) and can help prove beyond reasonable doubt that an individual is either guilty or innocent of a crime (Gill 2002). The discrimination power of SGM+ is no more than that found with earlier methods of VNTR DNA profiling. The technique has proved more successful due to the increased sensitivity achieved by PCR amplification, along with the ability to compare profiles within a database as the precise size and locus of origin can be determined. In identification cases, DNA profiling can be used to ascertain the likelihood of a sample belonging to the individual in question, either through reference samples or through samples from relatives (Jeffreys *et. al.* 1992; Gill *et. al.* 1994; Olaisen *et. al.* 1997; Crespillo *et. al.* 2000; Holland *et. al.* 2003; Leclair *et. al.* 2004; Budowle *et. al.* 2005; Butler 2005b).

The 21-SNP multiplex was developed to use in situations where current DNA profiling methods fail to produce a result, or only produce partial profiles due to the degradation of the DNA. The current profiling method of choice with difficult sample types is LCN SGM+ (Gill *et. al.* 2000b; Whitaker *et. al.* 2001; Gill 2001b; Gill 2002), however the amplification of the high molecular weight loci >200bp in length is hindered in highly degraded samples increasing the likelihood of gaining a partial profile. LCN SGM+ profiling is also used routinely in casework samples thought to contain minimal DNA due to the nature of the sample. The biological make-up of the sample type may mean it contains very little DNA, for example, bones and hair shafts; or there may only be a minimal amount of sample available for testing (such as minute blood stains found at a crime scene).

As a final assessment of the 21-SNP multiplex, a number of casework samples were profiled using the technique. These samples had previously been analysed using LCN SGM+ DNA profiling methods and had either failed to amplify or had only produced a partial STR profile.

The likelihood ratio (LR) is based on a comparison of two hypotheses – 'what is the probability that the suspect left the crime stain (H_p) against the probability that the crime stain was left by a random, unrelated, individual (H_d)?'. It is defined by the following equation:

$$LR = \frac{\Pr(E|H_p)}{\Pr(E|H_d)}$$

Where Pr(E|H_p) is calculated from the probability of the crime sample and the suspect sample matching <u>given</u> that the prosecution hypothesis is true, i.e. in simple scenarios this is equal to 1. Pr(E|H_d) is the match probability calculation, i.e. the probability of the profile given that the defence hypothesis is true. This is the same as the probability of observing the profile in the general population.

All crime scene samples were compared to reference samples to assess the likelihood of a sample coming from a suspected individual. Reference samples were obtained from various sources such as a toothbrush head and saliva residue of a pillow. Kinship analysis was used in some cases due to the lack of a reference sample from the individual in question. Kinship analysis was carried out using DNA from one or both parents. The LR values obtained in these cases were lower than those gained when a reference sample from the suspected individual was available but nonetheless allowed a value to be assigned to the data.

7.2 Materials & Methods

7.2.1 Casework DNA sample data

Sample DNA extracts from six different closed[8] cases were supplied from The Forensic Science Service® low copy number DNA profiling laboratory in Wetherby (Table 7.1). DNA extracts consisted of both reference samples, taken from items belonging to the deceased individual or buccal swabs from parents of the deceased; and crime scene samples.

Case ID	Casefile number	Sample ID	Sample type	Sample origin
1	300205756	85933300	toothbrush head	deceased individual
		QE03.5043.1	blood	crime scene
2	300100756	85298665	buccal	mother of deceased
		85298678	buccal	father of deceased
		QE03.3529.1	bone	crime scene
3	300091460	84477900	buccal	father of deceased
		85284421	buccal	mother of deceased
		QE03.3074.1	bone	crime scene
4	300110837	85287616	buccal	father of deceased
		85287661	muscle	crime scene
5	300065124	85276633	buccal	mother of deceased
		85376643	blood	crime scene
6	300227014	85160851	buccal	mother
		85160860	buccal	father
		85370230	saliva	deceased individual
		85369994	blood from hatchback car light	crime scene
		85407677	blood from towel	crime scene
		85407691	blood from circular saw	crime scene

Table 7.1 Casework DNA sample data.

All DNA samples had been extracted using Chelex® methods (Walsh *et. al.* 1991) and reference samples were quantified using Quantiblot® Human DNA Quantitation kits (Cat. No. N808-0114) according to the manufacturer's protocol. Crime scene samples were assumed to be at low copy number levels and were therefore not quantified.

[8] A 'closed' case is one that has been taken through the criminal justice system and a verdict has been reached.

An SGM+ DNA profile had been obtained for each sample collected. Reference samples from parents had been amplified using standard 28 cycle SGM+ parameters. Reference samples from the deceased individuals' belongings and crime scene samples had been amplified using 34 cycles LCN SGM+ DNA profiling. Genotype data was supplied from the reporting officer casefiles. LCN SGM+ results were run in duplicate and the resulting profile comprised a consensus of the two amplifications. Only alleles appearing in both amplification reactions were considered to be true alleles (Gill *et. al.* 2000b).

7.2.2 21-SNP multiplex DNA profiling

PCR amplification was carried out as described in chapter 3. All crime scene DNA extracts were added at a maximum volume of 12 µL. Quant values were used to estimate the amount of extract to add for the reference samples (Table 7.2).

PCR products were run on a capillary electrophoresis sequencer as described in chapter 3. Results were analysed using AB Genescan™ and Genotyper™ software before being interpreted using Celestial™ software (chapter 4).

All genotype results for LCN SGM+ and the 21-SNP multiplex were analysed and compared.

Sample ID	DNA quant value (ng/μL)	Amount added to PCR reaction (max. 12μL)
85933300	0.04	12.0
QE03.5043.1	-	12.0
85298665	0.12	10.0
85298678	0.14	12.0
QE03.3529.1	-	12.0
84477900	0.50	2.0
85284421	0.90	1.0
QE03.3074.1	-	12.0
85287616	2.50	1.0
85287661	-	12.0
85276633	0.90	1.0
85376643	-	12.0
85160851	4.40	1.0 [1:4 dilution]
85160860	1.40	1.0
85370230	0.08	12.0
85369994	-	12.0
85407677	-	12.0
85407691	-	12.0

Table 7.2 DNA quantification values (ng/μL) and PCR volumes for crime scene and reference samples.

7.2.3 Likelihood ratio calculations

Likelihood ratios (LRs) for crime scene samples compared to reference samples for the deceased individuals were calculated using STRipe™ (in-house computer program) for SGM+ profiles and Celestial™ for SNP profiles. Kinship analysis was used for LR calculations where the reference samples were derived from the parent/s of the deceased individual. LR values for SGM+ data were obtained from the casefiles. These had been calculated using a commercially available software package "Kinship" developed by Charles Brenner (Brenner 1997; Leclair *et. al.* 2004). The allele frequency arrays used for SNP kinship analyses are given in appendices IX and X.

7.3 Results

7.3.1 Case 1 – 300205756

The reference sample in this case (85933300) came from DNA extracted from the head of a toothbrush found in the bathroom of the deceased's living accommodation. A low quantification value of 0.04ng/µL was obtained from the DNA extract and a maximum volume of 20µL had been added to the SGM+ PCR reaction. PCR amplification was carried out at standard 28 cycle parameters and was analysed accordingly (Table 7.3). A speck of blood found at the crime scene was extracted (QE03.5043.1) and amplified using LCN SGM+ parameters.

Sample ID	D3		VWA		D16		D2		AMELO		D8		D21		D18		D19		THO		FGA	
REF 85933300	11R	14	16	18	12	13	17	22	X	Y	12	15	29	30	13	15	14	14	9.3	9.3	20	21
QE03.5043.1a	*11R*	*14*	*16*	*18*	*12*	*13*	*17*	*22*	*X*	*Y*	*12*	*15*	*29*	*30*	*(13)*	*(15)*	*(14)*	-	*9.3*	*9.3*	*20*	*21*
QE03.5043.1b	*11R*	*14*	*16*	*18*	*12*	*13*	*17*	*22*	*X*	*Y*	*12*	*15*	*29*	*30*	*13*	*15*	*(14)*	-	*9.3*	*9.3*	*20*	*21*
QE03.5043.1 consensus	11R	14	16	18	12	13	17	22	X	Y	12	15	29	30	13	15	14	F	9.3	9.3	20	21

Table 7.3 SGM+ DNA profiling results for case 1 – 300205756. Designations in (brackets) indicate peaks with heights falling below 50rfu. An 'F' designation indicates a locus where only one peak was seen but allele dropout may have occurred due to the low peak height. 'R' designation indicates a rare allele.

Both extracts provided were amplified at maximum volumes with the 21-SNP multiplex and genotyping results were run through Celestial™ to produce a SNP profile (Table 7.4).

Sample ID	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
REF 85933300	X/Y	T/T	T/T	A/A	T/T	G/G	T/T	C/G	A/F	C/C	C/C	C/C	F/A	T/A	A/A	C/F	C/F	T/C	T/F	C/C	T/T
QE03.5043.1a	*X/Y*	*T/T*	*T/T*	*A/A*	*T/T*	*G/G*	*T/T*	*C/G*	*A/A*	*C/C*	*C/C*	*C/C*	*A/A*	*T/A*	*A/A*	*C/C*	*C/C*	*T/C*	*C/T*	*C/C*	*T/T*
QE03.5043.1b	*X/Y*	*T/T*	*A/T?*	*A/A*	*T/T*	*G/G*	*T/T*	*C/G*	*A/A*	*C/C*	*C/C*	*C/C*	*A/A*	*T/A*	*A/A*	*C/C*	*C/C*	*T/C*	*C/T*	*C/C*	*T/T*
QE03.5043.1 consensus	X/Y	T/T	T/F	A/A	T/T	G/G	T/T	C/G	A/A	C/C	C/C	C/C	A/A	T/A	A/A	C/C	C/C	T/C	C/T	C/C	T/T

Table 7.4 21-SNP multiplex genotyping results for case 1 – 300205756. 'F' designations indicate peaks falling below the homozygote threshold calculated for that locus.

The resulting LRs for the two DNA profiling methods, calculated from $LR = \frac{\Pr(E|H_p)}{\Pr(E|H_d)}$ were 2.7E+11 (using STRipe™) and 1.25E+08 (using Celestial™) for SGM+ and SNPs respectively.

The reference sample gave 100% profile using SGM+ but this was decreased to only 79% for the SNP profile. SGM+ amplification of the crime scene sample in this case had generated a full profile except for one locus (D19), which showed a peak below the homozygote threshold set for LCN interpretation. As well as this, the individual demonstrated a rare allele at locus D3, greatly increasing the result of the LR calculation. The 21-SNP multiplex also gave a full profile except for one locus (U6) that had shown a minor heterozygous peak in one of the duplicate PCR reactions, necessitating the use of an 'F' designation.

7.3.2 Case 2 – 300100756

The reference samples in this case were buccal scrapes provided by the parents of the deceased individual (8598665 / 85298678). A DNA extract was provided from a bone sample found at the crime scene. SGM+ was carried out using standard amplification (28 cycles) for the reference samples and LCN conditions (34 cycles) for the bone sample (Table 7.5).

Sample ID	D3		VWA		D16		D2S		AMELO		D8		D21		D18		D19		THO		FGA	
85298665	17	17	18	19	12	12	17	22	X	X	14	15	31.2	32.2	12	12	15	15	6	9.3	20	25
85298678	15	15	16	16	12	12	24	25	X	Y	12	14	29	29	15	16	14	14	8	9.3	20	25
QE03.3529.1a	*15*	*17*	*16*	*18*	*(12)*	-	*17*	*25*	*X*	*Y*	*12*	*14*	*29*	*31.2*	*12*	*15*	*14*	*15*	*6*	*9.3*	*(20)*	-
QE03.3529.1b	*15*	*17*	*16*	*18*	*(12)*	-	*17*	*25*	*X*	*Y*	*12*	*14*	*29*	*31.2*	*12*	*15*	*14*	*15*	*6*	*9.3*	*(20)*	-
														30?								
QE03.3529.1 consensus	15	17	16	18	12	F	17	25	X	Y	12	14	29	31.2	12	15	14	15	6	9.3	20	F

Table 7.5 SGM+ DNA profiling results for case 2 – 300100756.

Table 7.6 shows the 21-SNP multiplex DNA profiling results gained for these samples.

Sample ID	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
85298665	X/X	T/C	T/T	A/T	F/T	G/G	T/A	C/G	A	C/T	C/C	C/C	T/T	A/A	A/T	C/C	C/C	T/T	T/T	C/C	T/T
85298678	X/Y	C/C	T/T	A	A/F	G/G	T/A	G/G	A/T	C/C	G/C	C/C	T/A	T/T	A/A	C/C	C/C	T/T	C/C	C/G	T/T
QE04.3529.1a	*X/Y*	*T/F*	*T/T*	*A/T*	-	*G/G*	*A/A*	*G/G*	*A/A*	*T/F*	*G/C*	-	*T/F*	*T/F*	*A/T*	*C/F*	*C/C*	*T/T*	*C/T*	*C/C*	*T/T*
QE04.3529.1b	*X/Y*	*T/F*	*T/T*	*A/T*	-	*G/G*	*A/A*	*G/G*	*A/A*	*T/F*	*G/C*	-	*T/F*	*T/F*	*A/T*	*C/F*	*C/C*	*T/T*	*C/T*	*C/C*	*T/T*
QE04.3529.1 consensus	X/Y	T/F	T/T	A/T	-	G/G	A/A	G/G	A/A	T/F	G/C	-	T/F	T/F	A/T	C/F	C/C	T/T	C/T	C/C	T/T

Table 7.6 21-SNP multiplex genotyping results for case 2 – 300100756.

Using kinship analysis calculations for two parent analyses, the LR of the SNP profile was determined to be 185, i.e. it was 185 times more likely that the sample QE04.3529.1 was from the offspring of the reference parents than from a random, unrelated individual.

7.3.3 Case 3 – 300091460

As with case 2, case 3 reference samples were derived from the parents of the deceased (84477900 / 85284421) and the crime scene sample had been extracted from a piece of bone (QE03.3074.1). Results for SGM+ profiling were obtained from the casefile (Table 7.7), using 28 cycles for reference samples and 34 cycle amplification for the crime sample.

Sample ID	D3		VWA		D16		D2S		AMELO		D8		D21		D18		D19		THO		FGA	
85294421	15	16	15	15	12	12	17	23	X	X	13	13	30	30	12	14	14	15.2	7	9	20	23
84477900	15	17	15	19	9	12	20	25	X	Y	10	14	30	30	15	20	15	15	7	9.3	22	23
QE03.3074.1a	*15*	*17*	*15*	*15*	*12*	*12*	*23*	*25*	*X*	*Y*	*10*	*13*	*30*	*30*	*14*	*15*	*15*	*15.2*	*7*	*9.3*	*22*	*23*
QE03.3074.1b	*15*	*17*	*15*	*15*	*12*	*12*	*23*	*25*	*X*	*Y*	*10*	*13*	*30*	*30*	*14*	*15*	*15*	*15.2*	*7*	*9.3*	*22*	*23*
														31								
QE03.3074.1 consensus	15	17	15	15	12	12	23	25	X	Y	10	13	30	30	14	15	15	15.2	7	9.3	22	23

Table 7.7 SGM+ DNA profiling results for case 3 – 300091460.

All three DNA extracts were amplified using the 21-SNP multiplex (Table 7.8).

Sample ID	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
84477900	X/Y	T/C	A/T	A/T	-	G/G	T/A	C/C	A/T	C/T	C/C	T/T	T/A	T/T	A/A	C/C	C/C	T/T	C/C	C/G	T/T
85284421	X/F	T/C	T/T	A/T	-	G/C	T/T	C/C	A/A	T/T	G/C	-	T/T	T/T	A/A	C/C	C/A	T/T	C/C	G/G	T/T
QE04.3074.1a	*X/Y*	*T/C*	*T/T*	*T/F*	-	*G/C*	*T/T*	*C/C*	*A/T*	*T/T*	*C/C*	-	*T/A*	*T/F*	*A/A*	-	-	*T/T*	*C/C*	*G/F*	*T/T*
QE04.3074.1b	*X/Y*	*T/C*	*T/T*	*T/T*	-	*G/C*	*T/T*	*C/C*	*A/T*	*T/T*	*C/C*	-	*T/A*	*T/T*	*A/A*	-	-	*T/F*	*C/F*	*G/G*	*T/T*
QE04.3074.1 consensus	X/Y	T/C	T/T	T/F	-	G/C	T/T	C/C	A/T	T/T	C/C	-	T/A	T/F	A/A	-	-	T/F	C/F	G/F	T/T

Table 7.8 21-SNP multiplex genotyping results for case 3 – 300091460.

Using kinship analysis calculations, the LR of the SNP profile was determined to be 42, i.e. it was 42 times more likely that the sample QE04.3074.1 was from the offspring of the reference parents than from a random, unrelated individual.

7.3.4 Case 4 – 300110837

The father of the deceased individual had provided a single reference sample in this case (85287616). The crime scene DNA extract was submitted from a section of deep muscle (85287661). SGM+ DNA profiles (28 cycles for the reference and 34 cycles for the crime sample) were provided from the casefile (Table 7.9) and 21-SNP multiplex results were collated (Table 7.10). Only one LCN SGM+ profile was obtained for the muscle sample, rather than the customary duplicate profiles.

Sample ID	D3		VWA		D16		D2		AMELO		D8		D21		D18		D19		THO		FGA	
REF 85287616	15	15	18	18	11	13	17	19	X	Y	14	14	31	32.2	13	18	13	15	7	9.3	22	23
85287661	15	19	-	-	-	-	-	-	X	Y	8?	-	-	-	-	-	13	14	7	(9.3)	-	-

Table 7.9 SGM+ DNA profiling results for case 4 - 300110837.

Sample ID	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
REF 85287616	X/Y	C/C	A/A	T/F	A/F	G/C	T/A	C/C	T/T	C/T	G/G	C/F	T/T	T/T	A/T	C/C	C/C	T/T	C/C	C/C	T/T
85287661.1a	*X/Y*	*T/C*	*A/A*	-	-	*G/C*	*T/T*	*C/F*	-	*C/C*	*G/C*	*C/C*	*T/F*	*T/F*	-	*C/C*	*C/C*	*T/T*	*C/C*	-	*T/T*
85287661.1b	*X/Y*	*T/C*	*A/A*	-	-	*G/C*	*T/T*	*C/C*	-	*C/C*	*G/C*	*C/F*	*T/T*	*T/T*	-	*C/C*	*C/C*	*T/T*	*C/C*	-	*T/T*
85287661 consensus	X/Y	T/C	A/A	-	-	G/C	T/T	C/F	-	C/C	G/C	C/F	T/F	T/F	-	C/C	C/C	T/T	C/C	-	T/T

Table 7.10 21-SNP multiplex genotyping results for case 4 – 300110837.

Using kinship analysis for a single parent comparison, the LR of the SNP profile was determined to be 719, i.e. it was 719 times more likely that the sample 85287661 was from the offspring of the reference parents than from a random, unrelated individual.

7.3.5 Case 5 – 300065124

The mother of the deceased individual provided the reference sample in this case (85276633). The questioned DNA extract came from a bloodstain found at the suspected crime scene (85376643). SGM+ DNA profiling results were given in the casefile (Table 7.11), using 28 cycles amplification for the reference sample and 34 cycles for the crime sample.

Sample ID	D3		VWA		D16		D2S		AMELO		D8		D21		D18		D19		THO		FGA	
REF 85276633	15	16	17	18	11	12	19	20	X	X	10	13	30	30	13	14	15	15	6	8	22.2	24
85276643.1a	*15*	*15*	*18*	*18*	*12*	*13*	*19*	*19*	*X*	*Y*	*10*	*14*	*30*	*30*	*13*	*14*	*13*	*15*	*8*	*9.3*	*24*	*25*
85276643.1b	*15*	*15*	*18*	*18*	*12*	*13*	*19*	*19*	*X*	*Y*	*10*	*14*	*30*	*30*	*13*	*14*	*13*	*15*	*8*	*9.3*	*24*	*25*
85276643 consensus	15	15	18	18	12	13	19	19	X	Y	10	14	30	30	13	14	13	15	8	9.3	24	25

Table 7.11 SGM+ DNA profiling results for case 5 – 300065124.

Both DNA extracts were genotyped using the 21-SNP multiplex, the crime scene extract being run in duplicate (Table 7.12).

Sample ID	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
85276633	X/F	C/C	A/T	-	-	G/G	T/T	C/C	A/A	C/T	C/C	C/C	T/A	T/A	A/T	C/C	C/A	T/C	C/C	C/C	A/T
85376643.1a	*X/Y*	*C/C*	*A/A*	*A/A*	-	*G/G*	*T/T*	-	*A/A*	*T/T*	-	*C/C*	-	*A/A*	*T/T*	*C/C*	*C/A*	*T/C*	*C/C*	*C/C*	*T/T*
85376643.1b	*X/Y*	*C/C*	*A/A*	*A/A*	*A/A*	*G/G*	*T/T*	*C/G*	*A/A*	*T/T*	*G/C*	*C/C*	*T/A*	*A/A*	*T/T*	*C/C*	*C/A*	*T/C*	*C/C*	*C/C*	*T/T*
85376643 consensus	X/Y	C/C	A/A	A/A	-	G/G	T/T	-	A/A	T/T	-	C/C	-	A/A	T/T	C/C	C/A	T/C	C/C	C/C	T/T

Table 7.12 21-SNP multiplex genotyping results for case 5 – 300065124.

Kinship analysis, using formulae for one parent, was carried out on the consensus SNP profile and an LR of 82 was calculated, i.e. it was 82 times more likely that the sample 85376643 was from the offspring of the reference parent than from a random, unrelated individual.

7.3.6 Case 6 – 300227014

Reference samples were obtained from both parents of the deceased individual (85160851 / 85160860) as well as a saliva sample taken from a pillowcase in the deceased individuals' house. DNA had been extracted from bloodstains acquired from three individual crime scenes. These had been found on a hatchback light (85369994), a towel (85407677) and a circular saw (85407691). Only one LCN SGM+ DNA profile was submitted from each crime scene sample, instead of the standard duplicate profiles (Table 7.13). All six DNA extracts were amplified using the 21-SNP multiplex, crime scene samples being run in duplicate (Table 7.14).

Sample ID	D3		VWA		D16		D2S		AMELO		D8		D21		D18		D19		THO		FGA	
REF 85160851	16	16	16	20	10	13	19	25	X	X	10	14	31.2	31.2	16	16	14	15	7	9	20	23
REF 85160860	15	16	14	18	8	8	25	25	X	Y	13	16	32.2	32.2	16	18	13	16	6	9	22	23
REF 85370230	15	16	18	20	8	10	19	25	X	Y	14	16	31.2	32.2	16	18	13	14	9	9	22	23
85369994	15	16	18	20	8	10	-	-	X	Y	14	16	-	-	-	-	13	14	9	F	-	-
85407677	15	16	18	20	8	10	-	-	X	Y	14	16	-	-	-	-	13	14	9	9	-	-
85407691	15	16	18	20	8	10	-	-	X	Y	14	16	31.2	F	-	-	13	14	9	F	22	23

Table 7.13 SGM+ DNA profiling results for case 6 – 300227014.

Sample ID	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
REF 85160851	X/F	C	T	T	-	G	T	C	A	C	G/C	C/T	T/A	T	A	A	C	T	C	C/G	T
REF 85160860	X/Y	T/C	A/T	A/A	-	G	T	C/G	A/T	C/T	C	T	T/A	T/A	A/T	C/A	C	T	C	C	T
REF 85370230	X/Y	C	A/T	A/T	-	G	T	C	A/T	C/T	G/C	T	T/A	T	A/T	A	C	T	C	C/G	T
85369994.1a	X/Y	-	A/F	-	-	G	T	C/F	-	C/T	G/C	F/T	T/F	T	A/T	A	-	T/F	C/F	-	-
85369994.1b	X/Y	-	A/F	-	-	G	T	C/F	-	C/T	G/C	F/T	T/F	T	A/T	A	-	T/F	C/F	-	-
85369994 consensus	X/Y	-	A/F	-	-	G	T	C/F	-	C/T	G/C	F/T	T/F	T	A/T	A	-	T/F	C/F	-	-
85407677.1a	X/Y	C	A/T	T/F	A/F	G	T	C	A/T	C/T	G/C	F/T	T/A	T	A/T	A	C	T	C	C/G	T
85407677.1b	X/Y	C	A/T	T/F	A/F	G	T	C	A/T	C/T	G/C	F/T	T/A	T	A/T	A	C	T	C	C/G	T
85407677 consensus	X/Y	C	A/T	T/F	A/F	G	T	C	A/T	C/T	G/C	F/T	T/A	T	A/T	A	C	T	C	C/G	T
85407691.1a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
85407691.1b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
85407691 consensus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 7.14 21-SNP multiplex genotyping results for case 6 – 300227014.

STRipe™ was used to calculate an LR for the SGM+ crime scene profiles compared to the reference saliva sample, using the formula:

$$LR = \frac{\Pr(E|H_p)}{\Pr(E|H_d)}$$

The samples gave LRs of 2.9E+07, 2.15E+08 and 2.03E+09 for the bloodstain off the car light, the blood from the towel and the blood from the circular saw respectively. An LR was calculated in Celestial™ for each SNP consensus profile compared to the reference saliva sample obtained in this case. The bloodstain from the hatchback light gave an LR of 1.21E+05 and the bloodstain from the towel gave an LR of 4.29E+07. The stain collected from the circular saw failed to produce a profile in either of the duplicate SNP amplifications.

Kinship analysis was used for evaluating the strength of the evidence given the SNP parental data. An LR of 131,579 was obtained from the hatchback light. This LR had been greatly increased by the presence of a rare homozygous T/T genotype at locus J2. The sample from the hatchback light gave an LR of 28 and the towel sample gave an LR of 3333. The rare allele in these cases had fallen below the homozygous threshold and was subsequently labelled with an 'F' designation, greatly decreasing the LR for that locus.

7.3.7 Comparison of percentage profiles and match probabilities

The percentage profiles and LR data for each crime scene sample was calculated using either kinship analysis (Table 7.15a) or allele frequency data (Table 7.15b). This allowed all profiles to be compared to each other, regardless of statistical calculations relating to identity. These results are shown graphically (Figure 7.1).

Case ID	Sample ID	Sample type	SGM+ % profile	LR (SGM+)	SNPs % profile	LR (SNPs)
300100756	QE03.3529 cons	Bone	91	3.00E+08	79	185
300091460	QE03.3074 cons	Bone	100	1.00E+09	69	41.7
300110837	85287661 cons	Muscle	36	4.00	67	714
300065124	85276643 cons	Blood stain	100	6670	81	83.3
300227014	85369994	Blood stain	59	8.00E+06	52*	27.8
"	85407677	Blood stain	64	5.32E+07	93*	3330
"	85407691	Blood stain	73	2.03E+09	-	-

Table 7.15a Percentage profiles and LR data for casework crime scene samples, using kinship analysis. * indicates SNP profiles obtained from two duplicate amplifications when SGM+ was only obtained from one amplification.

Case ID	Sample ID	Sample type	SGM+ % profile	LR (SGM+)	SNPs % profile	LR (SNPs)
300205756	QE03.5043 cons	Blood stain	95	2.70E+11	98	1.25E+08
300227014	85369994	Blood stain	59	2.90E+07	52*	1.21E+05
"	85407677	Blood stain	64	2.15E+08	93*	4.29E+07
"	85407691	Blood stain	73	2.03E+09	-	-

Table 7.15b Percentage profiles and LR data for casework crime scene samples, based on allele frequency data. * indicates SNP profiles obtained from two duplicate amplifications when SGM+ was only obtained from one amplification.

Three out of eight crime scene samples gave an increased percentage profile when amplified using the 21-SNP multiplex. One sample (85407691) failed to amplify using SNPs, in either of the duplicate PCR reactions; therefore five samples gave increased percentage profiles using LCN SGM+ amplification.

The interpretation criteria used for SNPs were very stringent due to the high sensitivity of the technique (chapter 4). The percentage profiles obtained were lower for SNPs due to the presence of more 'F' designations in the genotypes. The 'F' designations were also detrimental to the calculation of the LR as an 'F' could have indicated either a homozygous or heterozygous locus, decreasing the power of the result.

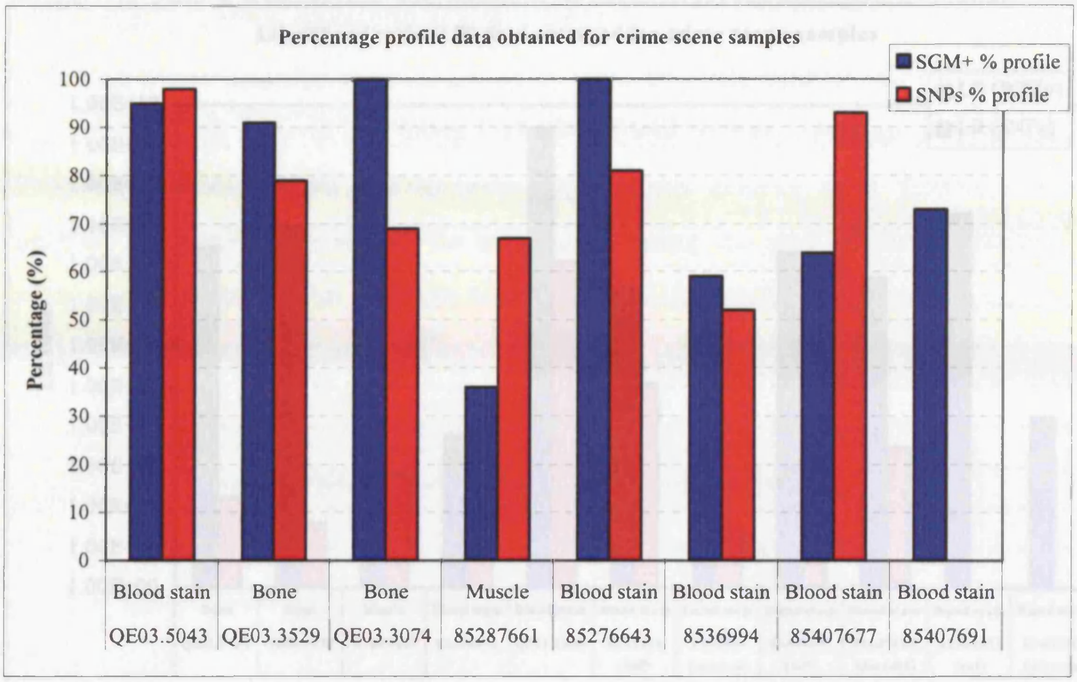


Figure 7.1 Percentage profile data obtained for crime scene casework samples using LCN SGM+ DNA profiling and the 21-SNP multiplex.

The amount of DNA extract added to the 21-SNP multiplex PCR reactions was 12μL. LCN SGM+ profiling routinely uses a maximum volume of 20μL. At LCN levels the amount of stochastic variation seen within a DNA extract is high and it is essential to add as much extract as possible to the PCR reaction to increase the likelihood of amplifying the few molecules that are present. The decreased amount of extract added for SNP amplification could have contributed to the lower success rate seen with the technique.

LR data was shown to be highly variable across the set of casework samples (Figure 7.2). An increased likelihood ratio was seen in cases where reference DNA samples were provided, as opposed to parent DNA samples. The use of kinship analysis allowed an LR to be gained in the absence of a reference DNA profile (Balding and Nichols 1994; Brenner 1997; Leclair *et. al.* 2004). LR values from kinship analysis varied from 42 to more than 130,000, dependent on the percentage profiles seen and the genotype data obtained.

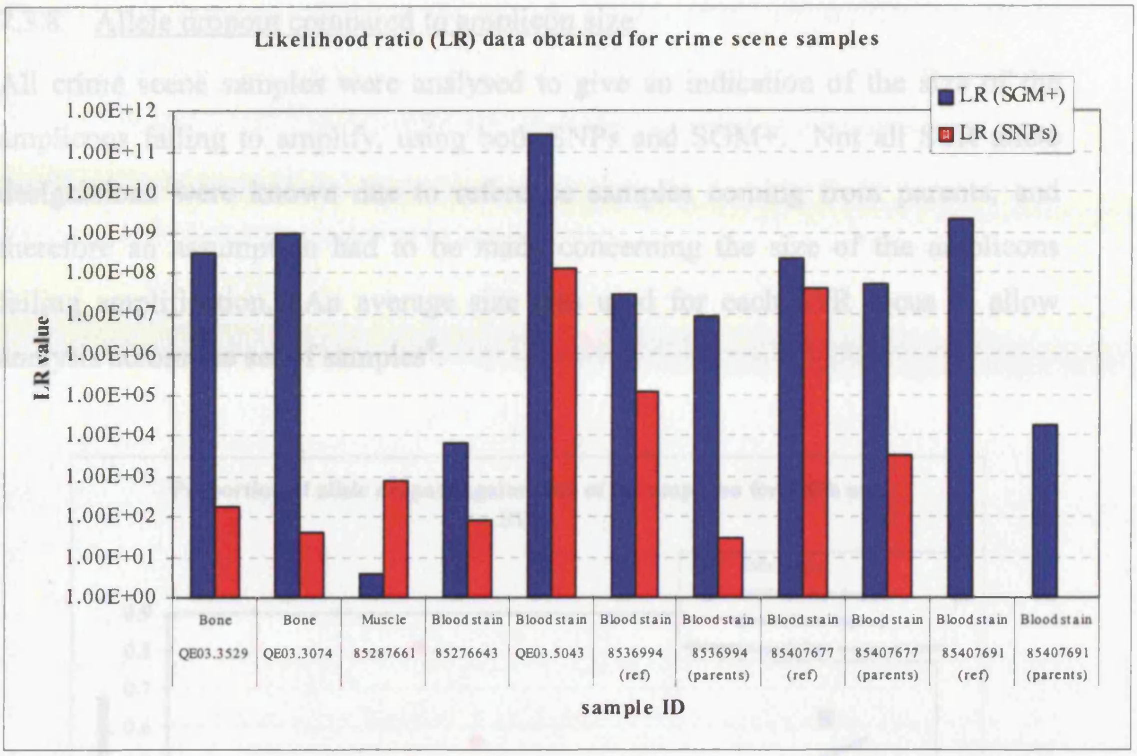


Figure 7.2 LR data obtained from a comparison of the genotype data gained for each crime scene sample.

In case 300227014, LR values were 10-fold higher using reference sample DNA profiles for comparison to the crime scene sample, as opposed to parent profiles. The 21-SNP multiplex LR values in this case were higher than that generally estimated for SNP analysis (4.5 million) due to the presence of a rare allele at locus TSCO J8. This increased the LR to 1 in 43 million, even though the profile obtained was incomplete (93%).

Overall, only one crime scene sample (85287661) showed an increased LR value when using the 21-SNP multiplex compared to LCN SGM+ profiling. The DNA extract in this case was obtained from muscle and was the only example of this sample type tested. Muscle is known to degrade at a faster rate than other body tissues after death has occurred due to the enzymes and chemicals present in muscle cells (Johnson and Ferris 2002). The increased success at profiling this sample type (67% SNP profile compared to 36% LCN SGM+ profile) using SNPs was suggestive of an increased amplification efficiency using the smaller target sites. A much larger sample set would need to be tested to clarify this observation.

7.3.8 Allele dropout compared to amplicon size

All crime scene samples were analysed to give an indication of the size of the amplicons failing to amplify, using both SNPs and SGM+. Not all STR allele designations were known due to reference samples coming from parents, and therefore an assumption had to be made concerning the size of the amplicons failing amplification. An average size was used for each STR locus to allow analysis across the set of samples[9].

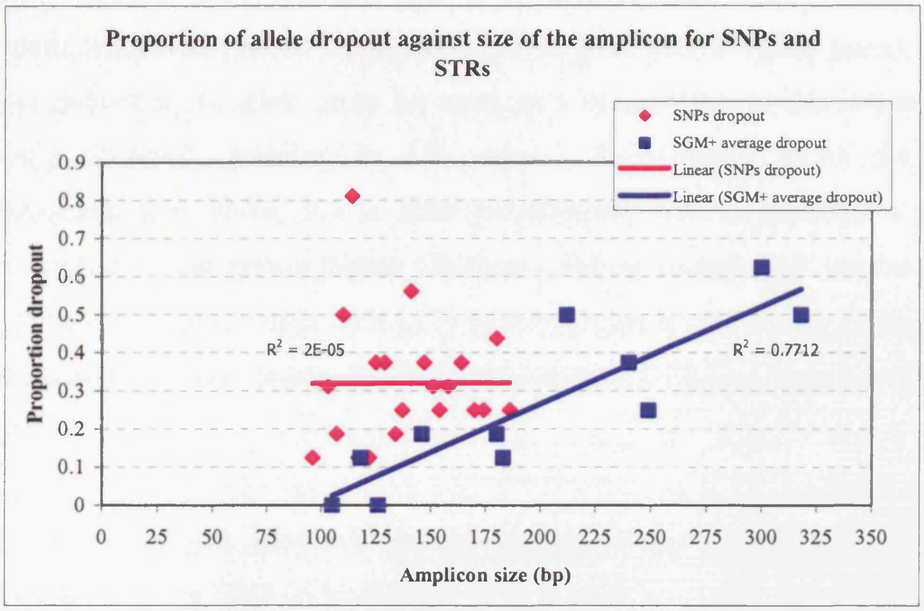


Figure 7.3 Scattergraph showing the proportion of allele dropout seen relative to the size of the target amplicon, using both the 21-SNP multiplex and LCN SGM+ DNA profiling.

The 21-SNP multiplex showed no relationship between the proportion of allele dropout compared to amplicon size (R^2 = 2.0E-05), an observation also seen in the artificially degraded samples previously genotyped using the technique (chapter 3). R-squared analysis indicated a positive regression when looking at STR allele dropout against amplicon size (R^2=0.7712). The graph clearly shows an increase in the amount of dropout seen with the higher molecular weight STR loci (Figure 7.3).

[9] Average STR locus size: Amelo 105bp; D19 118bp; D3 126bp; D8 146bp; vWA 180bp; THO 183bp; D21 212bp; FGA 240bp; D16 249bp; D18 300bp; D2 318bp.

The pattern of dropout observed for the crime scene LCN samples ties in with the observations seen for artificially degraded samples. This has a two-fold effect in that it strengthens the use of artificially degraded samples as a validation of DNA profiling techniques in the research environment, as well as allowing a study of the process of DNA degradation in general.

7.4 Discussion

Six sets of casework samples were received from The Forensic Science Service® laboratory in Wetherby. DNA extracts from reference samples and crime scene samples were provided for amplification using the 21-SNP multiplex, to supply enough information for a comparative study using SNPs and LCN SGM+ profiling.

Reference samples were obtained from either articles pertaining to the deceased individual, from both parents of the deceased, or from an individual parent. Each of these reference samples could be used as a comparison to the crime scene sample(s) obtained, yielding an LR value. Individually STRs are more discriminating than SNPs, due to their polymorphic nature; therefore a partial SGM+ profile would give a higher LR than a full or partial SNP profile. This study proved this observation with LCN SGM+ giving higher match probabilities for all samples bar one, even when only two or three STR loci were successfully amplified.

The 21-SNP multiplex gave a higher percentage profile and LR value for one sample tested. This DNA extract came from a muscle sample and indicated the need to define the degradation process within different sample types in more detail to give a more educated rationale behind the choice of DNA profiling technique to use to gain the best result.

DNA profiling using STRs is highly discriminating and is highly successful in identifying individuals from biological samples. The National DNA Database® currently holds over three million samples from both crime scenes and from individuals arrested for crimes. In 2004 individual samples on the database provided matches with more than 41,000 crime scenes and linked a further 4,500. Problems are encountered when the biological samples from which the DNA is obtained for profiling are compromised in some way, either by degradation or by the nature of the sample type. Bones, teeth and hair shaft contain limited amounts of nuclear DNA, making STR profiling difficult. Degraded DNA is fragmented, meaning the larger STR loci may fail to amplify in the PCR reaction, leading to partial DNA profiles being obtained.

Current methods for obtaining DNA profiles from compromised sample types include low copy number DNA profiling, whereby the number of amplification cycles is increased to target the smaller number of molecules available (Gill *et. al.* 2000b); and mitochondrial DNA profiling, targeting the increased number of mitochondria present in remaining cells (Butler and Levin 1998; Holland and Parsons 1999). Both of these methods require specialised techniques for analysis and suffer from sensitivity issues, meaning there is an increased chance of contamination being encountered. Mitochondrial DNA has a decreased discrimination power due to the maternal lineage of the DNA and is incompatible with current DNA databases, however it can be used for intelligence purposes with a high degree of success. LCN DNA profiling has become the preferred method of amplification for compromised samples with strict guidelines for interpretation of profiles, including duplicate amplifications and the generation of consensus profiles, allowing a result to be gained in samples which would previously have failed to amplify (Gill *et. al.* 2000b). Although routinely used in casework in the UK, LCN SGM+ profiling has not been readily accepted by the European and US community. Problems with contamination and the ability to profile trace amounts of DNA has led to the possibility of innocent transfer events, e.g. primary and secondary transfer from holding hands or objects, giving a false positive result (Gill 2002; Lowe *et. al.* 2002; Butler 2005b).

The results of the casework comparative study suggested that LCN DNA profiling was more successful than SNPs, mainly due to the higher discrimination power gained from fewer STR loci. The 21-SNP multiplex failed to give a result for some casework samples and only gave partial profiles for others. Samples had previously been stored frozen and there was a variable time lapse between the LCN DNA profiling and the 21-SNP multiplex amplification. This may have made a difference to the integrity of the sample and periods of freeze-thawing and transit of the samples via the mail system may have caused further degradation of the DNA. The samples had been extracted using Chelex® (Walsh *et. al.* 1991) and this may have caused inhibition of the PCR amplification as it is known to interact with the Taq polymerase, causing a decrease in amplification efficiency. The complexity of the 21-SNP multiplex may have made it more sensitive to PCR inhibition by the Chelex® particles.

Analysis of allele dropout for both SNPs and STRs indicated that SNPs showed a random pattern of dropout consistent with amplification inefficiency as opposed to the size of the amplicon. The STR loci showed a positive regression with the higher molecular weight loci. This result is consistent with the other studies on amplicon size carried out in this thesis and suggests that targeting smaller size DNA molecules could result in a greater chance of amplifying degraded DNA molecules, given an optimised multiplex system.

8 General Discussion

DNA profiling was first used in criminology in 1986 and, once accepted in court, quickly became established worldwide as a technique for human identification. Today, DNA profiling systems used for national DNA databases utilise polymorphic short tandem repeat (STR) sequences to gain a DNA profile of an individual. The most significant problem facing the forensic analyst in general casework is that approximately 50% of crime scene samples yield a partial DNA profile or no profile at all (P. Gill, *per comms*). This is often the result of the material being highly degraded and limited in quantity; consequently insufficient intact DNA molecules are available for profiling using the current multiplex systems. The resulting discriminating power of a partial profile may only be of the order of one in thousands to one in a million and when such profiles are used to interrogate national DNA databases, multiple matches may be found (Butler 2005b).

The effect of DNA degradation in forensic DNA profiling is most apparent in mass disaster situations, such as fires (Clayton *et. al.* 1995a), air crashes (Ballantyne 1997), terrorist attacks (Holland *et. al.* 2003), tsunamis and earthquakes (Alonso *et. al.* 2005). Dependent on the type of disaster, victims will have been subjected to a wide range of extreme conditions including ultra-high temperatures and high levels of humidity, causing degradation of the DNA molecules. Partial profiles obtained from degraded DNA samples consist mainly of the STR loci with lower amplicon sizes (Golenberg *et. al.* 1996; Wiegand and Kleiber 2001; Butler *et. al.* 2003; Gill *et. al.* 2006) making it increasingly difficult to obtain a DNA profile that can be successfully used for identification purposes.

Research was carried out into the use of single nucleotide polymorphisms (SNPs) as a possible adjunct to current DNA profiling systems. Autosomal SNP analysis had been dismissed from high-throughput DNA profiling in the early 1990s in favour of STRs, as STR loci were more amenable (at that time) to automated techniques (P. Gill, *per comms*). Specialist techniques were developed for some specific SNP applications, including mitochondrial (Hagelberg *et. al.* 1991; Holland *et. al.* 1993; Gill *et. al.* 1994; Butler and Levin 1998; Holland and Parsons 1999), Y-chromosome (Di Gaetano *et. al.* 2004; Hammer *et. al.* 2005; Lessig *et. al.* 2005; Brion 2005a; Brion *et. al.* 2005b) and red hair SNP analysis

(Grimes *et. al.* 2001). Before the introduction of low copy number (LCN) STR profiling, mitochondrial DNA (mtDNA) analysis was routinely used for genotyping degraded samples and samples containing little DNA, such as bone, hair and teeth. Mitochondria are present within cells in high copy number (approximately 500-2000 per cell (Parsons and Coble 2001)), hence mtDNA is more likely to survive intact for longer periods. This has made mtDNA analysis the primary method of genotyping ancient DNA samples (Cooper *et. al.* 2001; von Wurmb-Schwark *et. al.* 2003; Willerslev and Cooper 2005), although the technique is highly sensitive and results are not always distinguishable from contamination. MtDNA is maternally inherited; therefore all offspring of a female will have the same mtDNA sequence, making it less discriminating than STRs for forensic DNA analysis. Current mtDNA analysis involves complete sequence determination of two hypervariable regions (HV1 and HV2) within the mitochondrial control region. The control region is the only significant portion of mtDNA that doesn't encode for genes (Parsons and Coble 2001) and, as such, shows high variation within the population. Full sequencing of mtDNA is labour-intensive and attempts have been made to develop techniques that are less time-consuming such as mini-sequencing (Tully *et. al.* 1996; Quintans *et. al.* 2004) and other primer extension assays (Vallone *et. al.* 2004; Divne and Allen 2005). Mini-sequencing was also used to develop an analysis method for identification of hair colour, targeting twelve known SNP loci within the melanocortin 1 receptor gene (*MC1R*) (Grimes *et. al.* 2001). Homozygosity at a locus, or compound heterozygosity at two loci, was used as an indicator that an individual would have red hair. Although it is difficult to identify any individual gene for a physical characteristic, further research is ongoing into the use of SNPs for other physical traits, such as iris colour (Frudakis *et. al.* 2003) and skin colour (Bonilla *et. al.* 2005). Y-chromosome SNPs have been used for determination of ethnicity, using allele-hybridisation biochemistry in a commercially available kit, "Signet™ Y-SNP" (Marligen Biosciences Inc.). PCR products are detected by hybridisation to colour-coded beads using the Luminex®100™ System (Taylor *et. al.* 2001; Wetton *et. al.* 2005). The Y-chromosome is particularly useful as an indicator of ancestral origin due to the lack of recombination along the majority of the chromosome. This has resulted in "*an accumulation of mutations in slowly evolving SNP haplogroups that reflect the progressive diversification of Y* *chromosome lineages during the expansion of human populations*" (Wetton *et. al.* 2005). As with autosomal loci, STR sequences within the Y-chromosome have an increased discrimination power to SNPs, and these have been more widely researched and developed for forensic use in recent years (Corach *et. al.* 2001; Hall and Ballantyne 2003; Daniels *et. al.* 2004; Hanson and Ballantyne 2004; Mulero *et. al.* 2006).

The development of microarray technology re-introduced autosomal non-coding SNPs into the forensic field, as the possibility of high-throughput genotyping was realised (Southern 1996a; Southern 1996b). This development coincided with the introduction of the National DNA Database® in 1995. Not all samples successfully amplified by standard DNA profiling methods, due to degradation and/or low DNA copy number. SNP loci comprise one base, therefore it was suggested that the size of PCR amplicons could be reduced, increasing the chance of amplifying the smaller fragments of DNA found in degraded samples. Microarray technology was utilised, using fluorescent detection methods, for detection of SNP loci. Microarrays were originally developed for gene expression profiling due to the ability to analyse thousands of different samples and / or different genes in a single run (Southern 1995; Southern 1996b; Bowtell 1999; Vente *et. al.* 1999; Southern 2001). It was envisaged that the development of high-throughput automated microarray technology, in conjunction with the smaller amplicon size generated from SNP loci, would enable SNPs to be targeted for forensic DNA profiling. A computer program (ASGOTH) was developed for automated SNP genotyping. This allowed a precise measurement of the number of control samples and negative samples needed to allow accurate genotyping of each SNP locus. ASGOTH proved to be efficient to genotype SNP loci correctly, however a decision was made to continue the development of a SNP multiplex system that utilised the Universal Reporter Primer principle to amplify all loci in one large multiplex for detection using capillary electrophoresis (Hussain *et. al.* 2003; Dixon *et. al.* 2005a). The main drive behind this decision was the inability to produce large multiplexes for detection using fluorescent probes. Due to the presence of primer-dimer formations, probes were found to bind to the negative control spots on a microarray glass slide, causing high levels of background fluorescence. A multiplex of five SNP loci was developed that enabled accurate genotyping, but larger multiplexes exhibited high levels of background fluorescence in the negative controls (Long 2005). Detection using capillary electrophoresis separated the SNP amplicons according to size, allowing primer-dimer peaks to be easily separated from the genuine SNP loci. A recent study has demonstrated the feasibility of using of Microarrays for forensic SNP analysis (Divne and Allen 2005), however the technique requires large amounts of DNA (10-50ng) to be successful and it remains difficult to quantify because of the use of a primer extension reaction. More work is required to increase the robustness of the system to allow a smaller quantity of DNA to be successfully amplified and detected.

An assessment of the STR system used on the UK National DNA Database® (AMP*Fl*STR® SGMplus™ (SGM+)) compared to SNP multiplexes (detected using capillary electrophoresis) indicated an advantage of targeting smaller amplicons, leading to the development of a 21-SNP multiplex with amplicon sizes below 150 bp in length. The discrimination power of the 21-SNP multiplex was approximately one in four million, although this varied according to the genotypes gained for an individual sample. This discrimination power was lower than that obtained from a full STR multiplex result (circa one in a thousand million) (Cotton *et. al.* 2000); therefore its use would be limited to selected evidence types that failed to give an informative STR profile. In relation to parentage analysis and family reconstruction, STRs have proven to be highly successful in the past, e.g. the Waco and World Trade Centre disasters (Whitaker *et. al.* 1995; Clayton *et. al.* 1995a; Clayton *et. al.* 1995b; Holland *et. al.* 2003); however, a second system such as a SNP multiplex could prove advantageous in some sample types where only partial profiles may be obtained due to the degraded state of the samples.

Studies were performed on blood and saliva samples degraded *in situ* using multiplexes that targeted STRs, SNPs or mini-STRs. Degradation was achieved by maintaining samples at 100% humidity in a 37°C environment (Pulker 2004) allowing biochemical degradation by both cellular and microbial enzymes (Lindahl 1993; Poinar 2003). It was hypothesised that the length of DNA available for amplification could be related to the primary level of DNA coiling around a nucleosome complex[10]. The work carried out in this study suggested that smaller amplicon sizes increased the likelihood of gaining a DNA profile using SNP loci, but there was no direct comparison between this and the nucleosome theory. The interaction between histones and the DNA molecule is confined to the phophodiester backbone of the double helix and it is generally considered that no sequence-specificity exists for the regions of the DNA protected within the core particle (Suck 1992). If the coiling of DNA around the histones is random, then the sequences found within the linker DNA will also be random, and SNPs cannot be selected due to their positioning within the nucleosome structure. However other work has been carried out suggesting that there may be obligate sites within the DNA for nucleosome positioning (Sewack and Hansen 1997; Attema *et. al.* 2002). Thåström *et. al.* (2004) suggested the *physical* characteristics of particular DNA sequences, rather than the sequences themselves, were responsible for improved binding to the nucleosome complex (Thåström *et. al.* 2004a; Thåström *et. al.* 2004b) and assigned these as 'nucleosome positioning sequences'. Levitsky *et. al.* (2005) are compiling data through the 'Nucleosome Positioning Region Database', to identify sites within the DNA that may have higher affinity for nucleosome binding (Levitsky *et. al.* 2005). Although research is, as yet, unsubstantiated, further work will be carried out to identify SNPs that may lie in these regions, allowing a new multiplex to be developed with loci more resistant to DNA degradation. A panel of SNPs with known resistance to degradation could greatly increase the chance of successful amplification with degraded DNA samples, if the nucleosomal protection theory proves to be correct.

In order to accurately calculate match probabilities and likelihood ratios, it was necessary to assess the effect of genetic drift on SNP loci. The allele frequencies

[10] The nucleosome unit was defined in the 1970s as eight histone molecules interacting with approximately 200 bp DNA (Kornberg 1974). The nucleosome units were attached to each other by lengths of linker DNA, forming a 'beads on a string' effect. Micrococcal nucleases preferentially targeted the unprotected linker DNA, leaving 146bp of DNA attached to the nucleosome core particle (van Holde et. al. 1975; Noll and Kornberg 1977). A further ten base pairs protruding from each end of the nucleosome were shown to be more readily digestible than those protected within the histone octamer, leaving approximately 125 bp lengths of DNA protected by the nucleosome structure (Read and Crane-Robinson 1985b). The inability of the enzymes to digest the nucleosome/DNA complex suggested that *in vivo* cellular enzymes may also preferentially target the linker DNA leaving only 125 bp fragments of DNA after a prolonged period of degradation.

of loci within sub-populations vary due to different levels of drift and inbreeding within ethnic groups. Allele frequency data from three main ethnic groups (White Caucasian, British Afro-Caribbean and Indian sub-continent) are used for match probability calculations on the National DNA Database® and corrections must be made to compensate for the sub-structuring that occurs within these large population sets (Nichols and Balding 1991; Gill and Evett 1995a). The Balding-Nichols (BN) equation is used in casework to calculate match probabilities and likelihood ratios for STR DNA profiles (Balding and Nichols 1994; Balding and Nichols 1995; NRCII 1996; Gill *et. al.* 2003) and a large number of population studies have been carried out across the forensic community to assess STR allele frequencies within different populations (Balding *et. al.* 1996; Goodwin *et. al.* 2001; Ruitberg *et. al.* 2001; Shimada *et. al.* 2002; Gill *et. al.* 2003; Okamoto *et. al.* 2003; Overall *et. al.* 2003; Yoshida *et. al.* 2003; Konjhodžić *et. al.* 2004; Soltyszewski *et. al.* 2005). A database of DNA samples of sufficient size was unavailable for testing population sub-structure for the 21-SNP multiplex and so computer simulations were run to demonstrate the effectiveness of using the BN equation compared to true allele frequencies found in the simulated sub-structured population. Ideally it would be better to use a real database of samples from isolated populations so the true effect of genetic drift could be characterised, however these samples are difficult to obtain, and large data sets were unavailable for this study. The simulations demonstrated the applicability of the BN equation to SNPs as well as STRs, with minimal reduction in discrimination power. This equation could therefore be used in calculations of match probabilities and likelihood ratios for the 21-SNP multiplex.

A brief search on the Internet demonstrates the large number of government agencies and private companies across the world using STR multiplex systems for parentage analysis. Only accredited companies can provide a service that is acceptable in a court of law, but all companies can legitimately provide DNA information from a sample. This study demonstrated that SNPs could be used for exclusion of an individual as the true father of a child by analysis of the number of mismatched loci present between the alleged father and offspring. However, current STR methods of paternity testing are more discriminating than the 21-SNP multiplex, due to the higher PI values and probability of exclusion that can be calculated. The use of SNPs for paternity testing should not be discouraged as these loci generally have a lower mutation rate than the STR loci used for forensic identification (Chakraborty *et. al.* 1999; Amorim and Pereira 2005; Ayres 2005). This means calculations of exclusion can be based solely on the genotypes identified in each individual, without correction for mutation. To produce a set of SNP loci for paternity testing, each locus selected should have an allele frequency close to 0.5 and approximately 50 SNPs would be needed to match the discrimination of current STR systems (Chakraborty *et. al.* 1999; Gill 2001a). The 21-SNP multiplex developed for this study was not originally designed for paternity analysis, therefore not all of the selected SNPs conformed to the criteria required for a maximised discrimination power. With an increased amount of data now available in the public domain, the opportunity exists to rapidly create SNP multiplexes with loci of known allele frequency. In cases of disputed paternity, DNA can be obtained directly from the individual via blood donation or buccal scrape; therefore optimal amounts can be used for PCR amplification. This means primer extension detection methods, such as SNaPshot™ or UHT SNPStream™, could be used for a multiplex SNP paternity kit without compromising the accuracy of genotyping. Both methods have been used to successfully develop SNP multiplexes comprising up to 39 loci (Bell *et. al.* 2002; Inagaki *et. al.* 2004; Brion *et. al.* 2005b). The main drawback of any primer extension assay is the secondary extension step, which can be variable in efficiency, causing disparity between the peak balance of the amplified alleles. This is less pronounced in samples containing optimal amounts of DNA; therefore an assay for paternity analysis could be more easily developed than one for compromised forensic samples. As STR evidence is already readily accepted in court, it may not be beneficial to alter the system to one that does not give significantly better results. Nevertheless SNPs could be used as an adjunct to STR methods, providing further evidence in cases demonstrating mismatched STR loci between a child and alleged father.

The URP amplification method was developed to be quantitative but the addition of more loci to the multiplex decreased the robustness of the system as the number of primers within the multiplex increased. This meant the amplification efficiency of each locus was different and highly variable for heterozygous balance and homozygous threshold levels, even when using an optimal amount of DNA template. The low level DNA templates would have suffered from competition between the target DNA and the high number of primers present in the amplification reaction. The initial idea of the autosomal SNP project was to develop a multiplex containing 50 loci, allowing the system to maintain the discrimination power of current STR multiplexes (Gill *et. al.* 2000a). The URP biochemistry was used to simplify the design of the multiplex whilst decreasing the likelihood of non-specific binding of the primer sets (Hussain *et. al.* 2003), nevertheless the presence of multiple primers in the reaction decreased the efficiency of the method. It may prove impossible to create a multiplex with larger numbers of loci using the URP method of amplification, due to interaction between the primer sets used. Primer extension assays, such as SNaPshot™, do allow identification of high numbers of SNP loci in one amplification (Inagaki *et. al.* 2004; Quintans *et. al.* 2004; Divne and Allen 2005) and should be considered for further study. Strict interpretation guidelines would be necessary for accurate genotyping, but this is true for any DNA profiling method targeting LCN samples.

To minimise operator variability an in-house computer program, Celestial™, was used to genotype the data obtained from the 21-SNP multiplex, based on a set of rules formulated from dilution series experiments. The interpretation criteria were unique in that each peak was characterised according to the rules laid out for each particular SNP locus. Other DNA profiling systems use interpretation criteria generated from data for all loci to genotype sample data (Gill *et. al.* 1997; Cotton *et. al.* 2000; Gill *et. al.* 2000b). Celestial™ was used for genotyping all the sample data generated in this study. The criteria were set by assessing a range of DNA samples of varying dilutions, more or less forcing alleles to 'drop out' of the DNA profile. For optimal DNA templates, the variation between heterozygous balance and the thresholds for homozygous peaks were a lot lower and a much less stringent rule-set could have been used with equal success. The URP multiplex system could be used for non-forensic purposes, such as medical diagnostics, with relative success as optimal amounts of DNA are readily available. These diagnostic tests are much more amenable to high throughput primer extension techniques, due to increased DNA template levels, and are well-established in the gene expression community (Wang *et. al.* 1998; Heller 2002; Pusch *et. al.* 2003; Dudbridge and Koeleman 2004).

LCN casework samples were amplified with variable success using the 21-SNP multiplex. A comparison of LCN STR and SNP percentage profiles and likelihood ratios indicated that LCN DNA profiling had a greater chance of gaining a result, even though the DNA in the samples was degraded. The discrimination power gained from one STR locus is much higher than that gained from one SNP locus, due to the presence of many alleles at each STR site (Chakraborty *et. al.* 1999; Gill 2001a; Ayres 2005), and a partial STR profile will give an increased likelihood ratio compared to a partial SNP profile. Validation of the multiplex indicated that if LCN STR DNA profiling failed then the 21-SNP multiplex would also fail to amplify. This is most likely due to the amount of template DNA being too low to exponentially amplify, or there may not have been any DNA present in the sample collected. As a consequence of this observation, implementation of the technique into casework was carried out with direction to only use the technique in situations where a partial LCN DNA profile had been obtained.

Validation of the 21-SNP multiplex demonstrated the problems associated with targeting SNP loci as opposed to STRs. The SNP loci selected for use in the multiplex were biallelic, meaning there were only two alleles per locus. Some identified SNP loci are triallelic and it may be more beneficial to target these in future multiplexes (Phillips *et. al.* 2004). The match probability of each biallelic SNP locus is much reduced compared to the multi-allelic STR loci, so more loci need analysing to gain an increased discrimination power (Chakraborty *et. al.* 1999; Gill 2001a; Phillips *et. al.* 2004). The biallelic nature of the selected SNPs also makes it difficult to determine the presence of a mixture in a DNA sample. By developing a technique that is highly quantitative, mixture analysis would be feasible by observing the heterozygous balance of a locus, however no technique currently exists that is quantitative enough to assess mixtures (Gill *et. al.* 2004a; Dixon *et. al.* 2005a; Dixon *et. al.* 2005b). Mixture analysis can be determined by examination of the number of heterozygous loci in a single profile. An excess of heterozygotes can be indicative of a mixed DNA profile; however it is impossible to separate the two genotypes into single profiles due to the presence of only two alleles, and the profile could feasibly have come from one individual. Consequently, another recommendation for the implementation of the system into casework was to only submit discrete sample types, i.e. sample types that were unlikely to contain a mixture, such as deep muscle and bone. The 21-SNP multiplex was found to be of a similar sensitivity to the current method of LCN STR DNA profiling. Due to the problems associated with SNP analysis, along with the average performance of the 21-SNP multiplex (Dixon *et. al.* 2005b), a decision was made to research other methods for analysis of degraded DNA.

A recent extensive collaborative research study by the ENFSI DNA Working Group and EDNAP demonstrated that the success rate for analysis of degraded samples can be substantially improved by testing shorter ("mini") STRs (Dixon *et. al.* 2005b). Current STR loci can be redesigned to produce shorter amplicons, allowing a new mini-STR system to be compatible with current national DNA databases (Butler *et. al.* 2003; Chung *et. al.* 2004; Drabek *et. al.* 2004). The smaller mini-STR sequences benefit from being able to target smaller, more fragmented DNA molecules, whilst maintaining a discrimination power equal to that already gained from conventional STR systems. Research carried out by the National Institute of Standards and Technology (NIST) has considerably furthered the field of mini-STR multiplex development over the last few years (Ruitberg *et. al.* 2001; Butler *et. al.* 2003; Chung *et. al.* 2004; Drabek *et. al.* 2004). STR primer sets used in current multiplex systems are designed up and downstream of the STR locus, a suitable distance away from the polymorphic region. This method had the advantage of being able to design highly efficient primers, as well as making the amplicon sizes such that they could be easily separated using gel (and later capillary) electrophoresis. Mini-STR primers are simply targeted closer to the polymorphic region. This has the advantage of decreasing the length of amplified DNA but can be problematic as the region immediately up and downstream of the STR is highly polymorphic and primer-binding site mutations can be present in some individuals. The presence of primer-binding site mutations and, in some cases, deletions can cause discordance between mini-STR genotypes compared to STR genotypes derived from current profiling methods (Drabek *et. al.* 2004). Full sequencing of STR loci is used to identify any polymorphisms within these regions that could be detrimental to amplification. Primers can be designed with alternative bases, such as inosines, to allow binding within a known polymorphic region.

All existing studies confirm that there are substantial advantages to be obtained by converting current STR systems to low molecular weight mini-STRs (Butler *et. al.* 2003; Chung *et. al.* 2004; Drabek *et. al.* 2004; Graham 2005; Dixon *et. al.* 2005b; Gill *et. al.* 2006). The discriminating power of current systems would be maintained and mixture analysis remains feasible, an essential element when considering highly sensitive DNA profiling techniques which are more likely to yield low level DNA mixtures. The idea is to construct a "mini" STR multiplex, based on a set of core loci identified by analysis of all the systems currently in use across Europe. This mini-STR multiplex could be easily incorporated into the current DNA profiling systems used, and would be compatible with existing STR national DNA databases (Gill *et. al.* 2006). It would also be designed to follow the recommendations of the House of Commons Science and Technology Committee, as outlined in a Home Office response note from Gill *et. al.* in 2005 (http://www.publications.parliament.uk/pa/cm200506/cmselect/cmsctech/427/427.pdf; Gill *et. al.* 2005a). As the number of profiles loaded onto the National DNA Database® increases, the Committee recommends the introduction of a sixteen-locus STR system. Heeding the advice of Sir Alec Jeffreys: "*the consequences of even one false match leading to a conviction that was subsequently overturned could be severe for the DNA database and its public acceptability*"; more loci could be used to decrease the chances of an adventitious match. Gill (2005) outlines the development of a mini-STR system that will increase the likelihood of successful amplification on degraded samples, whilst increasing the discriminating power and improving the comparability to European databases (Gill *et. al.* 2005a; Gill *et. al.* 2006).

This study highlighted the benefits and drawbacks of using a SNP multiplex for forensic identification. The limited amount of research conducted on the forensic use of SNPs suggests it is unlikely that SNPs will replace STRs as the DNA profiling method of choice in the near future, due to incompatibility with current national DNA databases and difficulties in mixture analysis (Gill *et. al.* 2004a). The cost of upgrading a database to SNP DNA profiles far outweighs the benefits of such a system. Although disregarded from widespread use, there is still a requirement for a validated SNP multiplex system in the forensic community, to act as an adjunct to current methods. The ability to design small amplicons allows fragmented DNA to be targeted, however a more robust system is required to cope with the stochastic variation recognised at low DNA template levels. Research is ongoing into primer extension methods of detection but, to date, the 21-SNP multiplex is the only validated SNP multiplex in the forensic community.

Appendices

APPENDIX I – 21-SNP MULTIPLEX DATA

(Amelogenin is omitted from the data)

SNP internal ID	TSC identifier	Chromosome	Polymorphism	Amplicon size (bp)
TSCO D	0252540	3	C/T	103
TSCO U6	0746324	5	A/T	107
TSCO B6	1342445	3	A/T	110
TSCO N4	1156239	18	A/T	114
TSCO Y3	0846740	7	C/G	117
TSCO P5	0176551	1	A/T	122
TSCO A4	0421768	8	C/G	125
TSCO O6	1588825	5	A/T	129
TSCO Z2	0086795	6	C/T	134
TSCO K3	0078283	21	C/G	137
TSCO J2	0156245	19	C/T	141
TSCO Y6	0627632	5	A/T	147
TSCO P7	0897904	6	A/T	151
TSCO J8	0709016	3	A/T	154
TSCO X	0031988	8	A/C	158
TSCO F	0155410	10	A/C	164
TSCO G	0154197	11	C/T	170
TSCO L2	00384808	17	C/T	174
TSCO W3	0820041	9	C/G	180
TSCO H8	0131214	14	A/T	186

APPENDIX II – THE COMPUTER PROGRAM ASGOTH

```
Dim intUnknownSamples As Integer
Dim intUnknownSpots As Integer
Dim RowstoSelect As Integer
Dim SamplesUsed As Integer
Dim intControlSpots As Variant
Dim UnknownsUsed As Integer

Sub ASGOTH2002()

Application.StatusBar = "ASGOTH running..... Please wait..."
Application.ScreenUpdating = False
     Worksheets("collection").Select
     Range("A1").Select

SamplesUsed = Application.InputBox("how many control samples have been used?", "specify numbers", 0, ,
, , , 1)
UnknownsUsed = Application.InputBox("how many unknown samples are there?", "specify unknown sample
number", 0, , , , , 1)
RowstoSelect = Application.InputBox("how many spots do you want to use in the validation?", "select
number of spots", 0, , , , , 1)

  controls
  unknowns

 For theloop = 1 To 1000

  forbootstrappingcontrols
  forbootstrappingunknowns
  comparison
  collectionofdata
  resetforms

Next

Application.StatusBar = False

End Sub
```

```
Sub controls()

Worksheets("controls").Select
Range("F2").Select

intControlSamples = SamplesUsed

Er2:     intControlSpots = Application.InputBox("how many replicate spots are there per control sample?",
"control spots", 0, , , , , 1 + 2)
If intControlSpots = False Then Exit Sub
         If intControlSpots = "" Then
MsgBox "You must enter a valid number of spots", vbOKOnly + vbExclamation
                  GoTo Er2
         End If

Err:     txtGenotype = Application.InputBox("what is the genotype of the first sample?" & (Chr(13) &
Chr(10)) & (Chr(13) & Chr(10)) & " type '1' for Homozygote 1" & (Chr(13) & Chr(10)) & " type '2' for
Heterozygote" & (Chr(13) & Chr(10)) & " type '3' for Homozygote 2", "genotypes", , , , , , 1)

If txtGenotype = False Then Exit Sub
         If txtGenotype = 1 Then
                  For Cellinuse = 1 To intControlSpots
                           ActiveCell.Value = 1
                           ActiveCell.Offset(1, 0).Select
                  Next
         ElseIf txtGenotype = 2 Then
```

```
                For Cellinuse = 1 To intControlSpots
                        ActiveCell.Value = 2
                        ActiveCell.Offset(1, 0).Select
                Next
        ElseIf txtGenotype = 3 Then
                For Cellinuse = 1 To intControlSpots
                        ActiveCell.Value = 3
                        ActiveCell.Offset(1, 0).Select
                Next
        Else
MsgBox "You must enter a genotype 1, 2 or 3", vbOKOnly + vbExclamation, Attention
                        GoTo Err
        End If

 counting = 1

For intSample = 1 To ((intControlSpots * intControlSamples) - (intControlSpots)) Step intControlSpots

        counting = counting + 1

Err2:   txtGenotype = Application.InputBox("what is the genotype of the next sample?" & (Chr(13) &
Chr(10)) & " "" " & (Chr(13) & Chr(10)) & " type 1 for Homozygote 1" & (Chr(13) & Chr(10)) & " type 2
for Heterozygote" & (Chr(13) & Chr(10)) & " type 3 for Homozygote 2", "genotypes", , , , , 1)

If txtGenotype = False Then Exit Sub
                If txtGenotype = 1 Then
                        For Cellinuse = 1 To intControlSpots
                ActiveCell.Value = 1
                        ActiveCell.Offset(1, 0).Select
                        Next
        ElseIf txtGenotype = 2 Then
                        For Cellinuse = 1 To intControlSpots
                                ActiveCell.Value = 2
                                ActiveCell.Offset(1, 0).Select
                Next
                ElseIf txtGenotype = 3 Then
                        For Cellinuse = 1 To intControlSpots
                                ActiveCell.Value = 3
                                ActiveCell.Offset(1, 0).Select
                        Next
        Else
MsgBox "You must enter a genotype 1, 2 or 3", vbOKOnly + vbExclamation, Attention 'if any other value is
added this box comes up
                GoTo Err2
                End If
Next

Range("E2").Select

For intSample = 1 To (intControlSpots * intControlSamples)

If ActiveCell.Offset(0, -4).Value >= Range("K18").Value And ActiveCell.Offset(0, -2).Value >=
Range("L18").Value Then
If ActiveCell.Offset(0, -1).Value = 0 Or ActiveCell.Offset(0, -3).Value = 0 Then
ActiveCell.Value = ""
                ActiveCell.Offset(0, 1).Value = ""
ActiveCell.Offset(1, 0).Select
                Else
                        ActiveCell.Formula = "=LOG10(RC[-3]/RC[-1])"
ActiveCell.Offset(1, 0).Select
                End If
Else
                ActiveCell.Value = ""
ActiveCell.Offset(0, 1).Value = ""
ActiveCell.Offset(1, 0).Select
End If
Next
End Sub
```

Sub unknowns()

```
Worksheets("unknowns").Select
Range("A2").Select

intUnknownSamples = UnknownsUsed

intUnknownSpots = Application.InputBox ("how many replicate spots are there per unknown sample?",
"control spots", 0, , , , , 1)

If intUnknownSpots = False Then Exit Sub

Range("E2").Select

For intSample = 1 To (intUnknownSpots * intUnknownSamples)
If ActiveCell.Offset(0, -1).Value = 0 Or ActiveCell.Offset(0, -3).Value = 0 Then
                ActiveCell.Value = ""
                ActiveCell.Offset(1, 0).Select
        Else
                ActiveCell.Formula = "=LOG10(RC[-3]/RC[-1])"
ActiveCell.Offset(1, 0).Select
        End If
Next

End Sub
```

Sub forbootstrappingunknowns()

```
Worksheets("bootstrapunknowns").Select
Range("A2").Select

Worksheets("unknowns").Select

sample = RowstoSelect

Randomize

For intRow = 2 To ((UnknownsUsed * intUnknownSpots) + 1) Step intUnknownSpots

    For intSpot = 1 To RowstoSelect

      intRowstoPick = Int(24 * Rnd + intRow)

        Cells(intRowstoPick, 1).Select
        strStartcell = ActiveCell.Address(False, False)
        strEndcell = ActiveCell.Offset(0, 5).Address(False, False)
        strSelection = strStartcell & ":" & strEndcell
        Range(strSelection).Select
        Selection.Copy

            Worksheets("bootstrapunknowns").Select
            ActiveCell.Select
            Selection.PasteSpecial Paste:=xlValues
            ActiveCell.Offset(1, 0).Select
            Worksheets("unknowns").Select
    Next
Next

Worksheets("bootstrapunknowns").Select

Range("A:A").Select
Selection.Copy
Worksheets("compare").Select
Range("A1").Select
Selection.PasteSpecial Paste:=xlValues
```

```
Worksheets("bootstrapunknowns").Select
Range("C:C").Select
Selection.Copy
Worksheets("compare").Select
Range("B1").Select
Selection.PasteSpecial Paste:=xlValues

Worksheets("bootstrapunknowns").Select
Range("E:E").Select
Selection.Copy
Worksheets("compare").Select
Range("C1").Select
Selection.PasteSpecial Paste:=xlValues

End Sub
```

Sub forbootstrappingcontrols()

```
Worksheets("bootstrapcontrols").Select
Range("A2").Select

Worksheets("controls").Select

sample = RowstoSelect

Randomize

For intRows = 2 To ((SamplesUsed * intControlSpots) + 1) Step intControlSpots

    For intSpots = 1 To RowstoSelect

Again:

        intRowtoPick = Int(24 * Rnd + intRows)

        If Cells(intRowtoPick, 5).Value = "" Then GoTo Again

          Cells(intRowtoPick, 1).Select
          strStartcell = ActiveCell.Address(False, False)
          strEndcell = ActiveCell.Offset(0, 5).Address(False, False)
          strSelection = strStartcell & ":" & strEndcell
          Range(strSelection).Select
          Selection.Copy

              Worksheets("bootstrapcontrols").Select
              ActiveCell.Select
              Selection.PasteSpecial Paste:=xlValues
              ActiveCell.Offset(1, 0).Select
              Worksheets("controls").Select
    Next
Next

Worksheets("bootstrapcontrols").Select
Columns("E:F").Select
Selection.Copy
Sheets("bins").Select
Range("A1").Select
Selection.PasteSpecial Paste:=xlValues

intHom1 = 0
intHet = 0
intHom2 = 0

Range("A2").Select

For intsamples = 1 To (SamplesUsed * RowstoSelect)
```

```
If ActiveCell.Offset(0, 1).Value = 1 Then
intHom1 = intHom1 + 1
Cells(intHom1, 4).Value = ActiveCell.Value
ElseIf ActiveCell.Offset(0, 1).Value = 2 Then
intHet = intHet + 1
Cells(intHet, 5).Value = ActiveCell.Value
ElseIf ActiveCell.Offset(0, 1).Value = 3 Then
intHom2 = intHom2 + 1
Cells(intHom2, 6).Value = ActiveCell.Value
Else
End If

ActiveCell.Offset(1, 0).Select

Next

Range("H1:S4").Select
Selection.Copy
Sheets("compare").Select
Range("G8").Select
Selection.PasteSpecial Paste:=xlValues

End Sub
```

Sub comparison()

```
Worksheets("table").Select
Range("A1").Select
Worksheets("compare").Select
Range("C1").Select

For inttotalSamples = 0 To ((RowstoSelect * UnknownsUsed) - RowstoSelect) Step RowstoSelect

  ActiveCell.Offset(inttotalSamples + 1, 0).Select

  counter = 1
  counter2 = counter2 + 1

  For intSample = 1 To RowstoSelect

If ActiveCell.Offset(0, -2).Value > Range("I17").Value And ActiveCell.Offset(0, -1).Value >
Range("J17").Value Then
                    counter = counter + 1
                    Cells(counter, 5).Value = ActiveCell.Value
Else
End If

        ActiveCell.Offset(1, 0).Select

  Next

        If Range("I2").Text = "#NUM!" Then
Range("S2").Value = "FAIL"
GoTo next1
        ElseIf Range("I2").Value >= Range("M9") Then
                    Range("S2").Value = 1
ElseIf Range("I2").Value >= Range("M10") And Range("I2").Value <= Range("N10") Then
                    Range("S2").Value = 2
ElseIf Range("I2").Value <= Range("N11") Then
                    Range("S2").Value = 3
End If

        If Range("I2").Value <= Range("M9") And Range("I2").Value >= Range("N10") Then
Range("S2").Value = "POSSIBLE TYPE 1 OR 2"
        End If

If Range("I2").Value <= Range("M10") And Range("I2").Value >= Range("N11") Then
```

```
            Range("S2").Value = "POSSIBLE TYPE 2 OR 3"
        End If

next1:  Range("G2").Value = counter2

        Range("G2:S2").Select
        Selection.Copy
        Worksheets("table").Select
        ActiveCell.Offset(1, 0).Select
        Selection.PasteSpecial Paste:=xlValues

        Worksheets("compare").Select
        Range("E:E").Select
        Selection.ClearContents
Range("C1").Select

Next

Worksheets("table").Select
Range("A1").Value = "Sample Number"

Range("O2").Select

For samples = 1 To intUnknownSamples

If ActiveCell.Offset(0, -1).Value = ActiveCell.Offset(0, -2).Value Then
ActiveCell.Value = 1
ActiveCell.Offset(1, 0).Select
ElseIf ActiveCell.Offset(0, -2).Value = "POSSIBLE TYPE 1 OR 2" Then
ActiveCell.Offset(0, 2).Value = 1
ActiveCell.Offset(1, 0).Select
ElseIf ActiveCell.Offset(0, -2).Value = "POSSIBLE TYPE 2 OR 3" Then
ActiveCell.Offset(0, 2).Value = 1
ActiveCell.Offset(1, 0).Select
ElseIf ActiveCell.Offset(0, -2).Value = "FAIL" Then
ActiveCell.Offset(0, 2).Value = 1
ActiveCell.Offset(1, 0).Select
Else
ActiveCell.Offset(0, 1).Value = 1
ActiveCell.Offset(1, 0).Select
End If
Next

End Sub
```

Sub collectionofdata()

```
Worksheets("collection").Select
Worksheets("table").Select
Range("O915:Q915").Select
Selection.Copy
Worksheets("collection").Select
ActiveCell.Offset(1, 0).Select
Selection.PasteSpecial Paste:=xlValues

End Sub
```

Sub resetforms()

```
Worksheets("table").Select
Range("A2:M4000").Select
Selection.ClearContents
Range("O2:Q900").Select
Selection.ClearContents

End Sub
```

APPENDIX III – SNP 27-PLEX PRIMER SEQUENCES

9, 11 or 13 denotes the Universal sequence found at the 5' end of the primer sequences. Within the multimix, the locus-specific primers are found at concentrations varying from 50nM to 200nM. Universal primers with fluorescent labels are used at 2μM.

Forward primers Universal 9 tail (CGACGTGGTGGATGTGCTAT)

Amelo X	UNI9-CCAGATGTTTCTCAAGTGGTCCTG	44 mer
TSCO D/9	UNI9-GGGAAACTGCTGGGTCTGT	39 mer
TSCO B6/9	UNI9-GGGAGACAGGCCCATGCA	38 mer
TSCO N4/9	UNI9-CAGAAAAGGCAGGAACCTGGACA	43 mer
TSCO Y3/9	UNI9-ACCAACCCCACAAAGCAGG	39 mer
TSCO A4/9	UNI9-GATGCCTCTTGCATTGTGAACG	42 mer
TSCO O6/9	UNI9-GAGCCAAGAATCGCAGGGAA	40 mer
TSCO Z2/9	UNI9-CATTGTGTTTCAAACGCGTGCC	42 mer
TSCO K3/9	UNI9-TGCCACTCTGACACTGATGCTTG	43 mer
TSCO J2/9	UNI9-CTGCCTTGGCTCCCAGCC	38 mer
TSCO Y6/9	UNI9-CAAGATTCCTGGCCCCTGGTAA	42 mer
TSCO J8/9	UNI9-CAGGGAATGACAGGGAACCACTA	43 mer
TSCO X/9	UNI9-CTGTGCATCCACTGCGCC	38 mer
TSCO F/9	UNI9-CCTGGAGCATGXGCTGACCAC	41 mer
TSCO G/9	UNI9-CCATGCCTCACCTCCTGCATT	41 mer
TSCO L2/9	UNI9-GCATGCCATTGCCAAATTCC	40 mer
TSCO W3/9	UNI9-GCCAACCAGACCTCCCAGG	39 mer
TSCO H8/9	UNI9-CTCAGTTGGGTGCTTACGTGCA	42 mer
TSCO L6/9	UNI9-TGTGCATGTTCCCTGGTGTTCA	42 mer
TSCO K4/9	UNI9-GCGGGAGGAAGGAAGGGAGG	40 mer
TSCO X7/9	UNI9-TTTACCATTTGCTCCACAGGGAA	43 mer
TSCO U6/9	UNI9-GCAAGGCCCAAAGCAAAGAA	40 mer
TSCO W5/9	UNI9-AGGACAGTGGCTTCTGTACTGCTA	44 mer
TSCO U5/9	UNI9-CTGGAAGGGCTTTGTTTGCCAA	42 mer
TSCO V4/9	UNI9-CTGGGGAGGAAGGCTGGAGA	40 mer
TSCO P7/9	UNI9-CTCTTCCAGCAGGCACCATGA	41 mer
TSCO P5/9	UNI9-GGGGGTACTGGGGAGACCAA	40 mer

Forward primers Universal 11 tail (TGACGTGGCTGACCTGAGAC)

Amelo Y	UNI11-AAAGTGGTTTCTCAAGTGGTCCCA	44 mer
TSCO D/11	UNI11-GGGAAACTGCTGGGTCTGC	39 mer
TSCO B6/11	UNI11-GGGAGACAGGCCCATGCT	38 mer
TSCO N4/11	UNI11-CAGAAAAGGCAGGAACCTGGACT	43 mer
TSCO Y3/11	UNI11-ACCAACCCCACAAAGCAGC	39 mer
TSCO A4/11	UNI11-GATGCCTCTTGCATTGTGAACC	42 mer
TSCO O6/11	UNI11-GAGCCAAGAATCGCAGGGAT	40 mer
TSCO Z2/11	UNI11-CATTGTGTTTCAAACGCGTGCT	42 mer
TSCO K3/11	UNI11-TGCCACTCTGACACTGATGCTTC	43 mer
TSCO J2/11	UNI11-CTGCCTTGGCTCCCAGCT	38 mer
TSCO Y6/11	UNI11-CAAGATTCCTGGCCCCTGGTAT	42 mer
TSCO J8/11	UNI11-CAGGGAATGACAGGGAACCACTT	43 mer
TSCO X/11	UNI11-CTGTGCATCCACTGCGCA	38 mer
TSCO F/11	UNI11-CCTGGAGCATGXGCTGACCAA	41 mer
TSCO G/11	UNI11-CCATGCCTCACCTCCTGCATC	41 mer
TSCO L2/11	UNI11-GCATGCCATTGCCAAATTCT	40 mer
TSCO W3/11	UNI11-GCCAACCAGACCTCCCAGC	39 mer
TSCO H8/11	UNI11-CTCAGTTGGGTGCTTACGTGCT	42 mer
TSCO L6/11	UNI11-TGTGCATGTTCCCTGGTGTTCT	42 mer
TSCO K4/11	UNI11-GCGGGAGGAAGGAAGGGAGC	40 mer
TSCO X7/11	UNI11-TTTACCATTTGCTCCACAGGGAT	43 mer
TSCO U6/11	UNI11-GCAAGGCCCAAAGCAAAGAT	40 mer
TSCO W5/11	UNI11-AGGACAGTGGCTTCTGTACTGCTT	44 mer
TSCO U5/11	UNI11-CTGGAAGGGCTTTGTTTGCCAT	42 mer
TSCO V4/11	UNI11-CTGGGGAGGAAGGCTGGAGT	40 mer
TSCO P7/11	UNI11-CTCTTCCAGCAGGCACCATGT	41 mer
TSCO P5/11	UNI11-GGGGGTACTGGGGAGACCAT	40 mer

Reverse primers Universal 13 tail (CAAGCTGGTGGCTGTGCAAG)

Amelo/rev	UNI13- TGCTTAAACTGGGAAGCTGXTGGT	44 mer
TSCO D/rev	UNI13- AATGACCTGCCCCACAGGAG	40 mer
TSCO B6/rev	UNI13- GCCATTCAGAACTAACTAGTCTGGGA	46 mer
TSCO N4/rev	UNI13- CGACGGGGGTTGAGTGGTTCAG	42 mer
TSCO Y3/rev	UNI13- ATTAGAGCAGCCAAGTCCTGACCA	44 mer
TSCO A4/rev	UNI13- GCTCAACAGCACAACTCTGCTACAGC	46 mer
TSCO O6/rev	UNI13- GCTAAAGCAGCTCTGAAACCCA	42 mer
TSCO Z2/rev	UNI13- GGATCAGAGAAAGTGCAGCTGGT	43 mer
TSCO K3/rev	UNI13- AATGGGGAGATTGGCTTGGAC	41 mer
TSCO J2/rev	UNI13- CCTGAACATCCCTGAAGGTATTTCG	45 mer
TSCO Y6/rev	UNI13- GATTTGGGAXTTTAGTGACATCTGCA	46 mer
TSCO J8/rev	UNI13- CTGTACATCTTTTAAGACCAACTCCTT	47 mer
TSCO X/rev	UNI13- TCTAGGCTGGTGCCAGCCC	39 mer
TSCO F/rev	UNI13- GGCTCTGAAGAACAATGGGGAG	42 mer
TSCO G/rev	UNI13- CAATCCTGTTTGCAGAGTTCCAG	43 mer
TSCO L2/rev	UNI13- TGAGCCAAGGTGTGGGGA	38 mer
TSCO W3/rev	UNI13- TTACACAGGTCTCCAGCTTGAGCAA	45 mer
TSCO H8/rev	UNI13- AAGAGGGAGCACTGTGGGACTG	42 mer
TSCO L6/rev	UNI13- AACGGCCTTGCTTCGCTGA	39 mer
TSCO K4/rev	UNI13- GGGCAGGTCAGGATGGAGCAG	41 mer
TSCO X7/rev	UNI13- CACCTTGCTGCATCCTGCTG	40 mer
TSCO U6/rev	UNI13- TGGATAGATGATCAGTCTGCGTTC	44 mer
TSCO W5/rev	UNI13- GGCCAGCAGAGATTCACACTGT	42 mer
TSCO U5/rev	UNI13- GATGGAATCACTGTCCTTGCCCT	43 mer
TSCO V4/rev	UNI13- AGCCAAGATCGCACCACTGTA	41 mer
TSCO P7/rev	UNI13- AGTGGTTTGCTGCATGAGTCCA	42 mer
TSCO P5/rev	UNI13- CGGAGGAGATTTTGCCCTGCA	41 mer

Universal 9	CGACGTGGTGGATGTGCTAT	20 mer	5’-JOE-6 dye label
Universal 11	TGACGTGGCTGACCTGAGAC	20 mer	5’-FAM-6 dye label

X = Inosine

APPENDIX IV - SNP 21-PLEX PRIMER SEQUENCES

9, 11 or 13 denotes the Universal sequence found at the 5' end of the primer sequences.

Within the multimix, the locus-specific primers are found at concentrations varying from 50nM to 200nM. Universal primers with fluorescent labels are used at 2μM.

Forward primers Universal 9 tail (CGACGTGGTGGATGTGCTAT)

Amelo X	UNI9-CCAGATGTTTCTCAAGTGGTCCTG	44mer
TSC0 D/9	UNI9-GGGAAACTGCTGGGTCTGT	39mer
TSCO U6/9	UNI9-GCAAGGCCCAAAGCAAAGAA	40mer
TSCO B6/9	UNI9-GGGAGACAGGCCCATGCA	38mer
TSCO N4/9	UNI9-CAGAAAAGGCAGGAACCTGGACA	43mer
TSCO Y3/9	UNI9-ACCAACCCCACAAAGCAGG	39mer
TSCO P5/9	UNI9-GGGGGTACTGGGGAGACCAA	40mer
TSCO A4/9	UNI9-GATGCCTCTTGCATTGTGAACG	42mer
TSCO O6/9	UNI9-GAGCCAAGAATCGCAGGGAA	40mer
TSCO Z2/9	UNI9-CATTGTGTTTCAAACGCGTGCC	42mer
TSCO K3/9	UNI9-TGCCACTCTGACACTGATGCTTG	43mer
TSCO J2/9	UNI9-CTGCCTTGGCTCCCAGCC	38mer
TSCO Y6/9	UNI9-CAAGATTCCTGGCCCCTGGTAA	42mer
TSCO P7/9	UNI9-CTCTTCCAGCAGGCACCATGA	41mer
TSCO J8/9	UNI9-CAGGGAATGACAGGGAACCACTA	43mer
TSCO X/9	UNI9-CTGTGCATCCACTGCGCC	38mer
TSCO F/9	UNI9-CCTGGAGCATGXGCTGACCAC	41mer
TSCO G/9	UNI9-CCATGCCTCACCTCCTGCATT	41mer
TSCO L2/9	UNI9-GCATGCCATTGCCAAATTCC	40mer
TSCO W3/9	UNI9-GCCAACCAGACCTCCCAGG	39mer
TSCO H8/9	UNI9-CTCAGTTGGGTGCTTACGTGCA	42mer

Forward primers Universal 11 tail (TGACGTGGCTGACCTGAGAC)

Amelo Y	UNI11-AAAGTGGTTTCTCAAGTGGTCCCA	44mer
TSC0 D/11	UNI11-GGGAAACTGCTGGGTCTGC	39mer
TSCO U6/11	UNI11-GCAAGGCCCAAAGCAAAGAT	40mer
TSCO B6/11	UNI11-GGGAGACAGGCCCATGCT	38mer
TSCO N4/11	UNI11-CAGAAAAGGCAGGAACCTGGACT	43mer
TSCO Y3/11	UNI11-ACCAACCCCACAAAGCAGC	39mer
TSCO P5/11	UNI11-GGGGGTACTGGGGAGACCAT	40mer
TSCO A4/11	UNI11-GATGCCTCTTGCATTGTGAACC	42mer
TSCO O6/11	UNI11-GAGCCAAGAATCGCAGGGAT	40mer
TSCO Z2/11	UNI11-CATTGTGTTTCAAACGCGTGCT	42mer
TSCO K3/11	UNI11-TGCCACTCTGACACTGATGCTTC	43mer
TSCO J2/11	UNI11-CTGCCTTGGCTCCCAGCT	38mer
TSCO Y6/11	UNI11-CAAGATTCCTGGCCCCTGGTAT	42mer
TSCO P7/11	UNI11-CTCTTCCAGCAGGCACCATGT	41mer
TSCO J8/11	UNI11-CAGGGAATGACAGGGAACCACTT	43mer
TSCO X/11	UNI11-CTGTGCATCCACTGCGCA	38mer
TSCO F/11	UNI11-CCTGGAGCATGXGCTGACCAA	41mer
TSCO G/11	UNI11-CCATGCCTCACCTCCTGCATC	41mer
TSCO L2/11	UNI11-GCATGCCATTGCCAAATTCT	40mer
TSCO W3/11	UNI11-GCCAACCAGACCTCCCAGC	39mer
TSCO H8/11	UNI11-CTCAGTTGGGTGCTTACGTGCT	42mer

Reverse primers Universal 13 tail (CAAGCTGGTGGCTGTGCAAG)

Amelo/13	UNI13-TGCTTAAACTGGGAAGCTGXTGGT	44mer
TSCO D/13	UNI13-AATGACXTGCCCCACAGGAG	40mer
TSCO U6/13	UNI13-ACAAAGCCCCAAGGCAGAG	39mer
TSCO B6/13	UNI13-GCCATTCAGAACTAACTAGTCTGGGA	46mer
TSCO N4/13	UNI13-CGACGGGGGTTGAGTGGTTCAG	42mer
TSCO Y3/13	UNI13-ATTAGAGCAGCCAAGTCCTGACCA	44mer
TSCO P5/13	UNI13-AGGCGGATCCTGGAGGG	37mer
TSCO A4/13	UNI13-GCTCAACAGCACAACTCTGCTACAGC	46mer
TSCO O6/13	UNI13-GCTAAAGCAGCTCTGAAACCCA	42mer
TSCO Z2/13	UNI13-GGATCAGAGAAAGTGCAGCTGGT	43mer
TSCO K3/13	UNI13-AATGGGGAGATTGGCTTGGAC	41mer
TSCO J2/13	UNI13-CCTGAACATCCCTGAAGGTATTTCG	45mer
TSCO Y6/13	UNI13-TAGCCTTAGGACATGGTGATTACAGA	46mer
TSCO P7/13	UNI13-GATTTGGGAXTTTAGTGACATCTGCA	46mer
TSCO J8/13	UNI13-CTGTACATCTTTTAAGACCAACTCCTT	47mer
TSCO X/13	UNI13-TCTAGGCTGGTGCCAGCCC	39mer
TSCO F/13	UNI13-GGCTCTGAAGAACAATGGGGAG	42mer
TSCO G/13	UNI13-CAATCCTGTTTGCAGAGTTCCAG	43mer
TSCO L2/13	UNI13-TGAGCCAAGGTGTGGGGA	38mer
TSCO W3/13	UNI13-TTACACAGGTCTCCAGCTTGAGCAA	45mer
TSCO H8/13	UNI13-AAGAGGGAGCACTGTGGGACTG	42mer

Universal 9	CGACGTGGTGGATGTGCTAT	20 mer	5'-JOE-6 dye label
Universal 11	TGACGTGGCTGACCTGAGAC	20 mer	5'-FAM-6 dye label

X = Inosine

APPENDIX V – PCR AMPLIFICATION PARAMETERS

SNP multiplex amplification

	Temperature	*Time*
	95°C	11 min
6 cycles	94°C	30 sec
	60°C	15 sec
	72°C	15 sec
	60°C	15 sec
	72°C	15 sec
	60°C	15 sec
	72°C	30 sec
29 cycles	94°C	30 sec
	76°C	105 sec
3 cycles	94°C	60 sec
	60°C	30 sec
	76°C	60 sec
	60°C	45 min
	4°C	Hold

SGM+ amplification

	Temperature	*Time*
	95°C	11 min
28 cycles (standard) / 34 cycles (low copy number)	94°C	60 sec
	59°C	60 sec
	72°C	60 sec
	60°C	45 min
	4°C	Hold

APPENDIX VI – PERCENTAGE PROFILES FOR BOILED DNA EXTRACTS

		SNPs %			SGM+ %		
time of boiling (min)	Sample ID	full 27-plex profile	LMW SNPs only	HMW SNPs only	full SGM+ profile	LMW SGM+ STRs	HMW SGM+ STRs
0	CAS	96	100	89	100	100	100
15	CAS	94	100	83	100	100	100
30	CAS	94	100	83	100	100	100
45	CAS	94	100	83	100	100	100
60	CAS	94	100	83	100	100	100
75	CAS	94	100	83	100	100	100
90	CAS	94	100	83	100	100	100
105	CAS	87	100	56	100	100	100
120	CAS	81	100	39	100	100	100
150	CAS	80	100	33	100	100	100
180	CAS	69	94	11	100	100	100
210	CAS	69	94	11	95	100	90
240	CAS	63	92	6	86	100	70
0	DRJ	98	100	94	100	100	100
15	DRJ	98	100	94	100	100	100
30	DRJ	98	100	94	100	100	100
45	DRJ	98	100	94	100	100	100
60	DRJ	96	100	89	100	100	100
75	DRJ	96	100	89	100	100	100
90	DRJ	96	100	89	100	100	100
105	DRJ	91	100	67	100	100	100
120	DRJ	93	100	72	100	100	100
150	DRJ	89	100	61	100	100	100
180	DRJ	83	100	44	91	100	80
240	DRJ	78	100	33	77	100	50
0	SHM	100	100	100	100	100	100
15	SHM	100	100	100	100	100	100
30	SHM	100	100	100	100	100	100
45	SHM	100	100	100	100	100	100
60	SHM	98	100	94	100	100	100
75	SHM	98	100	94	100	100	100
90	SHM	98	100	94	100	100	100
105	SHM	96	100	83	100	100	100
120	SHM	94	100	83	100	100	100
150	SHM	91	100	72	91	100	80
180	SHM	89	97	67	73	83	60
210	SHM	80	97	39	41	75	0
240	SHM	52	75	6	23	42	0
0	HER	93	100	78	100	100	100
15	HER	93	100	78	100	100	100
30	HER	93	100	78	100	100	100
45	HER	91	100	72	100	100	100
60	HER	89	97	72	100	100	100
75	HER	87	97	67	100	100	100
90	HER	81	94	56	100	100	100
105	HER	89	100	72	95	100	90
120	HER	89	100	72	91	100	80
150	HER	89	100	67	91	100	80
180	HER	85	100	56	91	100	80
210	HER	80	100	39	82	100	60
240	HER	76	100	28	77	100	50
300	HER	67	94	11	73	100	40
0	ST	89	100	67	100	100	100
15	ST	89	100	67	100	100	100
30	ST	89	100	67	100	100	100
45	ST	89	100	67	100	100	100
60	ST	89	100	67	100	100	100
75	ST	87	100	61	100	100	100
90	ST	83	97	50	100	100	100
105	ST	87	100	61	100	100	100
120	ST	87	100	61	100	100	100
150	ST	87	100	61	91	100	80
180	ST	83	100	50	91	100	80
210	ST	78	100	33	82	100	60
240	ST	74	100	22	73	100	40
300	ST	63	86	6	50	92	0

APPENDIX VII - CELESTIAL™ INTERPRETATION CRITERIA

(12 second data / 20 second data)

SNP ID	Heterozygous balance ($Hb\%_{min}$)	Homozygote threshold (Ht_{max}) (rfu)
Amelo	24/26	484/672
D	25/41	738/1031
U6	32/36	373/671
B6	24/24	394/540
N4	11/13	506/736
Y3	25/26	186/256
P5	34/32	509/740
A4	25/23	583/895
O6	16/16	182/738
Z2	26/25	456/954
K3	17/14	703/994
J2	23/24	204/539
Y6	27/30	545/631
P7	15/15	229/329
J8	35/41	401/1020
X	42/40	468/655
F	33/33	860/1282
G	32/35	320/574
L2	50/50	276/395
W3	13/18	290/641
H8	50/50	156/452

Heterozygous balance (*Hb%*) data were calculated using dilution series data. Where the lowest *Hb%* was greater than 50% the Celestial™ criteria was set at 50% to allow for sample variation. Homozygous threshold (Ht_{max}) criteria were the maximum observed value plus an additional 20% to allow for outliers. The negative baseline (*Bt*) was set at 100rfu and 200rfu for 12 and 20 second data respectively.

APPENDIX VIII - FULL 21-SNP MULTIPLEX GENOTYPING RESULTS FOR KUWAITI FAMILY SAMPLES

Genotypes in **bold** indicate rare alleles (allele frequency <0.1).
Genotypes in red indicate parent SNP profiles.
Genotypes in blue indicate offspring unrelated to the alleged father with mismatched loci highlighted in *italics*.
Genotypes in grey indicate the locus is uninformative due to the father being heterozygous.

	Sample ID		Am	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8	
Family 1	10094	Father 1	X/Y	C/C	A/T	A/A	A/T	G/C	T/A	C/G	A/A	C/T	G/C	C/C	T/A	T/A	A/A	C/C	C/A	T/C	C/C	C/C	T/T	Parent
	10095	Mother 1	X/X	T/T	A/T	A/T	A/A	G/G	T/T	C/G	A/A	C/C	C/C	T/T	T/T	T/A	A/A	C/C	C/C	T/T	C/C	C/G	T/T	Parent
	10096	Child 1A	X/Y	T/C	T/T	A/A	A/A	G/C	T/A	C/G	A/A	C/T	G/C	C/T	T/T	T/A	A/A	C/C	C/C	T/C	C/C	C/G	T/T	
	10097	Child 1B	X/X	T/C	A/T	A/T	A/A	G/C	T/T	C/C	A/A	C/T	G/C	C/T	T/A	T/A	A/A	C/C	C/A	T/C	C/C	C/C	T/T	
	10098	Child 1C	X/Y	T/C	A/T	A/A	A/T	G/C	T/T	C/C	A/A	C/C	C/C	C/T	T/T	T/A	A/A	C/C	C/C	T/T	C/C	C/G	T/T	
	10099	Child 1D	X/Y	T/C	T/T	A/A	A/A	G/G	T/A	C/C	A/A	C/T	G/C	C/T	T/A	T/A	A/A	C/C	C/A	T/C	C/C	C/G	T/T	
	10100	Child 1E	X/Y	T/C	A/T	A/A	A/A	G/G	T/A	C/G	A/A	C/T	G/C	C/T	T/T	T/T	A/A	C/C	C/C	T/T	C/C	C/C	T/T	
Family 2	10218	Father 2	X/Y	C/C	A/T	A/T	A/A	G/G	T/A	C/C	A/T	C/C	G/C	C/C	T/A	T/T	A/T	C/C	C/C	T/T	C/C	C/C	T/T	Parent
	10219	Mother 2	X/X	T/C	T/T	T/T	A/T	G/G	A/A	C/G	T/T	C/T	C/C	C/C	T/A	T/A	A/A	C/C	C/C	T/C	C/C	C/C	T/T	Parent
	10212	Child 2A	X/Y	T/C	T/T	T/T	A/A	G/G	T/A	C/G	A/T	C/T	C/C	C/C	T/T	T/A	A/T	C/C	C/C	T/C	C/C	C/C	T/T	
	10213	Child 2B	X/Y	C/C	A/T	A/T	A/T	G/G	A/A	C/C	T/T	C/T	G/C	C/C	A/A	T/A	A/A	C/C	C/C	T/C	C/C	C/C	T/T	
	10214	Child 2C	X/Y	T/C	T/T	A/T	A/T	G/G	T/A	C/C	A/T	C/T	G/C	C/C	T/T	T/T	A/T	C/C	C/C	T/T	C/C	C/C	T/T	
	10215	Child 2D	X/X	C/C	A/T	T/T	A/A	G/G	T/A	C/C	T/T	C/C	G/C	C/C	T/A	T/A	A/A	C/C	C/C	T/C	C/C	C/C	T/T	
	10216	Child 2E	X/X	C/C	T/T	A/T	A/T	G/G	T/A	C/G	T/T	C/T	C/C	C/C	A/A	T/A	A/T	C/C	C/C	T/T	C/C	C/C	T/T	
	10217	Child 2F	X/X	C/C	A/T	T/T	A/T	G/G	T/A	C/C	T/T	C/T	G/C	C/C		T/T	A/A	C/C	C/C	T/T	C/C	C/C	T/T	
Family 3	10244	Father 3	X/Y	C/C	A/T	A/T	A/A	G/G	T/T	C/G	T/T	C/C	G/C	C/T	T/A	T/T	A/A	C/C	C/A	T/T	C/C	C/C	T/T	Parent
	10245	Mother 3	X/X	C/C	T/T	A/T	A/T	G/C	A/A	C/C	A/T	C/T	G/C	C/C	T/A	T/T		C/C	C/C	T/C	C/C	C/C	T/T	Parent
	10246	Child 3A	X/Y	C/C	A/T	A/T	A/T	G/C	T/A	C/G	A/T	C/C	G/G	C/T	T/A	T/T	A/A	C/C	C/C	T/T	C/C	C/C	T/T	
	10247	Child 3B	X/Y	C/C	T/T	A/T	A/A	G/G	T/A	C/G	T/T	C/C	C/C	C/T	T/T	T/T	A/A	C/C	C/C	T/C	C/C	C/C	T/T	
	10248	Child 3C	X/X	C/C	T/T	T/T	A/A	G/C	T/A	C/G	T/T	C/C	G/G	C/T	T/A	T/T	A/A	C/C	C/C	T/C	C/C	C/C	T/T	
	10249	Child 3D	X/Y	C/C	A/T	T/T	A/A	G/G	T/A	C/C	A/T	C/T	G/C	C/T	A/A	T/T	A/A	C/C	C/A	T/T	C/C	C/C	T/T	
	10250	Child 3E	X/Y	C/C	A/T	T/T	A/T	G/C	T/A	C/C	A/T	C/T	G/C	C/T	T/A	T/T	A/A	C/C	C/C	T/C	C/C	C/C	T/T	
Family 4	10466	Father 4	X/Y	T/C	T/T	A/T	A/T	G/C	T/T	C/C	A/A	C/C	C/C	C/C	T/A	T/A	A/T		C/A	T/T	C/C	C/C	A/T	Parent
	10467	Mother 4	X/X	T/T	T/T	A/A	A/T	G/G	T/A	C/G	A/T	C/C	G/C	C/C	T/A	T/T	A/T	C/C	C/C	T/C	C/C	C/C	T/T	Parent
	10468	Child 4A	X/Y	T/T	T/T	A/A	A/T	G/G	T/A	C/C	A/T	C/C	C/C	C/C	T/A	T/T	A/T	C/C	C/C	T/C	C/C	C/C	A/T	
	10469	Child 4B	X/Y	T/T	T/T	A/T	A/T	G/C	T/T	C/C	A/T	C/C	G/C	C/C	T/A	T/T	T/T	C/C	C/A	T/C	C/C	C/C	A/T	
	10470	Child 4C	X/Y	T/C	T/T	A/T	A/T	G/G	T/T	C/G	A/T	C/C	C/C	C/C	A/A	T/T	A/T	C/C	C/A	T/C	C/C	C/C	A/T	
	10471	Child 4D	X/Y	T/T	T/T	A/T	A/T	G/G	T/T	C/G	A/T	C/C	C/C	C/C	T/A	T/T	A/A	C/C	C/C	T/T	C/C	C/C	A/T	
	10472	Child 4E	X/X	T/T	T/T	A/T	A/A	G/C	T/A	C/G	A/A	C/C	C/C	C/C	T/A	T/A	A/T	C/C	C/C	T/T	C/C	C/C	A/T	
	10473	Child 4F	X/X	T/T	T/T	A/A	A/T	G/C	T/T	C/C	A/A	C/C	C/C	C/C	T/T	T/T	T/T	C/C	C/C	T/T	C/C	C/C	T/T	

	Sample ID		Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8	
Family 5	8290	Mother 5	X/X	T/C	T/T	A/T	A/T	G/C	T/T	C/C	A/A	C/C	C/C	C/C	T/A	T/A	A/A	C/C	C/C	T/T	C/C	C/G	T/T	Parent
	8291	Father 5	X/Y	T/T	A/A	A/A	A/T	G/G	T/A	C/G	T/T	C/C	G/C	C/C	T/A	T/T	A/A	C/A	C/C	T/C	C/C	C/C	T/T	Parent
	8292	Child 5A	X/Y	T/C	A/T	A/T	A/A	G/G	T/A	C/C	A/T	C/C	G/C	C/C	T/A	T/A	A/A	C/A	C/C	T/T	C/C	C/C	T/T	
	8293	Child 5B	X/Y	T/C	A/T	A/A	A/T	G/C	T/A	C/G	A/T	C/C	C/C	C/C	T/T	T/A	A/A	C/A	C/C	T/T	C/C	C/C	T/T	
	8294	Child 5C	X/X	T/T	A/T	A/T	A/T	G/C	T/A	C/G	A/T	C/C	C/C	C/C	A/A	T/A	A/A	C/A	C/C	T/C	C/C	C/C	T/T	
	8295	Child 5D	X/Y	T/C	A/T	A/A	A/T	G/G	T/A	C/G	A/T	C/C	G/C	C/C	T/A	T/A	A/A	C/C	C/C	T/C	C/C	C/C	T/T	
	8296	Child 5E	X/Y	T/T	A/T	A/A	T/T	G/C	T/A	C/G	A/T	C/C	C/C	C/C	A/A	T/T	A/A		C/C	T/C	C/C	C/C	T/T	
	8297	Child 5F	X/Y	T/T	A/T	A/T	A/A	G/C	T/A	C/G	A/T	C/C	G/C	C/C	T/A	T/A	A/A	C/C	C/C	T/C	C/C	C/G	T/T	
Family 6	8485	Father 6	X/Y	T/T	T/T	A/A	A/A	G/G	T/T	C/G	A/A	C/T	G/C	C/C	T/A	T/T	A/T	C/C	A/A	T/T	C/C	C/C	T/T	Parent
	8486	Mother 6	X/X	T/C	A/T	T/T	A/A	G/G	T/A	C/G	A/A	T/T	C/C	C/T	A/A	T/A	A/T	C/C	C/C	T/T	T/T	C/C	A/T	Parent
	8487	Child 6A	X/Y	T/T	T/T	A/T	A/A	G/G	T/T	C/C	A/A	T/T	G/C	C/T	A/A	T/A	A/T	C/C	C/A	T/T	C/T	C/C	T/T	
	8488	Child 6B	X/X	T/C		A/T	A/A	G/G	T/A	C/G	A/A	C/T	G/C	C/T	T/A	T/T	A/T	C/C	C/A	T/T	C/T	C/C	T/T	
	8489	Child 6C	X/Y	T/T	A/T	A/T	A/A	G/G	T/A	C/G	A/A	T/T	G/C	C/C	A/A	T/T	A/T	C/C	C/A	T/T	C/T	C/C	A/T	
	8490	Child 6D	X/Y	T/T	T/T	A/T	A/A	G/G	T/T	C/C	A/A	C/T	C/C	C/T	A/A	T/A	A/T	C/C	C/A	T/T	C/T	C/C	T/T	
	8491	Child 6E	X/X	T/T	T/T	A/T	A/A	G/G	T/T	C/G	A/A	C/T	C/C	C/T	A/A	T/A	A/T	C/C	C/A	T/T	C/T	C/C	A/T	
	8492	Child 6F	X/X	T/C	A/T	A/T	A/A	G/G	T/A	C/G	A/A	T/T	C/C	C/T	T/A	T/T	A/T	C/C	C/A	T/T	C/T	C/C	T/T	
	8493	Child 6G	X/Y	T/C	T/T	*T/T*	*A/T*	G/G	T/A	C/G	A/A	C/T	C/C	C/C	A/A	*A/A*	A/A	C/C	C/A	T/T	*T/T*	C/C	A/T	Different father
Family 7	8755	Father 7	X/Y	T/C	A/T	A/A	A/A	G/C	T/T	C/G	A/T	C/T	G/C	C/C	T/T	T/T	A/T	C/A	C/C	T/C	C/T	C/C	T/T	Parent
	8756	Mother 7	X/X	T/C	T/T	A/A	A/T	G/G	T/A	G/G	A/T	T/T	G/C	C/C	T/A	T/T	A/T	C/C	C/C	C/C	C/T	C/C	A/A	Parent
	8757	Child 7A	X/X	T/C	A/T	A/A	A/T	G/G	T/A	C/G	A/T	T/T	G/C	C/C	T/A	T/T	A/T	C/C	C/C	T/C	C/T	C/C	A/T	
	8758	Child 7B	X/X	T/C	A/T	A/A	A/T	G/C	T/A	C/G	T/T	T/T	G/G	C/C	T/A	T/T	A/A	C/C	C/C	C/C	T/T	C/C	A/T	
	8759	Child 7C	X/X	T/C	T/T	A/A	A/A	G/G	T/T	C/G	A/T	T/T	G/C	C/C	T/A	T/T	A/T	C/C	C/C	C/C	C/C	C/C	A/T	
	8760	Child 7D	X/Y	T/C	T/T	A/A	A/T	G/G	T/T	C/G	A/T	T/T	G/C	C/C	T/T	T/T	A/A	C/A	C/C	T/C	C/T	C/C	A/T	
	8761	Child 7E	X/X	T/C	T/T	A/A	A/A	G/C	T/T	C/G	A/T	T/T	G/C	C/C	T/A	T/T	A/T	C/A	C/C	T/C	C/C	C/C	A/T	
	8762	Child 7F	X/Y	C/C	T/T	A/A	A/T	G/G	T/A	C/G	T/T	C/T	G/C	C/C	T/T	T/T	T/T	C/A	C/C	T/C	C/T	C/C	A/T	
Family 8	8830	Father 8	X/Y	T/T	A/A	T/T	A/T	G/G	T/A	C/G	A/T	C/C	G/C	C/T	T/T	T/T		C/C	C/A	T/C	C/C	G/G	T/T	Parent
	8831	Mother 8	X/X	C/C	T/T	A/T	A/A	G/C	T/A	C/G	T/T	C/T	C/C	C/C	T/A	T/A		C/C	C/A	T/T	C/C	C/C	T/T	Parent
	8833	Child 8A	X/X	*C/C*	*T/T*	T/T	A/T	G/G	T/A	C/C	A/T	T/T	C/C	C/C	T/T	T/A	A/A	C/C	A/A	T/T	*C/T*	*C/C*	T/T	Different father
	8834	Child 8B	X/Y	T/C	A/T	T/T	A/T	G/G	T/A	C/G	T/T	C/C	C/C	C/T	T/T	T/A	A/A	C/C	C/A	T/C	C/C	C/G	T/T	
	8835	Child 8C	X/Y	T/C	A/T	T/T	A/A	G/G	A/A	C/C	T/T	C/C	C/C	C/C	T/A	T/T	A/A	C/C	C/C	T/C	C/C	C/G	T/T	
	8836	Child 8D	X/Y	T/C	A/T	A/T	A/A	G/G	T/A	C/G	A/T	C/T	G/C	C/C	*A/A*	T/T	A/A	*C/A*	C/A	T/T	C/C	*C/C*	T/T	Different father
	8837	Child 8E	X/Y	T/C	*T/T*	A/T	A/A	G/C	T/T	C/G	T/T	C/T	C/C	C/C	T/T	T/A	A/A	*C/A*	C/A	T/T	C/C	*C/C*	T/T	Different father

	Sample ID		Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8	
Family 9	9234	Father 9	X/Y	T/C	[illegible]	A/A	T/T	[illegible]	T/T	C/C	A/A	T/T	G/G	C/C	T/T	T/T		C/C	C/C	T/C	C/C	C/C	T/T	Parent
	9235	Mother 9	X/X	T/T	T/T	A/A	A/A	G/G	T/T	C/C	A/A	C/T	C/C	C/C	A/A	T/T	A/T	C/A	C/C	T/T	C/C	C/G	T/T	Parent
	9236	Child 9A	X/Y	T/C	A/T	A/A	A/T	G/G	T/T	C/C	A/A	C/T	G/C	C/C	T/A	T/T	A/A	C/A	C/C	T/T	C/C	C/G	T/T	
	9237	Child 9B	X/X	T/C	T/T	A/A	A/T	G/G	T/T	C/C	A/A	C/T	G/C	C/C	T/A	T/T	A/A	C/A	C/C	T/T	C/C	C/C	T/T	
	9238	Child 9C	X/X	T/T	A/T	A/A	A/T	G/G	T/T	C/C	A/A	T/T	G/C	C/C	T/A	T/T	A/T	C/C	C/C	T/T	C/C	C/G	T/T	
	9239	Child 9D	X/X	T/T	A/T	A/A	A/T	G/G	T/T	C/C	A/A	C/T	G/C	C/C	T/T	T/T	A/A	C/A	C/C	T/C	C/C	C/G	T/T	
	9240	Child 9E	X/Y	T/C	T/T	A/A	A/T	G/G	T/T	C/C	A/A	C/T	G/C	C/C	T/A	T/T	A/T	C/C	C/C	T/C	C/C	C/G	T/T	
Family 10	9516	Father 10	X/Y	T/C	A/T	A/A	A/A	G/G	T/T	C/C	A/T	C/C	C/C	C/T	T/T	T/A		C/A	C/A	T/T	C/C	C/C	T/T	Parent
	9517	Mother 10	X/X	T/T	A/T	A/A	A/A	G/G	T/T	G/G	A/A	C/C	C/C	C/C	T/T	T/A		C/C	C/C	C/C	C/C	C/C	A/T	Parent
	9518	Child 10A	X/X	T/C	A/T	A/A	A/A	G/G	T/T	C/G	A/T	C/C	C/C	C/T	T/T	A/A	A/A	C/C	C/C	T/C	C/C	C/C	A/T	
	9519	Child 10B	X/X	T/T	A/A	A/A	A/A	G/G	T/T	C/G	A/A	C/C	C/C	C/T	T/T	A/A	A/A	C/A	C/A	T/C	C/C	C/C	T/T	
	9520	Child 10C	X/Y	T/T	A/T	A/A	A/A	G/G	T/T	C/G	A/A	C/C	C/C	C/T	T/T	A/A	A/A	C/A	C/C	T/C	C/C	C/C	A/T	
	9521	Child 10D	X/Y	T/C	A/A	A/A	A/A	G/G	T/T	C/G	A/A	C/C	C/C	C/T	T/T	A/A	A/A	C/C	C/A	T/C	C/C	C/C	T/T	
	9522	Child 10E	X/Y	T/T	A/T	A/A	A/A	G/G	T/T	C/G	A/T	C/C	C/C	C/C	T/T	T/A	A/A	C/C	C/C	T/C	C/C	C/C	T/T	
	9523	Child 10F	X/X	T/C	T/T	A/A	A/A	G/G	T/T	C/G	A/T	C/C	C/C	C/T	T/T	A/A	A/A	C/A	C/C	T/C	C/C	C/C	A/T	
	9524	Child 10G	X/X	T/C	T/T	A/A	A/A	G/G	T/T	C/G	A/T	C/C	C/C	C/T	T/T	T/A	A/A	C/A	C/A	T/C	C/C	C/C	T/T	
Family 11	9613	Father 11	X/Y	T/T	A/T	A/A	A/A	G/C	T/A	C/C	A/T	C/T	G/C	C/C	A/A	T/A		C/C	C/C	T/C	C/C	C/C	T/T	Parent
	9614	Mother 11	X/X	T/C	T/T	A/A	A/A	G/C	T/A	C/C	A/A	T/T	G/C	C/C	T/A	T/A	T/T	C/C	C/C	T/C	C/C	C/G	A/T	Parent
	9615	Child 11A	X/Y	T/C	A/T	A/A	A/A	C/C	A/A	C/C	A/T	T/T	G/C	C/C	T/A	A/A	A/T	C/C	C/C	T/C	C/C	C/C	T/T	
	9616	Child 11B	X/X	T/C	A/T	A/A	A/A	C/C	T/A	C/C	A/A	T/T	G/C	C/C	A/A	T/A	A/T	C/C	C/C	C/C	C/C	C/G	T/T	
	9617	Child 11C	X/Y	T/T	A/T	A/A	A/A	C/C	T/T	C/C	A/A	C/T	G/C	C/C	T/A	T/A	A/T	C/C	C/C	T/C	C/C	C/C	A/T	
	9618	Child 11D	X/Y	T/C	T/T	A/A	A/A	G/C	A/A	C/C	A/A	T/T	G/C	C/C	A/A	T/T	A/T	C/C	C/C	C/C	C/C	C/C	T/T	
	9619	Child 11E	X/Y	T/T	A/T	A/A	A/A	G/C	T/A	C/C	A/A	C/T	G/C	C/C	T/A	T/A	A/T	C/C	C/C	C/C	C/C	C/G	T/T	
	9620	Child 11F	X/Y	T/C	T/T	A/A	A/A	G/C	T/A	C/C	A/A	T/T	G/C	C/C	T/A	T/T	A/T	C/C	C/C	C/C	C/C	C/C	T/T	
	9621	Child 11G	X/X	T/C	T/T	A/A	A/A	C/C	T/A	C/C	A/A	T/T	G/C	C/C	A/A	A/A		C/C	C/C	C/C	C/C	C/G	T/T	
Family 12	9645	Father 12	X/Y	T/C	T/T	T/T	T/T	G/G	T/T	G/G	A/A	T/T	G/C	C/C	T/T	T/A		C/A	C/C	T/T	C/C	C/C	T/T	Parent
	9646	Mother 12	X/X	T/T	T/T	A/T	A/T	G/C	T/A	C/G	A/T	C/T	G/C	C/T	T/T	T/T		C/C	C/A	T/C	C/T	C/C	T/T	Parent
	9647	Child 12A	X/X	T/C	T/T	A/T	A/T	G/G	T/T	C/G	A/A	T/T	G/G	C/C	T/T	T/T	A/A	C/A	C/C	T/C	C/C	C/C	T/T	
	9648	Child 12B	X/X	T/T	A/T	A/T	T/T	G/G	T/A	C/G	A/A	C/T	G/C	C/C	T/T	T/T	A/A	C/A	C/C	T/T	C/T	C/C	T/T	
	9649	Child 12C	X/X	T/T	T/T	A/T	T/T	G/G	T/A	C/G	A/A	T/T	G/G	C/T	T/T	T/T	A/A	C/A	C/A	T/C	C/C	C/C	T/T	
	9650	Child 12D	X/Y	T/T	T/T	A/T	T/T	G/G	T/T	C/G	A/A	T/T	G/G	C/C	T/T	T/T	A/A	C/A	C/A	T/T	C/T	C/C	T/T	
	9651	Child 12E	X/Y	T/C	T/T	A/T	T/T	G/C	T/A	C/G	A/A	C/T	C/C	C/T	T/T	T/T	A/A	C/A	C/C	T/T	C/T	C/C	T/T	
	9652	Child 12F	X/Y	T/C	T/T	A/T	A/T	G/G	T/A	G/G	A/A	T/T	C/C	C/T	T/T	T/T	A/A	C/C	C/A	T/C	C/C	C/C	T/T	
	9653	Child 12G	X/X	T/C	T/T	A/T	A/T	G/C	T/T	C/G	A/A	C/T	C/C	C/C	T/T	T/A	A/A	C/C	C/C	T/T	C/C	C/C	T/T	
	9654	Child 12H	X/X	T/C	T/T	A/T	T/T	G/C	T/T	C/G	A/A	T/T	G/G	C/T	T/T	T/A	A/A	C/C	C/A	T/T	C/T	C/C	T/T	

	Sample ID		Am	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8	
Family 13	9234	Father 13	X/X	T/T	A/T	A/T	A/A	G/G	T/T	C/C	A/A	C/T	C/C	C/C	A/A	T/A		C/A	C/C	T/T	C/C	C/C	T/T	Parent
	9235	Mother 13	X/Y	T/C	A/T	T/T	T/T	G/G	T/T	C/G	A/T	C/T	C/C	C/C	T/T	T/T	A/A	C/A	C/C	T/T	C/C	C/C	A/T	Parent
	9236	Child 13A	X/X	T/T	A/T	A/T	A/T	G/G	T/T	C/G	A/A	C/T	C/C	C/C	T/A	T/T	A/A	C/C	C/C	T/T	C/C	C/C	A/T	
	9237	Child 13B	X/X	T/C	A/A	A/T	A/T	G/G	T/T	C/G	A/T	C/C	C/C	C/C	T/A	T/T	A/A	C/A	C/C	T/T	C/C	C/C	A/T	
	9238	Child 13C	X/X	T/T	T/T	A/T	A/T	G/G	T/T	C/G	A/A	C/T	C/C	C/C	T/A	T/A	A/A	C/A	C/C	T/T	C/C	C/C	A/T	
	9239	Child 13D	X/Y	T/C	A/T	A/T	A/T	G/G	T/T	C/G	A/A	T/T	C/C	C/C	T/A	T/A	A/A	C/A	C/C	T/T	C/C	C/C	T/T	
	9240	Child 13E	X/Y	T/T	A/T	T/T	A/T	G/G	T/T	C/G	A/T	T/T	C/C	C/C	T/A	T/A	A/A	C/A	C/C	T/T	C/C	C/C	A/T	

APPENDIX IX - LIKELIHOOD RATIO CALCULATIONS FOR KINSHIP ANALYSIS

Parent 1	Parent 2	Body	Likelihood ratio calculation
aa	aa	**aa**	$1/a^2$
aa	aa	**aF**	1/(a x 1) = 1/a
aa	ab	**aa**	$1/2a^2$
aa	ab	**aF**	1/(2a x 1) = 1/2a
aa	ab	**ab**	1/4ab
ab	ab	**ab**	1/4ab
ab	ab	**aF**	1/(4a x 1) = 1/4a
ab	ab	**Fb**	1/(1 x 4b) = 1/4b
ab	ab	**aa**	$1/4a^2$
ab	ab	**bb**	$1/4b^2$
ab	ab	**bF**	1/(4b x 1) = 1/4b
aa	ab	**Fb**	1/(4b x 1) = 1/4b
aa	bb	**ab**	1/2ab
aa	bb	**aF**	1/(2a x 1) = 1/2a
aa	bb	**Fb**	1/(1 x 2b) = 1/2b
ab	bb	**ab**	1/4ab
ab	bb	**aF**	1/(4a x 1) = 1/4a
ab	bb	**Fb**	1/(1 x 4b) = 1/4b
ab	bb	**bb**	$1/2b^2$
bb	bb	**bb**	$1/b^2$
bb	bb	**bF**	1/(b x 1) = 1/b
aa	-	**aa**	1/a
aa	-	**ab**	1/2a
aa	-	**aF**	1/2a
aa	-	**Fb**	1
ab	-	**aa**	1/2a
ab	-	**ab**	(a + b)/4ab
ab	-	**bb**	1/2b
ab	-	**aF**	1/4a
ab	-	**Fb**	1/4b
bb	-	**ab**	1/2b
bb	-	**bb**	1/b
bb	-	**aF**	1

Where $LR = \frac{\Pr(E|H_p)}{\Pr(E|H_d)}$

Hp = The body is the biological child of M and/or F

Hd = The body is an unknown, unrelated individual

APPENDIX X - LIKELIHOOD RATIO ARRAY FOR KINSHIP ANALYSIS

	White Caucasian allele frequencies			Likelihood ratios based on parent genotype data *[parent 1 + parent 2 = body]*																				
SNP locus	Allele 1/ Allele 2	Allele 1 (a)	Allele 2 (b)	aa+aa=aa	aa+aa=aF	aa+ab=aa	aa+ab=aF	aa+ab=ab	ab+ab=ab	ab+ab=aF	ab+ab=Fb	ab+ab=aa	ab+ab=bb	ab+ab=bF	aa+ab=Fb	aa+bb=ab	aa+bb=aF	aa+bb=Fb	ab+bb=ab	ab+bb=aF	ab+bb=Fb	ab+bb=bb	bb+bb=bb	bb+bb=bF
D	T / C	0.52	0.48	3.6982	1.9231	1.8491	0.9615	1.0016	1.0016	0.4808	0.5208	0.9246	1.0851	0.5208	0.5208	2.0032	0.9615	1.0417	1.0016	0.4808	0.5208	2.1701	4.3403	2.0833
U6	A / T	0.37	0.63	7.3046	2.7027	3.6523	1.3514	1.0725	1.0725	0.6757	0.3968	1.8262	0.6299	0.3968	0.3968	2.1450	1.3514	0.7937	1.0725	0.6757	0.3968	1.2598	2.5195	1.5873
B6	A / T	0.64	0.36	2.4414	1.5625	1.2207	0.7813	1.0851	1.0851	0.3906	0.6944	0.6104	1.9290	0.6944	0.6944	2.1701	0.7813	1.3889	1.0851	0.3906	0.6944	3.8580	7.7160	2.7778
N4	A / T	0.57	0.43	3.0779	1.7544	1.5389	0.8772	1.0200	1.0200	0.4386	0.5814	0.7695	1.3521	0.5814	0.5814	2.0400	0.8772	1.1628	1.0200	0.4386	0.5814	2.7042	5.4083	2.3256
Y3	G / C	0.92	0.08	1.1815	1.0870	0.5907	0.5435	3.3967	3.3967	0.2717	3.1250	0.2954	39.0625	3.1250	3.1250	6.7935	0.5435	6.2500	3.3967	0.2717	3.1250	78.1250	156.2500	12.5000
P5	T / A	0.72	0.28	1.9290	1.3889	0.9645	0.6944	1.2401	1.2401	0.3472	0.8929	0.4823	3.1888	0.8929	0.8929	2.4802	0.6944	1.7857	1.2401	0.3472	0.8929	6.3776	12.7551	3.5714
A4	C / G	0.71	0.29	1.9837	1.4085	0.9919	0.7042	1.2142	1.2142	0.3521	0.8621	0.4959	2.9727	0.8621	0.8621	2.4284	0.7042	1.7241	1.2142	0.3521	0.8621	5.9453	11.8906	3.4483
O6	A / T	0.75	0.25	1.7778	1.3333	0.8889	0.6667	1.3333	1.3333	0.3333	1.0000	0.4444	4.0000	1.0000	2.0000	2.6667	0.6667	2.0000	1.3333	0.3333	1.0000	8.0000	16.0000	4.0000
Z2	C / T	0.56	0.44	3.1888	1.7857	1.5944	0.8929	1.0146	1.0146	0.4464	0.5682	0.7972	1.2913	0.5682	1.1364	2.0292	0.8929	1.1364	1.0146	0.4464	0.5682	2.5826	5.1653	2.2727
K3	G / C	0.31	0.69	10.4058	3.2258	5.2029	1.6129	1.1688	1.1688	0.8065	0.3623	2.6015	0.5251	0.3623	0.7246	2.3375	1.6129	0.7246	1.1688	0.8065	0.3623	1.0502	2.1004	1.4493
J2	C / T	0.92	0.08	1.1815	1.0870	0.5907	0.5435	3.3967	3.3967	0.2717	3.1250	0.2954	39.0625	3.1250	6.2500	6.7935	0.5435	6.2500	3.3967	0.2717	3.1250	78.1250	156.2500	12.5000
Y6	T / A	0.63	0.37	2.5195	1.5873	1.2598	0.7937	1.0725	1.0725	0.3968	0.6757	0.6299	1.8262	0.6757	1.3514	2.1450	0.7937	1.3514	1.0725	0.3968	0.6757	3.6523	7.3046	2.7027
P7	T / A	0.62	0.38	2.6015	1.6129	1.3007	0.8065	1.0611	1.0611	0.4032	0.6579	0.6504	1.7313	0.6579	1.3158	2.1222	0.8065	1.3158	1.0611	0.4032	0.6579	3.4626	6.9252	2.6316
J8	A / T	0.77	0.23	1.6866	1.2987	0.8433	0.6494	1.4116	1.4116	0.3247	1.0870	0.4217	4.7259	1.0870	2.1739	2.8233	0.6494	2.1739	1.4116	0.3247	1.0870	9.4518	18.9036	4.3478
X	C / A	0.79	0.21	1.6023	1.2658	0.8012	0.6329	1.5069	1.5069	0.3165	1.1905	0.4006	5.6689	1.1905	2.3810	3.0139	0.6329	2.3810	1.5069	0.3165	1.1905	11.3379	22.6757	4.7619
F	C / A	0.78	0.22	1.6437	1.2821	0.8218	0.6410	1.4569	1.4569	0.3205	1.1364	0.4109	5.1653	1.1364	2.2727	2.9138	0.6410	2.2727	1.4569	0.3205	1.1364	10.3306	20.6612	4.5455
G	T / C	0.75	0.25	1.7778	1.3333	0.8889	0.6667	1.3333	1.3333	0.3333	1.0000	0.4444	4.0000	1.0000	2.0000	2.6667	0.6667	2.0000	1.3333	0.3333	1.0000	8.0000	16.0000	4.0000
L2	C / T	0.79	0.21	1.6023	1.2658	0.8012	0.6329	1.5069	1.5069	0.3165	1.1905	0.4006	5.6689	1.1905	2.3810	3.0139	0.6329	2.3810	1.5069	0.3165	1.1905	11.3379	22.6757	4.7619
W3	C / G	0.77	0.23	1.6866	1.2987	0.8433	0.6494	1.4116	1.4116	0.3247	1.0870	0.4217	4.7259	1.0870	2.1739	2.8233	0.6494	2.1739	1.4116	0.3247	1.0870	9.4518	18.9036	4.3478
H8	A / T	0.11	0.89	82.6446	9.0909	41.3223	4.5455	2.5536	2.5536	2.2727	0.2809	20.6612	0.3156	0.2809	0.5618	5.1073	4.5455	0.5618	2.5536	2.2727	0.2809	0.6312	1.2625	1.1236

Likelihood ratio array for all possibilities of parent – parent – child genotype combinations. Data is based on the formulae outlined in appendix IX. Only data for White Caucasian allele frequencies has been used as all casework samples were obtained from this ethnic group.

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Forensic Science International 154 (2005) 62–77

Forensic Science International

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Short communication

Validation of a 21-locus autosomal SNP multiplex for forensic identification purposes

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Received 28 September 2004; received in revised form 1 December 2004; accepted 3 December 2004
Available online 20 January 2005

Abstract

A single nucleotide polymorphism (SNP) multiplex has been developed to analyse highly degraded and low copy number (LCN) DNA template, i.e. <100 pg, for scenarios including mass disaster identification. The multiplex consists of 20 autosomal non-coding loci plus Amelogenin for sex determination, amplified in a single tube PCR reaction and visualised on the Applied Biosystems 3100 capillary electrophoresis (CE) system. Allele-specific primers tailed with shared universal tag sequences were designed to speed multiplex design and balance the amplification efficiencies of all loci through the use of a single reverse and two differentially labelled allele denoting forward universal primers. As the multiplex is intended for use with samples too degraded for conventional profiling, a computer program was specifically developed to aid interpretation. Critical factors taken into account by the software include empirically determined extremes of heterozygous imbalance (*Hb*) and the drop-out threshold (*Ht*) defined as the maximum peak height of a surviving heterozygous allele, where its partner may have dropped out. The discrimination power of the system is estimated at 1 in 4.5 million, using a White Caucasian population database. Comparisons using artificially degraded samples profiled with both the SNP multiplex and AMP*FI*STR® SGM plus™ (Applied Biosystems) demonstrated a greater likelihood of obtaining a profile using SNPs for certain sample types. Saliva stains degraded for 147 days generated an 81% complete SNP profile whilst short tandem repeats (STRs) were only 18% complete; similarly blood degraded for 243 days produced full SNP profiles but only 9% with STRs. Reproducibility studies showed concordance between SNP profiles for different sample types, such as blood, saliva, semen and hairs, for the same individual, both within and between different DNA extracts.

Keywords: Single nucleotide polymorphisms; Degraded DNA; Low copy number; Interpretation criteria

1. Introduction

The current method of DNA profiling used for the National DNA Database® (AMP*FI*STR® SGM plus™) exploits the polymorphic nature of short tandem repeat (STR) sequences to discriminate between both related and unrelated individuals [1]. The technique is highly discriminating but is limited by the size of the DNA fragments produced for detection (ranging from 100–360 bases in length). As DNA becomes degraded, the higher molecular weight STR loci fail to amplify [2] giving a 'partial' DNA profile that has a lower discrimination power.

During cell death by apoptosis or necrosis, endonucleases initially target unprotected linker DNA to leave monomeric nucleosomes with 146 base pairs of protected

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doi:10.1016/j.forsciint.2004.12.011

DNA [3]. As a consequence of this observation, it may be preferable to develop DNA profiling techniques that can target smaller lengths of DNA, to ensure an improved success rate with degraded samples.

We have developed a multiplex system using biallelic SNPs, selected from The SNP Consortium database [4]. By designing closer to the single polymorphic base, the likelihood of obtaining a result when STRs fail is increased. The discrimination power of SNPs increases with the number of loci multiplexed [5]. There are many different assays available for SNP genotyping [6–11], although these are generally used when there is ample DNA available, something not often encountered with forensic DNA profiling. To achieve a large stable multiplex we have used the Amplification Refractory Mutation System (ARMS) [12] combined with Universal reporter primers (URP) [13] in a two-phase PCR reaction (Fig. 1), to amplify DNA fragments ranging from 57 to 146 base pairs in length. During amplification, two 20 bp Universal reporter primer sequences are incorporated onto the ends of the DNA strand giving PCR products 40 bases longer than the original genome target size. The first phase amplifies individual loci using low concentrations of locus-specific primers to amplify all targets to equivalent levels, whilst simultaneously incorporating URP tags. The second phase of the reaction employs two different Universal primers (Uni-9 5′-JOE-6 dye label (green); Uni-11 5′-FAM-6 dye label (blue)) to fluorescently label the PCR products. Each product is detected on a capillary electrophoresis (CE) instrument and is visualised as either a green or blue peak for homozygous loci or both a green and blue peak for heterozygous loci.

To validate the SNP multiplex system we investigated segregation patterns, detection limitations, sample-to-sample reproducibility, species specificity and mock casework samples.

2. Materials and methods

2.1. DNA extraction and quantification

DNA was extracted from a variety of samples using Qiagen™ QiaAmp Mini-Kits (Cat. no. 51306) or Qiagen™ Genomic-Tip system (Cat. no. 10223, 20/G tips). Samples had been stored frozen at −20 °C and were thawed at room temperature prior to DNA extraction. The manufacturer's protocol for each sample type was used to obtain up to 2 ng/μL DNA (Mini-Kits) or 5–15 ng/μL DNA (Genomic-Tips), suspended in 1× TE Buffer (100 mM Tris, 1 mM EDTA disodium). Samples were quantified using PicoGreen [14] and/or a UV spectrophotometer (Biochrom Ltd., UK), according to the manufacturers' protocols.

2.2. SNP multiplex amplification

The SNP multimix for each amplification reaction consisted of oligonucleotide primers (synthesised by IBA, Germany) at varying concentrations (primer sequences are listed in Appendix A and on the NIST website [15]), 0.4 μg/μL bovine serum albumin (Boehringer Mannheim, Germany), 225 μM dNTPs (dATP, dCTP, dTTP, dGTP; Boehringer Mannheim, Germany), 1 × PCR Buffer II containing 1.5 mM $MgCl_2$ (Applied Biosystems, UK) and 5 units of AmpliTaq Gold® (Applied Biosystems, UK). DNA was added up to maximum of 1 ng DNA template.

DNA extracts were amplified in a total reaction volume of 25 μL in 0.2 mL tubes, without mineral oil, on a thermal cycler (Applied Biosystems GeneAmp PCR system 9600) using the following conditions: 95 °C Taq activation for 11 mins; 6 cycles of 94 °C/30 s, 60 °C/15 s, 72 °C/15 s, 60 °C/15 s, 72 °C/15 s, 60 °C/15 s, 72 °C/30 s; 29 cycles of 94 °C/30 s, 76 °C/105 s; 3 cycles of 94 °C/60 s, 60 °C/30 s, 76 °C/60 s; 60 °C extension for 45 min followed by a 4 °C hold.

2.3. SGM plus™ PCR amplification

AMP*FI*STR® SGM plus™ kit (Applied Biosystems, UK) containing reaction mix, primer mix (for components see Applied Biosystems user manual), AmpliTaq Gold® DNA polymerase at 5 U/μL and AMP*FI*STR® control DNA heterozygous for all loci, in 0.05% sodium azide and buffer was used for amplification of STR loci. DNA extract was amplified in a total reaction volume of 50 μL without mineral oil on a 9600 thermal cycler (Applied Biosystems GeneAmp PCR system 9600) using the following conditions: 95 °C for 11 min, 28 cycles (or 34 cycles for LCN amplification) of 94 °C/60 s, 59 °C/60 s, 72 °C/60 s; 60 °C extension for 45 min; holding at 4 °C.

2.4. Detection of PCR products using capillary electrophoresis

A 1.1 μL of each PCR product and 10 μL GS-HD400 ROX size standard (Applied Biosystems, UK, Part no. 402985):HI-DI Formamide (Applied Biosystems) (ratio 1:37) was added to each well in a 96-well micro-titre plate. Samples were run on a CE sequencer (ABI model 3100) using Collection software v1.1 (ABI) according to the manufacturer's protocol. SNP amplification products were run with two alternative injection times—12 and 20 s. A 12-s injection was sufficient for samples with optimal DNA amplification, samples with lower amounts of starting DNA material (<0.5 ng) were injected for a longer time period (20 s) which increased peak heights but also raised the baseline noise.

2.5. Analysis and Interpretation of results

Sample data from the 3100CE instrument was analysed using ABI Prism™ Genescan™ Analysis v3.7.1 and ABI

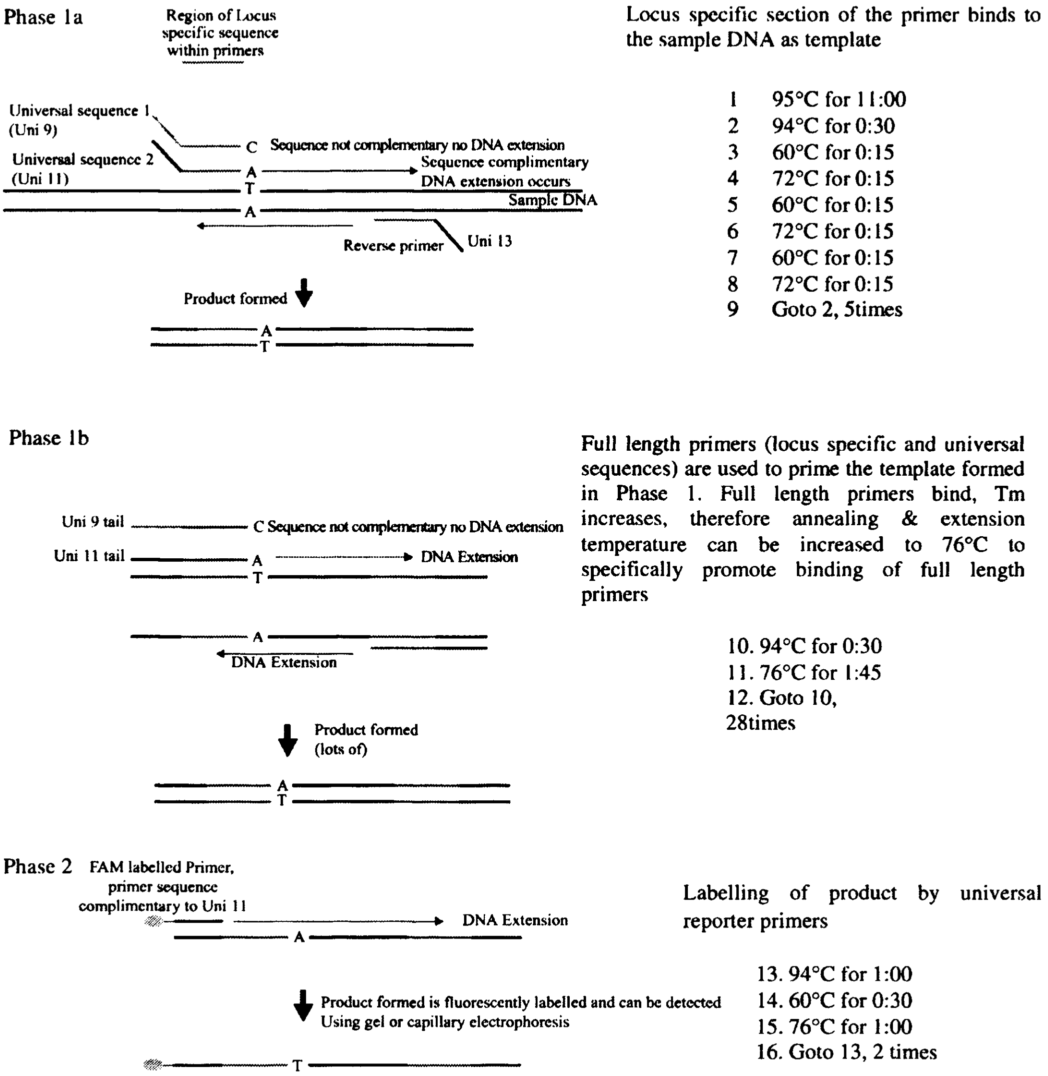
Phase 1a
Region of Locus specific sequence within primers
Locus specific section of the primer binds to the sample DNA as template
Universal sequence 1 (Uni 9)
Universal sequence 2 (Uni 11)
C Sequence not complementary no DNA extension
A
T
A
Sequence complimentary DNA extension occurs
Sample DNA
Reverse primer
Uni 13
Product formed
A
T
1 95°C for 11:00
2 94°C for 0:30
3 60°C for 0:15
4 72°C for 0:15
5 60°C for 0:15
6 72°C for 0:15
7 60°C for 0:15
8 72°C for 0:15
9 Goto 2, 5times
Phase 1b
Uni 9 tail
C Sequence not complementary no DNA extension
Uni 11 tail
A
DNA Extension
T
Full length primers (locus specific and universal sequences) are used to prime the template formed in Phase 1. Full length primers bind, Tm increases, therefore annealing & extension temperature can be increased to 76°C to specifically promote binding of full length primers
A
DNA Extension
Product formed (lots of)
A
T
10. 94°C for 0:30
11. 76°C for 1:45
12. Goto 10, 28times
Phase 2
FAM labelled Primer, primer sequence complimentary to Uni 11
DNA Extension
A
Labelling of product by universal reporter primers
Product formed is fluorescently labelled and can be detected Using gel or capillary electrophoresis
T
13. 94°C for 1:00
14. 60°C for 0:30
15. 76°C for 1:00
16. Goto 13, 2 times

Fig. 1. Diagrammatical representation of the URP/ARMS Principle. The amplification technique has two distinct phases: phase 1a uses the locus-specific portion of the ~40-mer primers to provide sufficient template with Universal tails for amplification in phase 2. The increase in T_m observed in phase 1b allows the whole length of the long primers to bind to the template, dependent on the Universal tail present. By phase 2, all long primers have been exhausted and the annealing temperature is reduced to allow the 20-mer fluorescently labelled Universal primers to anneal and extend.

Prism™ Genotyper™ software v3.7 NT. The ROX size standard peaks were used to determine the size (bp) of peaks present. Data extracted from Genotyper™ (peak height, peak area, scan number, size in bases) were transformed into *.csv format and analysed by Celestial™ (The Forensic Science Service® proprietary software used to designate alleles). Based on a number of predetermined interpretation criteria (Appendix B) for each SNP locus, genotypes were allocated for each sample.

SNP loci are identified by an arbitrary internal ID reference. The SNP Consortium (TSC) [4] identification numbers are given in Appendix A.

3. Results

3.1. Population studies

Sub-populations, comprising 201 White Caucasian, 71 British Afro-Caribbean and 86 Indian sub-continent DNA samples were genotyped using the SNP multiplex. Allele frequencies were derived from the data (Table 1) and a number of tests were carried out for SNP characterisation including Hardy–Weinberg equilibrium tests, Exact tests for linkage disequilibrium and Monte-Carlo simulations on Exact test data [16,17]. Approximate discrimination powers

Table 1
Allele frequencies for each of the 20 SNP loci used in the multiplex for each race group studied and combined likelihood ratios for each group.

SNP locus	Allele 1 (green)/allele 2 (blue)	White Caucasian		British Afro-Caribbean		Indian sub-continent	
		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
D	T/C	0.52	0.48	0.27	0.73	0.49	0.51
U6	A/T	0.37	0.63	0.10	0.90	0.32	0.68
B6	A/T	0.64	0.36	0.77	0.23	0.57	0.43
N4	A/T	0.57	0.43	0.51	0.49	0.53	0.47
Y3	G/C	0.92	0.08	0.96	0.04	0.94	0.06
P5	T/A	0.72	0.28	0.59	0.41	0.80	0.20
A4	C/G	0.71	0.29	0.51	0.49	0.66	0.34
O6	A/T	0.75	0.25	0.82	0.18	0.77	0.23
Z2	C/T	0.56	0.44	0.45	0.55	0.45	0.55
K3	G/C	0.31	0.69	0.25	0.75	0.39	0.61
J2	C/T	0.92	0.08	0.94	0.06	0.94	0.06
Y6	T/A	0.63	0.37	0.57	0.43	0.53	0.47
P7	T/A	0.62	0.38	0.73	0.27	0.79	0.21
J8	A/T	0.77	0.23	0.86	0.14	0.72	0.28
X	C/A	0.79	0.21	0.89	0.11	0.78	0.22
F	C/A	0.78	0.22	0.85	0.15	0.80	0.20
G	T/C	0.75	0.25	0.64	0.36	0.59	0.41
L2	C/T	0.79	0.21	0.94	0.06	0.90	0.10
W3	C/G	0.77	0.23	0.87	0.13	0.77	0.23
H8	A/T	0.11	0.89	0.10	0.90	0.15	0.85
Multiplex likelihood ratio		4,460,764		364,761		3,173,898	

for each population group were calculated using the method outlined by Jones (1972) [18].

Hardy–Weinberg equilibrium tests demonstrated no significant deviation from expectation ($p > 0.05$) for all 20 SNPs in the White Caucasian and Afro-Caribbean populations and for 19 out of 20 for the Indian sub-continent population, with Bonferroni correction [19] only locus K3 (Indian sub-continent) gave a $p < 0.05$. K3 data showed an excess of heterozygotes within the population set suggesting that the deviation may be due to sampling error, rather than a genetic or biochemical abnormality, such as primer binding site mutation or population sub-structuring effect—both of which would appear to increase the homozygosity.

Exact tests for linkage disequilibrium were carried out on the population data using genetic data analysis (GDA) software [19,20] to detect associations between alleles at different loci. Probability data (p-values), calculated from each locus–locus association, were plotted against a random number matrix, as a probability (P–P) plot (Fig. 2a–c). All data fitted within the random number bins for each race group indicating that the SNP loci were behaving as expected within a randomly-mating population with little or no linkage disequilibrium [21,22]. To demonstrate the effectiveness of the test an artificial sub-structured population was created using data from the White Caucasian and Indian sub-continent populations. This generated p-values deviating from expected values as would be expected due to the Wahlund Effect [23] (Fig. 2d).

3.2. *Linkage mapping*

Using mapping data from The SNP Consortium [4] (Table 2), each pair of SNPs lying on the same chromosome was assessed for the likelihood of linkage. On chromosomes 6 and 8, one SNP lay on the short arm (P7 = 6p23; X = 8p23) and one on the long arm (Z2 = 6q27; A4 = 8q21). Linkage disequilibrium can typically extend up to a few megabases [24]; however, the shortest distance between any two multiplexed SNPs in this study was more than 33 Mb (D and J8 on chromosome 3). This was sufficient distance to ensure that multiple chromosomal recombination events would result in linkage equilibrium between any pair of loci [25]. Consequently, the assumption of independence was reasonable with regard to physical linkage.

3.3. *BigDye^{TM} terminator cycle sequencing*

A control panel was constructed from five individuals, plus Cambio^{TM} foetal placental male and female DNA, to ensure there was a heterozygous individual represented at every SNP locus. Sequencing of each SNP allele and its flanking region was carried out using Applied Biosystems BigDye^{TM} terminator cycle sequencing according to the manufacturer's protocol. Each locus was sequenced in duplicate using primers complementary to both the forward and reverse strand, giving a total of four read sequences for each individual for each SNP. Sequenced SNPs were shown to be fully concordant with the electrophoretic results (n = 28 for each of the 20 loci).

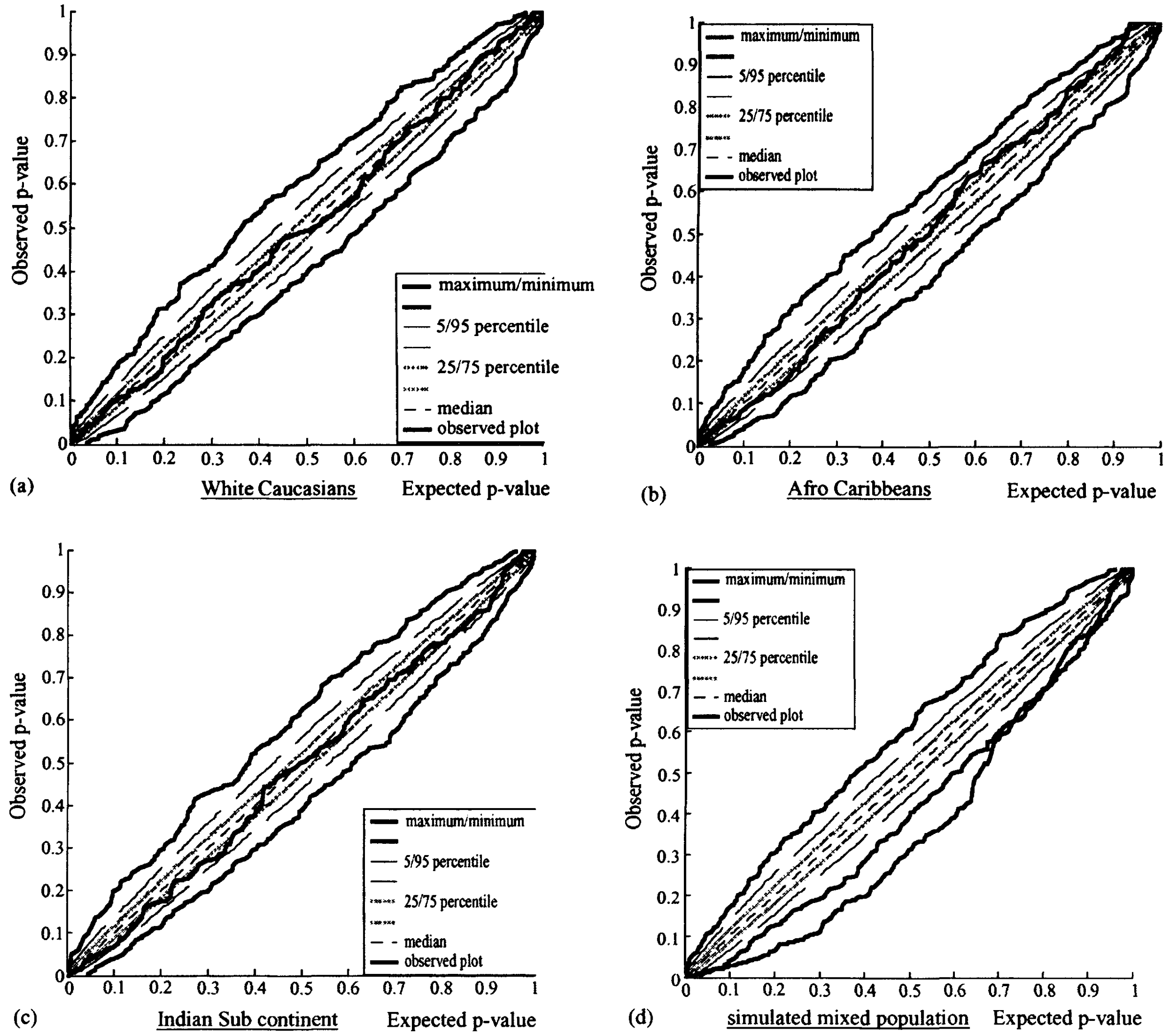

Fig. 2. Exact test linkage disequilibrium P–P plots for all three population databases (a–c) and an artificially mixed population (d) (x values = expected p-values, y values = observed values). Single race group plots (a–c) show all p-values amalgamate within the random distribution bins, i.e. there is no bias or deviation in the data from that expected in a randomly mating population. The artificially mixed population (d) shows deviation away from a random distribution indicating the influence of the Wahlund effect.

Table 2
SNP loci that lie on the same chromosome

SNP locus	TSC code	Band	Distance from p telomere (kb)	Distance from closest SNP used in multiplex (kb)
D	252540	3p25	9,092	33,572
J8	709016	3p21	42,664	36,437
B6	1342445	3p13	79,101	36,437
O6	1588825	5p15	8,346	44,489
Y6	627632	5q11	52,835	44,489
U6	746324	5q35	170,140	117,305
P7	897904	6p23	14,070	153,973
Z2	86795	6q27	168,043	153,973
X	31988	8p23	238	91,431
A4	421768	8q21	91,669	91,431

SNPs were selected based on a maximised distance away from other SNPs on the same chromosome to minimise the effects of linkage.

Comparison of sequencing results to The SNP Consortium [4] data revealed additional SNPs within the B6, A4 (C deletion) and J2 sequences that were not present in the Consortium sequences. Two additional SNPs were found within primer-binding regions for locus F and Y6 hence the primers were re-designed to include inosine bases at these positions as inosine is complementary to any A, G, C or T base.

The five control individuals, plus the Cambio^{TM} male and female DNA controls, were used as reference samples for all validation experiments.

3.4. 3100 CE instrument comparison

Control sample data was obtained from two different 3100 CE machines used during the validation experiments. Sizing data (base pairs) for each SNP was collated from Celestial^{TM} and examined for sequencer variation and variation between different samples. The maximum standard deviation seen between samples on each instrument was 0.08 bp (P5-green, $n = 90$) and 0.13 bp (P5-green, $n = 90$) and between instruments for all samples was 0.23 bp (W3-blue, $n = 28$). The SNP multiplex was designed with an average gap of four base pairs between each SNP locus, with a minimum spacing of 2.7 base pairs between the P5 product (~122.7 bases) and the A4 product (~125.4 bases). P5 showed the maximum standard deviation on both instruments but the 2.7 base pair separation between P5 and A4 enabled the locus bin to be set to one base pair (i.e. 0.5 bases either side of an average value), without compromising the accuracy to designate alleles.

3.5. Interpretation criteria

Every biallelic SNP locus with alleles A and B was characterised in terms of heterozygous balance (Hb) relative to peak height (Section 3.5.1), where Hb (%) = (ϕ_S/ϕ_L) × 100 (ϕ_S = smallest peak height; ϕ_L = largest peak height), determined experimentally from analysis of control reference samples (Section 3.3) (Table 3). Samples were amplified using varying amounts of starting DNA template as follows: 0, 16, 31, 62, 125, 250, 500 pg, 1 ng. Hb data were combined with known allele drop-out data, where ϕA or ϕB falls below a threshold level (Ht), determined by the DNA control dilutions (peak height) for each SNP (Section 3.5.2) (Table 4). The minimum value of Hb ($Hb_{\min}$) and the maximum value of Ht ($Ht_{\max}$) was recorded for each locus. $Hb_{\min}$ and $Ht_{\max}$ were encoded into Celestial^{TM} (Sections 3.5.1 and 3.5.2) for interpretation purposes. If a calculated Hb was less than $Hb_{\min}$ in an experimental sample for a given SNP, i.e. showed signs of severe imbalance, it was used as an indication of contamination or PCR artefacts (Sections 3.7 and 3.13). If a homozygous allele fell below $Ht_{\max}$, the locus was given an 'F' designation [26] indicating that allele drop-out may have occurred and the locus might be heterozygous.

3.5.1. Heterozygous balance (Hb)

Data were collated for Hb for all SNPs at all PCR template levels. The data were tabulated and the greatest imbalance for each SNP at each PCR template concentration was noted (Table 3). At optimal DNA template levels (0.5–1.0 ng) the lowest balance exhibited was with Y3, at approximately 25%, for both the 12 and 20 s injection times from the same PCR amplification. The most balanced heterozygous SNPs at optimal PCR conditions were G and J2 at both the 12 and 20 s injection times with $Hb_{\min} > 68\%$, comparable to existing STR multiplex systems [27]. As DNA template level decreases, Hb decreases, due to stochastic variation seen at low levels. This is consistent with low copy number (LCN) STRs using systems such as SGM plus^{TM} where optimal DNA template gives $Hb > 0.6$ but at LCN levels the distribution of Hb is almost random as a consequence of stochastic effects [26]. SNP heterozygous imbalance was most markedly seen with $Hb_{\min}$ at 11.2% (12 s) for N4 and 15.4% (12 s) for P7 at a DNA template level of 125 pg, closely followed by O6 (15.6% (12 s) and 16.2% (20 s)) and K3 (16.5 and 13.6% for 12 and 20 s, respectively) at the sub-125 pg PCR template level. The most extreme $Hb_{\min}$, irrespective of DNA template level, was used for analysis in Celestial^{TM} (Appendix B).

3.5.2. Homozygous thresholds (Ht)

If a locus is heterozygous with alleles A and B and either allele is missing because of drop-out then Ht is defined as the experimentally observed maximum peak height of the remaining allele plus 20% to allow for unobserved extreme variation:

$$Ht^{\phi A=0} = \phi B_{\max} + 0.2(\phi B_{\max})$$

or

$$Ht^{\phi B=0} = \phi A_{\max} + 0.2(\phi A_{\max})$$

Ht for the known reference samples was estimated from the dilution series experiment. Data were tabulated to show Ht for each SNP allele for each instrument injection parameter (Table 4). At optimal DNA template amounts (0.5–1.0 ng), $Ht_{\max}$ was observed for J8 at both the 12-s injection time (334 rfu) and the 20-s injection time (850 rfu). At sub-125 pg template amounts the largest $Ht_{\max}$ was observed at locus F, at a height of 717 rfu (12 s) and 1068 rfu (20 s). This was at a template level below 125 pg and no allele drop-out was observed for this SNP at the higher template concentrations. Drop-out was not observed at G at the sub-125 pg level. Consequently the theoretical drop-out level for G was calculated based on the observed $Hb_{\min}$. An understanding of the behaviour of Ht is crucial for interpretation purposes and the most extreme data, plus 20% to allow for unobserved extremes, was used for Ht analysis in Celestial^{TM}.

Table 3
Heterozygous balance data [$Hb_{\mathrm{min}} = (\phi S/\phi L) \times 100$ (%)] collected from two runs of the AB 3100 capillary electrophoresis instrument at 12 and 20 s

SNP locus	Hb_{min} 12 s injection (ϕ_S/ϕ_L) × 100 (%)				Hb_{min} 20 s injection (ϕ_S/ϕ_L) × 100 (%)			
	Sub 125 pg	125 pg	250 pg	500–1000 pg	Sub 125 pg	125 pg	250 pg	500–1000 pg
Amelo	#	#	23.5	31.0	#	#	25.9	35.7
D	25.0	39.2	48.0	47.8	#	41.3	55.2	49.7
U6	#	#	31.5	45.0	#	#	36.0	39.4
B6	#	#	23.6	44.2	#	#	23.6	45.1
N4	#	11.2	37.5	40.3	#	13.0	34.7	41.6
Y3	#	#	68.4	25.1	#	63.4	67.3	24.4
P5	34.3	#	55.1	54.1	32.3	#	34.6	47.8
A4	#	24.8	41.3	41.6	#	23.1	36.8	39.6
O6	16.2	50.0	57.8	40.2	15.6	52.3	61.1	41.3
Z2	25.7	29.7	36.3	38.6	25.2	29.8	37.9	39.9
K3	16.5	29.7	29.9	33.2	13.6	28.9	29.3	32.0
J2	#	#	22.6	69.3	#	#	24.2	68.4
Y6	27.4	#	50.2	35.1	30.0	#	51.2	35.9
P7	#	15.4	24.8	32.0	#	14.6	22.1	27.7
J8	#	#	40.7	34.5	#	#	40.9	58.7
X	#	#	41.7	56.5	#	#	39.8	62.0
F	32.7	#	46.1	36.9	33.0	#	52.9	38.4
G	31.6	#	69.5	67.5	35.0	#	69.3	71.5
L2	#	#	51.8	53.0	53.0	#	#	53.6
W3	#	#	13.3	52.7	#	18.3	42.1	53.9
H8	#	#	61.1	79.7	#	#	57.7	81.7

(#) Indicates loci with either allele drop-out or total drop-out across all samples used, hence no heterozygous balance calculation.

Table 4
Observed homozygote peak heights (rfu) where allele drop-out has occurred

SNP locus	12 s injection (*Ht*)				20 s injection (*Ht*)			
	Sub 125 pg	125 pg	250 pg	500–1000 pg	Sub 125 pg	125 pg	250 pg	500–1000 pg
Amelo	403	#	#	#	560	#	#	527
D	615	#	#	#	859	#	#	#
U6	311	198	141	249	559	464	211	520
B6	328	207	#	#	450	439	#	#
N4	422	#	#	#	613	412	#	#
Y3	155	#	#	#	213	#	#	#
P5	424	#	#	#	617	#	#	#
A4	486	#	#	#	746	274	#	#
O6	152	#	#	#	615	#	#	#
Z2	380	#	#	#	795	#	372	#
K3	586	#	#	#	828	#	#	#
J2	170	#	#	#	#	449	#	#
Y6	454	#	#	#	526	#	#	#
P7	191	#	#	#	274	#	#	#
J8	160	#	#	334	#	372	#	850
X	390	191	#	#	546	334	#	221
F	717	#	#	#	1068	#	#	#
G	#	#	#	#	478	#	#	#
L2	230	#	#	#	329	#	#	#
W3	#	242	#	#	#	534	#	#
H8	134	#	#	#	#	377	#	#

Allele drop-out was identified from known control sample heterozygotes, from the same dataset used to generate heterozygous balance data (Table 3). (#) indicates heterozygous loci giving no allele drop-out, or complete locus drop-out, across all samples used.

3.6. Validation of new batches of multimix

One of the difficulties in preparing new multimix batches is the inevitability that no two batches will be identical because individual manufactured primer sets vary in consistency, making it important to balance new batches of multimix. This is not trivial, normally requiring successive rounds of altering primer concentrations in order to optimise the inter/intra locus balance. The problem becomes greater as more loci are added to the reaction, however the construction of multiplexes is greatly simplified by the use of URP biochemistry [13]. Nevertheless differences are still observed between multimixes and each one requires separate validation. Performance is dictated by the two parameters previously defined, *Hb* and *Ht*, since these are critical to the interpretation strategy. The aim of multimix validation is to ensure that these parameters (a) fall within defined criteria, and, because no two multimixes are the same, (b) to encode the parameters into Celestial™ so that they are effectively multimix specific.

3.7. Negative control thresholds

LCN is characterised by unavoidable allele drop-out and drop-in (laboratory contamination measured by reference to negative controls) [26]. A 96-well microtitre plate was prepared for SNP amplification using water controls as negatives instead of DNA samples. Drop-in peaks could be characterised as environmental contamination due to the lack of any positive DNA controls. The plate was processed through the system and any drop-in peaks were identified, for both a 12-s injection time and a 20-s injection time (Table 5). For a 12-s injection time the largest drop-in peak seen was at D (blue) at 81 rfu peak height. For 20 s, the largest peak was 150 rfu at G (green). The baseline level (*Bt*) was set according to the greatest experimental drop-in peak observed across loci plus ~25% to take account of batch variations.

$$Bt = Bt_{\max}^{\text{locus1}\ldots n} + 0.25(Bt_{\max}^{\text{locus1}\ldots n})$$

Consequently the thresholds for Celestial™ were set at 100 and 200 rfu for 12 and 20 s, respectively.

3.8. Reporting guidelines

Once *Hb* and *Ht* are known then match probability (P_{m}) calculations are carried out using the following algorithm that encompasses all possible scenarios. This algorithm is encoded into Celestial™ and is therefore automated (Fig. 3):

Table 5
Maximum peak height data (rfu) for allele drop-in peaks seen on a 96-well negative (deionised water) control plate for both 12 and 20 s injection times

SNP locus	12 s injection (peak height rfu)		20 s injection (peak height rfu)	
	Green peak	Blue peak	Green peak	Blue peak
Amelo		53 (1)	76 (1)	77 (1)
D	58 (1)	81 (1)	118 (6)	130 (3)
U6				
B6	57 (1)			73 (1)
N4			92 (2)	56 (1)
Y3				
P5			91 (1)	75 (1)
A4		51 (1)	79 (2)	72 (2)
O6	56 (1)			
Z2			133 (2)	
K3	52 (1)		76 (1)	
J2				
Y6			66 (1)	
P7				
J8			60 (1)	
X				
F			119 (1)	
G			150 (2)	
L2			87 (2)	
W3				
H8	56 (1)			

Numbers in brackets indicate total number of observations for each locus across all 96 wells.

If $\phi A > Ht$ and $\phi B < Bt$ then $P_{\text{m}_{\text{locus}}} = f(p_A^2)$ (allele *A* exceeds *Ht* and locus is homozygous),
else If $\phi B > Ht$ and $\phi A < Bt$ then $P_{\text{m}_{\text{locus}}} = f(p_B^2)$ (allele *B* exceeds *Ht* and locus is homozygous),
else If $\phi A > Ht$ and $\phi B > Bt$ then $P_{\text{m}_{\text{locus}}} = f(2p_Ap_B)$ (allele *A* exceeds *Ht* and allele *B* exceeds *Bt*; locus is heterozygous),
else if $\phi B > Ht$ and $\phi A > Bt$ then $P_{\text{m}_{\text{locus}}} = f(2p_Ap_B)$ (allele *B* exceeds *Ht* and allele *A* exceeds *Bt*; locus is heterozygous),
else If $\phi A < Ht$ and $\phi A > Bt$ and $\phi B > Bt$ then $P_{\text{m}_{\text{locus}}} = f(2p_Ap_B)$ (both alleles exceed *Bt*; locus is heterozygous),
else If $\phi B < Ht$ and $\phi B > Bt$ and $\phi A > Bt$ then $P_{\text{m}_{\text{locus}}} = f(2p_Ap_B)$ (both alleles exceed *Bt*; locus is heterozygous),

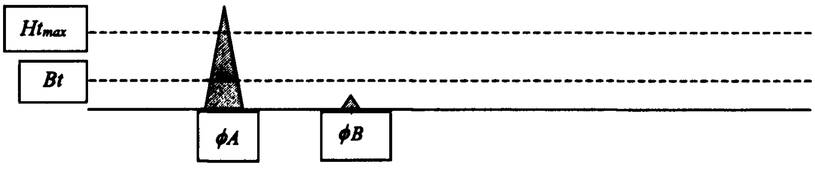

Fig. 3. An example of a SNP profile compared to thresholds $Ht_{\max}$ and Bt, respectively. In this example, allele *A* exceeds $Ht_{\max}$ – the low level *B* allele is below *Bt* and could be explained by background noise or by minor contamination. Consequently, the locus is reported as homozygous *A*.

Table 6
Genotypes generated in Celestial$^{\text{TM}}$, using interpretation guidelines indicated in Appendix B, for control DNA samples using varying amounts of starting template from 16 pg to 1 ng (reference profile)

FileName	Lane	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
CAS reference	A01	G	G/B	G/B	G/B	G/B	G	G	G/B	G	G/B	G/B	G	B	G	G	G/B	G	G	G	G	B
CAS 500pg	A02	G/F	G/B	G/B	G/B		G	G	G/B	G	G/B	G/B	G/F	F/B	G	G	G/B	G	G	G	G	B
CAS 250pg	A03	G	G/B	G/B	G/B	G/F	G	G	G/B	G	G/B	G/B	G/F	B	G	G	G/B	G	G	G	G	B
CAS 125pg	A04	G/F			G/B		G	G		G/F	G/B	G/B			G/F	G/F	F/B		G/F	G/F	G/F	
CAS 62pg	A05	G/F			G/B		G	G			G/B	G/B			G/F	G/F	F/B		G/F	G/F	G/F	
CAS 31pg	A06	G/F					G/F				F/B	G/B			G/F	G/F			G/F		G/F	
CAS 16pg	A07	G/F			G/B		G/F					G/B			G/F				G			B
DRJ reference	B01	G/B	G	G/B	G/B	G/B	G	G	G	G	G	B	G/B	G	G/B	B	G/B	G	G	G	G/B	B
DRJ 500pg	B02	G/B	G	G/B	G/B	G/B	G	G	G	G	G	B	G/B	G	G/B	B	G/B	G	G	G	G/B	B
DRJ 250pg	B03	G/B	G/F	G/F	G/B		G	G	G	G	G	B	F/B	G/F	G/B	B	G/B	G	G	G	G/B	B
DRJ 125pg	B04	G/F			G/B		G	G	G/F	G/F	G	B	F/B	G/F	G/B	B	F/B		G	G/F	G/F	B
DRJ 62pg	B05	G/B		G/F	G/B		G	G	G/F	G/F	G/F	B	F/B	G/F	G/B	B			G		G/B	B
DRJ 31pg	B06	G/F			G/B			G			G/F	F/B		G/F	G/B		F/B		G/F			
DRJ 16pg	B07	G/F			G/B							F/B			G/F	B					G/F	
HER reference	C01	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 500pg	C02	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 250pg	C03	G	G	G/B	G/B	G/B	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 125pg	C04	G	G/F		G/B	G/F	G	G/B	G/B	G/B	G	B	G	G/B	B	G	G/B	G/B	G	G	G	B
HER 62pg	C05	G	G/F	G/F	G/B		G	G/B	G/B	G/F	G	B	G/F	G/F	F/B	G	G/B	G/B	G	G	G	B
HER 31pg	C06	G/F			G/B	G/F	G/F	G/B	G/F	G/F	G	B	G/F		F/B	G	F/B	F/B	G		G	B
HER 16pg	C07	G/F			F/B		G/F				G	B	G/F		F/B				G		G/F	B
SHM reference	D01	G	G/B	B	G/B	G/B	G	G	G/B	G	G/B	G/B	G	G/B	G/B	G	G	G/B	G/B	G	G	B
SHM 500pg	D02	G	G/B	F/B	G/B	G/F	G	G	G/B	G	G/B	G/B	G	G/B	G/B	G	G	G/B	G/B	G	G	B
SHM 250pg	D03	G			G/B		G	G	F/B	G	G/B	G/B		G/F	G/B	G	G		G/B	G/F	G	B
SHM 125pg	D04	G/F	G/F		G/B		G	G	G/B	G	G/B	G/B	G/F	G/B	G/B	G	G		G/B	G	G	B
SHM 62pg	D05	G			G/B		G	G	F/B		G/B	G/F	G/F	G/F	G/F	G	G		G/B	G/F	G	B
SHM 31pg	D06	G/F			G/B		G/F	G			G/B	G/F		G/F	G/B				G/B		G/F	
SHM 16pg	D07	G/F			G/B						G/F	F/B			G/B				F/B		G/F	
ST reference	E01	G	G/B	G/B	G/B	G/B	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 500pg	E02	G	G/B	G/B	G/B	G/B	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 250pg	E03	G	G/F	G/F	G/B	G/F	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 125pg	E04	G	G/F		G/B	G/F	G/B	G	G	G	G	G/B	G	G/B	G	G	G/B	G	G	G	B	B
ST 62pg	E05	G/F			G/B		G/B	G	G/F	G	G	G/B	G/F	G/F	G	G	G/B	G/F	G	G/F	B	B
ST 31pg	E06	G/F			G/B		G/F	G			G	G/F			G/F	G	F/B		G/F		F/B	
ST 16pg	E07	G/F			F/B						G/F	F/B			G/F				G/F		F/B	
Cambio M reference	F01	G/B	G/B	B	G	G	G	G/B	G/B	G	G/B	G/B	G	B	G	G	G	G	G	G/B	G	B
Cambio M 500pg	F02	G/B	G/B	B	G	G	G	G/B	G/B	G	G/B	G/B	G	B	G	G	G	G	G	G/B	G	B
Cambio M 250pg	F03	G/B	G/B	F/B	G	G/F	G	G/B	G/B	G	G/B	G/B	G/F	F/B	G	G	G	G	G	G/B	G	B
Cambio M 125pg	F04	G/B	G/B	F/B	G	G/F	G	G/B	G/B	G	G/B	G/B	G/F	F/B	G	G	G	G	G	G/B	G	B
Cambio M 62pg	F05	G/F			G	G/F	G			G/F	G/B	G/B		F/B	G	G	G		G	F/B	G	B
Cambio M 31pg	F06	G/F			G		G/F				F/B	G/B			G/F	G	G/F		G/F		G	B
Cambio M 16pg	F07	G/F										G/B			G/F	G/F			G/F	F/B	G/F	
Cambio F reference	G01	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 500pg	G02	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 250pg	G03	G	G	G/B	G/B	G/B	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G	G/B	B
Cambio F 125pg	G04	G	G	G/B	G/B	G/F	G	G/B	G/B	G	G/B	B	G	G	G	G/B	G/B	G	G	G/F	G/B	B
Cambio F 62pg	G05	G/F	G/F		G/B	G/F	G	G/B	G/F	G/F	G/B	B	G/F	G/F	G	G/B	G/B	G	G	G/F	G/B	B
Cambio F 31pg	G06	G/F	G/F		G/B							B	G/F	G/F	G	G/F	F/B		G		G/B	B
Cambio F 16pg	G07	G/F			G/B							F/B		G/F	G/F				G/F		G/F	

'F' designations indicate single peaks falling below the homozygote threshold, suggesting that allele drop-out may have occurred and the locus might be heterozygous. Grey boxes indicate complete locus drop-out. Heterozygous genotypes are standardised with green peak base/blue peak base.

else If $\phi A < Ht$ and $\phi A > Bt$ and $\phi B < Bt$ then $P_{\mathrm{m_{locus}}} = f(p_A^2) + f(2p_Ap_B)$ (allele B may have dropped out), else If $\phi A < Bt$ and $\phi B < Ht$ and $\phi B > Bt$ then $P_{\mathrm{m_{locus}}} = f(p_B^2) + f(2p_Ap_B)$ (allele A may have dropped out), elseIf $\phi A < Bt$ and $\phi B < Bt$ then $P_{\mathrm{m_{locus}}} = 1$ (complete locus drop-out),

then $P_{\mathrm{m_{Genotype}}} = \prod_{\mathrm{locus}=1}^{n} P_{\mathrm{m_{locus}}}$

3.9. Limit of detection

Using interpretation guidelines based on the *Hb* and *Ht* data, a set of dilution series samples peak data were analysed

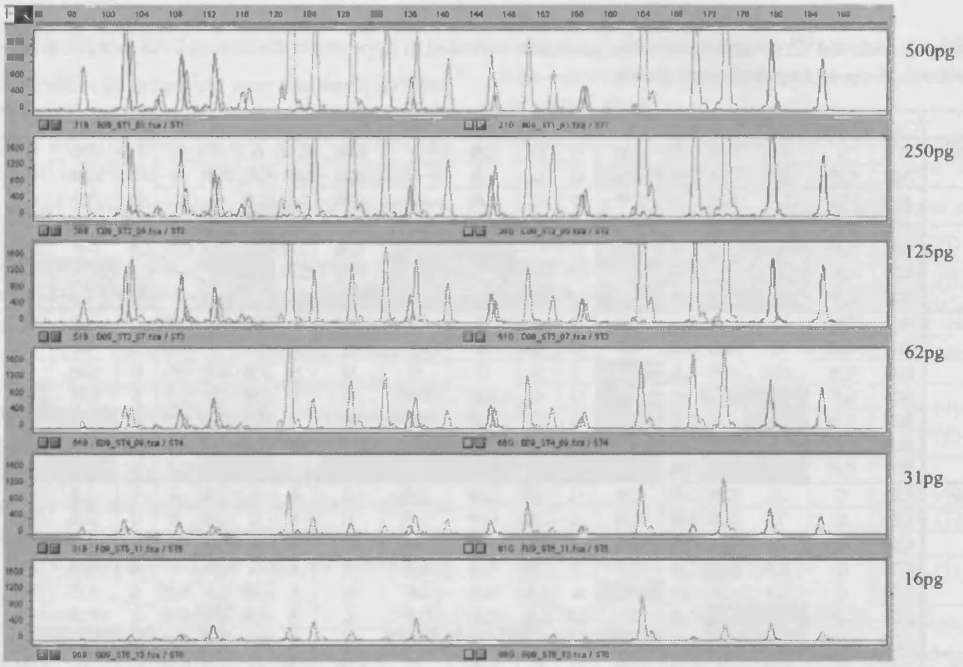

Fig. 4. Electropherograms showing SNP profiles obtained for the dilution series of ST control DNA template.

through Celestial^{TM} to generate genotypes for each sample. The SNP data were collated (Table 6) and an example of the SNP profile electropherograms is presented in Fig. 4.

Full profiles were observed for all samples using 1 ng of DNA template and six out of seven samples gave full profiles with 500 pg DNA template, although some homozygous peaks were <*Ht* and were subsequently labelled with an 'F' designation (Section 3.5). HER and ST gave full profiles down to 250 pg DNA template and Cambio^{TM} male and female samples were correctly genotyped using 125 pg DNA template. All seven samples gave partial DNA profiles down to the lowest DNA template level of 16 pg.

We tested a total of seven individual DNA samples at seven different DNA starting concentrations ($n = 49$). From these results it was demonstrated that all samples provided a full and correct, SNP profile at optimal DNA amounts (0.5–1.0 ng) and partial DNA profiles were obtained with a DNA template between 500 pg and 16 pg, lower levels were not tested. SGM plus^{TM} amplification routinely gives a full profile above 100 pg starting DNA material [1] and LCN SGM plus^{TM} is used to provide full or partial profiles at sub-125 pg DNA concentrations using LCN amplification conditions [26].

3.10. Testing the robustness and sensitivity of the SNP multiplex (artificially degraded samples)

Blood, saliva and semen samples were pipetted onto cotton squares and kept at 37 °C for a period of 243 days. At specific time intervals, a number of cotton squares for each sample were collected and stored at −20 °C. The DNA was extracted using the Qiagen^{TM} QiaAmp Mini-Kit, using the manufacturer's protocols for the different sample types. The degraded DNA samples were genotyped using both the SNP multiplex system and SGM plus^{TM} DNA profiling (28 cycles). Standard SGM plus^{TM} amplification methods were used as the sample concentrations exceeded 300 pg/μL using Picogreen quantification [14], suggesting plentiful DNA template, albeit in degraded form. The results for both SGM plus^{TM} and SNPs are tabulated in Tables 7a and 7b. Partial profiles were classed as those samples exhibiting either allele drop-out, i.e. 'F' designations due to peaks falling below *Ht*, or complete locus drop-out.

The saliva samples exhibited the highest level of DNA degradation using both profiling techniques. SGM plus^{TM} showed a lower amplification efficiency than the SNP multiplex, most noticeably after 147 days of degradation when SNP profiling still gave an 81% partial profile, whereas SGM plus^{TM} had decreased to only 18%. Similarly, the blood samples gave a full SNP profile at all degradation time intervals whereas SGM plus^{TM} gave only a 9% partial profile by 243 days. The semen samples showed little degradation in these experiments and a full SGM plus^{TM} profile and SNP profile was obtained at all time intervals except for one allele drop-out in SGM plus^{TM}.

The experiments performed using artificially degraded DNA suggested that the SNP multiplex is capable of giving results where SGM plus^{TM} DNA profiling failed. This may be because the genome target size is much smaller (the SNP multiplex ranges from 56–146 bases compared to 103–359 bases using SGM plus^{TM}).

Table 7a
SGM plus$^{\mathrm{TM}}$ (28 cycles) DNA profiles obtained from artificially degraded DNA samples

SGM plus™ profiles	Size range of STR loci (bp)	102-136		111-140		124-170		155-207		163-202		185-240		212-353		229-270		262-346		291-345	
	Days in humidifier	D19		D3		D8		VWA		THO1		D21		FGA		D16		D18		D2	
Saliva	0	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	F
	42	12	14	15	17	11	15	16	17	6	F	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
	62	12	14	15	17	11	15	16	17	6	F	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
	84	12	14	15	17	11	15	16	17	6	F	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
	147	▨	▨	15	F	11	F	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
	243	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Semen	0	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	42	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	62	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	84	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	147	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	23
	243	12	14	15	17	11	15	16	17	6	6	30	30.2	18	23	11	13	15	16	23	F
Blood	0	13	15	16	18	12	13	16	17	6	6	28	32.2	20	21	11	12	12	13	25	25
	42	13	15	16	18	12	13	16	17	6	6	28	32.2	20	21	11	12	12	F	25	F
	62	13	15	16	18	12	13	16	17	6	6	28	32.2	20	21	11	12	12	13	25	25
	84	13	15	16	18	12	13	16	17	6	6	28	32.2	20	21	11	12	▨	▨	25	F
	147	13	15	16	18	12	13	16	17	6	6	▨	▨	▨	▨	11	F	▨	▨	▨	▨
	243	13	F	F	18	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨

Grey boxes indicate complete locus drop-out. 'F' designations indicate single peaks falling below *Ht*, suggesting that allele drop-out may have occurred and the locus might be heterozygous.

3.11. Reproducibility studies

Various biological samples, including fresh blood and saliva, fingerprints, hairs, semen and post-coital vaginal swabs, from different individuals were used to assess the reproducibility of the SNP multiplex system. PCR was performed (in duplicate for LCN samples) and compared to results from SGM plus$^{\mathrm{TM}}$ profiling.

Blood, semen and post-coital extracts gave full profiles using the SNP multiplex. Saliva extracts showed variable allele drop-out (8% mean drop-out) between individuals but this is also seen for STRs [28]. LCN sample types (hair roots, hair shafts, latent fingerprints) gave variable results for SNPs, both within and between different samples. Each extract was amplified in duplicate following LCN methodology described for STRs [26] and, as expected, stochastic variation was seen between amplifications of the same extract. The results showed that samples with optimal DNA template available (0.5–1.0 ng) would routinely give a full SNP profile and different sample types and different extracts had no effect on the results gained for each individual (data not shown).

Table 7b
SNP profiles obtained from artificially degraded DNA samples

SNP profiles	Size of genome target (bp)	57	63	67	69	74	77	82	85	90	94	97	101	107	110	114	118	125	132	135	140	146
	Days in humidifier	Amelo	D	U6	B6	N4	Y3	P5	A4	O6	Z2	K3	J2	Y6	P7	J8	X	F	G	L2	W3	H8
Saliva	0	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	42	X/Y	T	F/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	62	X/Y	T	F/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	84	X/Y	T	▨	A/T	A	G	A	C/G	A	C	F/C	C	T/A	T/A	A/T	C/F	C/A	T/C	C	C	T
	147	X/Y	T	▨	A/T	A/F	G/F	A	C/G	A	C	F/C	C	T/A	T/A	F/T	C/F	C/A	T/C	C	C	T
	243	X/Y	T/F	▨	▨	▨	▨	F/A	▨	A/F	▨	▨	C/F	▨	▨	▨	▨	▨	▨	▨	▨	▨
Semen	0	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	42	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	62	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	84	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	147	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
	243	X/Y	T	A/T	A/T	A	G	A	C/G	A	C	C	C	T/A	T/A	A/T	C	C/A	T/C	C	C	T
Blood	0	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	42	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	62	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	84	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	147	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T
	243	X/X	T	A/T	A/T	T	G	T	C/G	A	C/T	C	C	T/A	T	A	C	C	T	C/T	C/G	T

Grey boxes indicate complete locus drop-out. 'F' designations indicate single peaks falling below *Ht*. Heterozygous genotypes are standardised with green peak base/blue peak base. Saliva and semen samples were provided by one donor and the blood sample by a different donor.

Allele drop-in was observed for both hair and latent fingerprint samples. Finger marks are most likely to show contamination from other individuals due to the nature of the sample type. Secondary transfer of DNA from one individual to another through touch is well documented [29]. SGM plus^{TM} can be used to assess DNA mixtures and contamination much more readily than SNPs and so, in these cases, STRs should be used in preference. Hair root samples for some individuals also showed some allele drop-in peaks, i.e. peaks not related to the reference sample.

3.12. Species specificity

The following samples were extracted for amplification using the SNP multiplex: dog, cat, guinea pig, ferret, horse, chicken, wolf, toad, rat, bull, deer, badger, pigeon and orangutan; along with three types of bacteria: *Micrococcus luteus*, *Escherichia coli* and *Clostridium perfringens*. Samples were amplified using the same method as for human samples with optimal DNA starting templates of 1 ng. PCR products were run on the 3100 CE instrument using 20 s injection parameters and data was analysed in Celestial^{TM}. A number of non-allelic peaks with low peak heights (<200 rfu) were observed in all samples, these were not characterised as they fell below the negative threshold baseline (*Bt*). Only four species samples produced peaks in allelic positions: a horse sample showed a blue peak at W3 (220 rfu), one cat sample gave a green peak at W3 (232 rfu); another cat sample gave a blue peak at D (288 rfu); and a toad sample showed a green peak at P5 (219 rfu). It should be noted that all peaks were below 300 rfu and may be attributable to stochastic mispriming events as they were not reproducible.

3.13. Artefacts

Artefact peaks were found to be associated with some SNP loci. Negative control logs allowed the possibility of contamination to be disregarded and full sequencing was carried out on samples showing artefact peaks to identify the sample genotypes. Full sequencing identified the samples as homozygotes enabling the peaks to be characterised as true artefacts, possibly derived from primer–primer or primer–sample DNA interactions, as opposed to low *Hb* peaks.

4. Discussion

There are a number of factors that have to be considered in the development of new SNP multiplexes for forensic identification purposes. They should have low molecular weight genomic targets, i.e. lower than current conventional STR systems with the amplicon size preferably be less than 150 bp. This size of amplicon coincides with the length of DNA wrapped around the octameric histone core of the nucleosome [3] and we hypothesise that this covalent attachment actually protects fragments less than this length, and consequently some SNP allele copies, from degradation by nucleases. The use of SNPs for analysis of very highly degraded samples, such as burned or heavily decomposed bodies, means that to maximise their efficiency we need to implement a stringent interpretation strategy. Degraded samples have a lower amplification efficiency, even when DNA template concentrations are not limiting, due to fragmentation of the DNA, which necessitates the use of LCN guidelines. Also the use of a SNP multiplex should act as an adjunct to current STR profiling techniques [30], allowing the same DNA extracts to be used for both systems.

In this paper, we have developed a candidate low molecular weight SNP multiplex that has been validated in order to be used in a comparative study with competing strategies such as conventional STR LCN methods and mini-STRs [31], both of which boost the sensitivity of DNA analysis. The SNP system that we have developed can detect DNA down to sub-100 pg levels in a single multiplex and is able to give a full or partial profile when SGM plus^{TM} fails to give a full profile. In contrast, alternative strategies have used several PCR reactions per sample to achieve large multiplex sizes, e.g. a 70-SNP analysis method using five separate 12 SNP multiplexes, [6], in addition requiring 1–2 ng of DNA in each reaction. Recently, a 39-plex analysis method using multiplex PCR followed by primer extension has been reported [7]. This initially employs a single tube reaction that is subsequently split into five tubes for a primer extension assay. The authors used 1 ng reactions in all analyses. The SNP multiplex presented in this paper is advantageous over other techniques as it is a single tube reaction which could be easily automated; interpretation of the results is possible using an automated software solution; multiplex preparation is standardised due to the use of URP biochemistry and the technique has been shown to work on degraded DNA samples.

To interpret mixtures, Gill [5] has detailed a theoretical way forward and the SNP multiplex described here can be used as a model to develop the strategy. Details of the limitations of the system will be outlined in a subsequent paper.

5. Conclusions

The 21-locus SNP multiplex was developed to act as an adjunct to the currently available methods of DNA profiling. Validation studies were carried out to verify the use of the technique for casework purposes. This paper indicates that the SNPs selected for use within the multiplex all conform to Hardy–Weinberg expectations and show no linkage disequilibrium. All genotypes obtained were verified using control samples within all PCR amplification batches. Interpretation guidelines were based on dilution series data identifying the threshold limits for both *Hb* and *Ht*, allowing both optimal

(0.5–1.0 ng) and sub-125 pg DNA template to be amplified and genotyped with confidence in the result. These parameters were encoded into software in order to automate the process of interpretation. Artificially degraded samples showed a greater level of amplification when looking at SNPs than STRs, due to targeting of smaller DNA fragment sizes between 57 and 146 bases in length. Genotypes obtained from SNPs were reproducible across a range of different sample types, and for different DNA extracts from the same sample types. This paper supports the use of SNPs for forensic identification purposes, for discrete sample types.

Appendix A. Primer sequences

TSC = The SNP Consortium identification number; () = Arbitrary internal reference code

Forward primers Universal 9 tail (CGACGTGGTGGATGTGCTAT)	
Amelo X (Am)	Uni9-CCAGATGTTTCTCAAGTGGTCCTG
TSC0252540/9 (D)	Uni9-GGGAAACTGCTGGGTCTGT
TSC0746324/9 (U6)	Uni9-GCAAGGCCCAAAGCAAAGAA
TSC1342445/9 (B6)	Uni9-GGGAGACAGGCCCATGCA
TSC1156239/9 (N4)	Uni9-CAGAAAAGGCAGGAACCTGGACA
TSC0846740/9 (Y3)	Uni9-ACCAACCCCACAAAGCAGG
TSC0176551/9 (P5)	Uni9-GGGGGTACTGGGGAGACCAA
TSC0421768/9 (A4)	Uni9-GATGCCTCTTGCATTGTGAACG
TSC1588825/9 (O6)	Uni9-GAGCCAAGAATCGCAGGGAA
TSC0086795/9 (Z2)	Uni9-CATTGTGTTTCAAACGCGTGCC
TSC0078283/9 (K3)	Uni9-TGCCACTCTGACACTGATGCTTG
TSC0156245/9 (J2)	Uni9-CTGCCTTGGCTCCCAGCC
TSC0627632/9 (Y6)	Uni9-CAAGATTCCTGGCCCCTGGTAA
TSC0897904/9 (P7)	Uni9-CTCTTCCAGCAGGCACCATGA
TSC0709016/9 (J8)	Uni9-CAGGGAATGACAGGGAACCACTA
TSC0031988/9 (X)	Uni9-CTGTGCATCCACTGCGCC
TSC0155410/9 (F)	Uni9-CCTGGAGCATGIGCTGACCAC
TSC0154197/9 (G)	Uni9-CCATGCCTCACCTCCTGCATT
TSC0384808/9 (L2)	Uni9-GCATGCCATTGCCAAATTCC
TSC0820041/9 (W3)	Uni9-GCCAACCAGACCTCCCAGG
TSC0131214/9 (H8)	Uni9-CTCAGTTGGGTGCTTACGTGCA
Forward primers Universal 11 tail (TGACGTGGCTGACCTGAGAC)	
Amelo Y	Uni11-AAAGTGGTTTCTCAAGTGGTCCCA
TSC0252540/11	Uni11-GGGAAACTGCTGGGTCTGC
TSC0746324/11	Uni11-GCAAGGCCCAAAGCAAAGAT
TSC1342445/11	Uni11-GGGAGACAGGCCCATGCT
TSC1156239/11	Uni11-CAGAAAAGGCAGGAACCTGGACT
TSC0846740/11	Uni11-ACCAACCCCACAAAGCAGC
TSC0176551/11	Uni11-GGGGGTACTGGGGAGACCAT
TSC0421768/11	Uni11-GATGCCTCTTGCATTGTGAACC
TSC1588825/11	Uni11-GAGCCAAGAATCGCAGGGAT
TSC0086795/11	Uni11-CATTGTGTTTCAAACGCGTGCT
TSC0078283/11	Uni11-TGCCACTCTGACACTGATGCTTC
TSC0156245/11	Uni11-CTGCCTTGGCTCCCAGCT
TSC0627632/11	Uni11-CAAGATTCCTGGCCCCTGGTAT
TSC0897904/11	Uni11-CTCTTCCAGCAGGCACCATGT
TSC0709016/11	Uni11-CAGGGAATGACAGGGAACCACTT
TSC0031988/11	Uni11-CTGTGCATCCACTGCGCA
TSC0155410/11	Uni11-CCTGGAGCATGIGCTGACCAA
TSC0154197/11	Uni11-CCATGCCTCACCTCCTGCATC
TSC0384808/11	Uni11-GCATGCCATTGCCAAATTCT
TSC0820041/11	Uni11-GCCAACCAGACCTCCCAGC
TSC0131214/11	Uni11-CTCAGTTGGGTGCTTACGTGCT

Appendix A. (*Continued*)

Reverse primers Universal 13 tail (CAAGCTGGTGGCTGTGCAAG)	
Amelo/13	Uni13-TGCTTAAACTGGGAAGCTGITGGT
TSC0252540/13	Uni13-AATGACITGCCCCACAGGAG
TSC0746324/13	Uni13-ACAAAGCCCCAAGGCAGAG
TSC1342445/13	Uni13-GCCATTCAGAACTAACTAGTCTGGGA
TSC1156239/13	Uni13-CGACGGGGGTTGAGTGGTTCAG
TSC0846740/13	Uni13-ATTAGAGCAGCCAAGTCCTGACCA
TSC0176551/13	Uni13-AGGCGGATCCTGGAGGG
TSC0421768/13	Uni13-GCTCAACAGCACAACTCTGCTACAGC
TSC1588825/13	Uni13-GCTAAAGCAGCTCTGAAACCCA
TSC0086795/13	Uni13-GGATCAGAGAAAGTGCAGCTGGT
TSC0078283/13	Uni13-AATGGGGAGATTGGCTTGGAC
TSC0156245/13	Uni13-CCTGAACATCCCTGAAGGTATTTCG
TSC0627632/13	Uni13-TAGCCTTAGGACATGGTGATTACAGA
TSC0897904/13	Uni13-GATTTGGGAITTTAGTGACATCTGCA
TSC0709016/13	Uni13-CTGTACATCTTTTAAGACCAACTCCTT
TSC0031988/13	Uni13-TCTAGGCTGGTGCCAGCCC
TSC0155410/13	Uni13-GGCTCTGAAGAACAATGGGGAG
TSC0154197/13	Uni13-CAATCCTGTTTGCAGAGTTCCAG
TSC0384808/13	Uni13-TGAGCCAAGGTGTGGGGA
TSC0820041/13	Uni13-TTACACAGGTCTCCAGCTTGAGCAA
TSC0131214/13	Uni13-AAGAGGGAGCACTGTGGGACTG

Appendix B. Celestial^{TM} interpretation criteria

(12 s data/20 s data)

SNP ID	Heterozygous balance (*Hb*) %	Homozygous threshold (*Ht*) (rfu)
Amelo	24/26	484/672
D	25/41	738/1031
U6	32/36	373/671
B6	24/24	394/540
N4	11/13	506/736
Y3	25/26	186/256
P5	34/32	509/740
A4	25/23	583/895
O6	16/16	182/738
Z2	26/25	456/954
K3	17/14	703/994
J2	23/24	204/539
Y6	27/30	545/631
P7	15/15	229/329
J8	35/41	401/1020
X	42/40	468/655
F	33/33	860/1282
G	32/35	320/574
L2	50/50	276/395
W3	13/18	290/641
H8	50/50	156/452

Heterozygous balance (*Hb*) data were calculated using dilution series data. Where $Hb_{\min}$ was greater than 50% the Celestial^{TM} criteria was set at 50% to allow for sample variation. Homozygous threshold criteria ($Ht_{\max}$) were the maximum observed value plus an additional 20% to allow for sample variation. An upper peak height threshold was set at 7000 rfu and the negative baseline (*Bt*) was set at 100 and 200 rfu for 12 and 20 s data, respectively.

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Analysis of artificially degraded DNA using STRs and SNPs—results of a collaborative European (EDNAP) exercise

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Received 23 August 2005; received in revised form 8 November 2005; accepted 8 November 2005

Abstract

Recently, there has been much debate about what kinds of genetic markers should be implemented as new core loci that constitute national DNA databases. The choices lie between conventional STRs, ranging in size from 100 to 450 bp; mini-STRs, with amplicon sizes less than 200 bp; and single nucleotide polymorphisms (SNPs). There is general agreement by the European DNA Profiling Group (EDNAP) and the European Network of Forensic Science Institutes (ENFSI) that the reason to implement new markers is to increase the chance of amplifying highly degraded DNA rather than to increase the discriminating power of the current techniques.

A collaborative study between nine European and US laboratories was organised under the auspices of EDNAP. Each laboratory was supplied with a SNP multiplex kit (Foren-SNPs) provided by the Forensic Science Service®, two mini-STR kits provided by the National Institute of Standards and Technology (NIST) and a set of degraded DNA stains (blood and saliva). Laboratories tested all three multiplex kits, along with their own existing DNA profiling technique, on the same sets of degraded samples. Results were collated and analysed and, in general, mini-STR systems were shown to be the most effective.

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doi:10.1016/j.forsciint.2005.11.011

Accordingly, the EDNAP and ENFSI working groups have recommended that existing STR loci are reengineered to provide smaller amplicons, and the adoption of three new European core loci has been agreed.

Keywords: Degraded DNA; Short tandem repeats; Single nucleotide polymorphisms; Mini-STRs

1. Introduction

Existing short tandem repeat (STR) systems used in European national DNA databases (NDNADBs) include seven core STR loci recommended by the European Network of Forensic Science Institutes (ENFSI) and agreed by Interpol [1]. The core loci are included in commercially available multiplexes. However, all current markers have relatively large amplicon sizes (between 150 and 450 bp) [2]. It has been demonstrated that smaller amplicons are much more likely to be amplified in samples containing degraded DNA [3–11]. There are two kinds of markers that can bring the size of the amplicon substantially below 150 bp: 'mini-STRs' that have short flanking regions to the tandem repeat sequence and single nucleotide polymorphisms (SNPs). See Butler [4] and Budowle [12] for an extensive review of existing technologies. A small number of validated SNP assays are used in casework and these include mini-sequencing assays for mitochondrial DNA (mtDNA) [13–16], Y chromosome [17], a red hair marker assay [18] and autosomal multiplexes [19]. There has been some debate about which is the best approach [20]. Some existing high molecular weight markers have already been converted into low molecular weight (<130 bp) 'mini-STR' multiplexes simply by moving the primer binding sites closer to the STR repeat region [3,7,21]. The advantage of this approach is that it is possible to maintain consistency with existing core loci that are used in NDNADBs. To achieve the ultimate lower limit of small amplicons (ca. 40 bp), SNPs are preferable, but the downside is that a panel of 45–50 loci would be needed to achieve match probabilities comparable with existing STR multiplexes [22,23]. Furthermore, the larger the multiplex, the more difficult it is to reliably and to reproducibly construct [12]; loss of amplification efficiency may ensue, effectively defeating the object of the exercise. To circumvent this problem, several SNP multiplexes of a dozen loci each can be used in concurrent multi-tube reactions, however, the sample size needs to be sufficient to allow this option [24,25]. Large amounts of DNA from, e.g., bones, can be analysed in this way, but the study of many small forensic stains is precluded as the amount of DNA extract available is limited. In addition, the binary nature of SNPs means that their statistical characteristics are not amenable to the interpretation of complex samples such as mixtures. A robust, highly quantitative SNP assay would be required to allow determination of mixtures using an interpretation strategy based on heterozygous balance and homozygous thresholds [22].

Accordingly, a collaborative EDNAP study was carried out to compare some different DNA profiling techniques for their usefulness in genotyping artificially degraded samples. The study was primarily designed to assess the effectiveness of new techniques (especially SNPs and mini-STRs).

2. Materials and methods

2.1. Degraded DNA samples

All laboratories were provided with sets of artificially degraded blood and saliva samples. Aliquots of 5 μl blood or 10 μl saliva were pipetted onto 4 mm^2 cotton squares and degraded at 37 °C in a 100% humid environment over a period of 12 and 16 weeks, for saliva and blood, respectively. After set periods of 0, 2, 8, 12 [saliva] and 16 [blood] weeks, degradation was suspended by storing the samples at −20 °C until the time course was complete. Laboratories extracted 3–4 stains at each time-interval, combining the extracts together. This protocol was used to average out variation that may be inherent between different stains.

2.2. Extraction and quantification

Standard protocols of laboratories carrying out the analyses were used (Table 1). Methods included: QIAamp or QIAshredder supplied by Qiagen^{TM} [26,27] and phenol–chloroform [28]. Quantification was carried out using Picogreen [29], Quantifiler^{TM} Human DNA Quantification kit (according to manufacturer's protocol) or Slot-blot methodology [30]. One laboratory performed quantification using a real-time quantitative PCR assay with a fluorogenic Taqman probe, targeting the human Alu repetitive sequence, with PCR primers adopted from Nicklas and Buel [31].

2.3. SNP and STR kits and protocols

The following STR kits were used in the study, according to manufacturer's protocol: AMP*Fl*STR$^{®}$ SGM Plus^{TM} (SGM+) (Applied Biosystems) [7 labs][32]; AMP*Fl*STR$^{®}$ Identifiler (Applied Biosystems) [1 lab] [33]; Powerplex$^{®}$16 system (Promega) [1 lab] [34]; plus mini-SGM and miniNC01 (National Institute of Standards and Technology (NIST), US) [9 labs] [3,6,21]. The 21 loci 'Foren-SNP^{TM}' multiplex kit (The Forensic Science Service$^{®}$, UK) was used as described by Dixon et al. [9 labs] [19].

Table 1
Extraction and quantification methods and results, provided by participants. Grey boxes indicate that no information was received. UND = undetermined data value

Lab ID	Extraction protocol	Quantification values (ng/uL)																
		Ref 1 blood					Ref 1 saliva				Ref 2 blood				Ref 2 saliva			
		Quant method	0 weeks	2 weeks	8 weeks	16 weeks	0 weeks	2 weeks	8 weeks	12 weeks	0 weeks	2 weeks	8 weeks	16 weeks	0 weeks	2 weeks	8 weeks	12 weeks
1	Qiagen (manual)	Picogreen	1.91	0.22	0.22	0.03	1.03	0.07	0.01	0.01	2.13	0.23	0.23	0.02	0.36	0.03	0.03	0.01
2	Qiagen (robot)	Quantifiler	0.63	0.01	0.00	0.00	0.82	0.00	0.00	0.00	0.72	0.01	0.01	0.00	0.36	0.00	0.00	0.00
3	Phenol–chloroform	Quantifiler	2.31	0.22	0.06	0.02	6.59	0.00	0.00	0.00	5.16	0.92	0.25	0.46	8.95	0.06	0.01	0.03
4	Phenol–chloroform	Quantifiler	2.18	1.29	0.00	0.00	UND	0.00	0.00	0.00	3.65	2.35	0.96	0.00	8.31	0.00	0.00	0.00
5	Phenol–chloroform	qPCR	9.67	0.93	0.53	0.12	19.00	0.10	0.03	0.03	10.29	1.64	1.97	1.58	15.84	0.06	0.15	0.06
6	Chelex	None																
7																		
8	Phenol–chloroform	Slot-blot	11.79	0.57	1.00	1.68	33.46	0.12	0.03	0.05	10.15	2.33	4.13	0.80	14.88	0.36	0.23	0.05
9	Qiagen (manual)	None																

In addition, two laboratories carried out low copy number AMP*Fl*STR® SGM Plus™ profiling (34 PCR amplification cycles) using the method described by Gill et al. [35]

All PCR products were electrophoresed on AB 3100 capillary electrophoresis (CE) sequencers (Applied Biosystems) with either POP-4 or POP-6 polymer. Results were analysed using Genescan™ and Genotyper™ analysis software (Applied Biosystems).

2.4. Data analysis

Each laboratory was given an identifier number and genotyping results for each DNA profiling system for each laboratory were collated on Microsoft® Excel spreadsheets.

Genotypes were analysed as percentages—e.g. for SGM+ a full genotype comprised 22 alleles, thus a profile with 11 alleles was 50% of a full profile. Converting into percentages allowed direct comparisons between different multiplex systems.

Data were analysed with Minitab™ Release 14 using ANOVA, box–whisker plots, and the median polish method [36]. Box–whisker plots are a convenient method to display the main features of a set of data and facilitate the comparison of multiple sets. A box–whisker plot comprises a box, whiskers and outliers. A line is drawn across the box to represent the median; the bottom of the box is the first quartile (Q_1) and the top is the third quartile (Q_3)—hence half of the data are represented in the inter-quartile (IQ) range Q_3–Q_1; 25% of the data values are less than or equal to the value of Q_1; and 75% are less than or equal to the value of Q_3. The whiskers are lines extending from the top and bottom of the box. The lower whisker extends to the lowest value within the lower limit, whilst the upper whisker extends to the highest value within the upper limit. The limits are defined by: $Q_1 - 1.5(Q_3 - Q_1)$ (lower limit) and $Q_3 + 1.5(Q_3 - Q_1)$ (upper limit). The outliers are unusually high or low data values that lie outside of the lower and upper limits, these are represented by asterisks.

Identifiler® and Powerplex®-16 were omitted from the final results analysis, except for the inter-laboratory comparison, because only one laboratory used each multiplex. Low copy number (LCN) SGM+ results were also disregarded from intra-laboratory analyses, because only two laboratories submitted data.

3. Results

3.1. Extraction methods

Details of extraction techniques and corresponding quantification values were submitted by six of the laboratories (Table 1). These ranged from 0 ng/μL for heavily degraded samples to 33 ng/μL for a reference sample stain (Fig. 1).

The inter-quartile (IQ) range for degraded saliva samples (≥2 weeks incubation) varied between 0.03 and 0.17 ng/μL, compared to 0.5–2.3 ng/μL for blood samples indicating that DNA in the saliva stains degraded much more rapidly than in blood.

In comparison, undegraded control (time zero) reference samples showed considerable variation in the amount of DNA recovered between laboratories. More DNA was recovered with phenol–chloroform compared to Qiagen™ but the variation was much greater in the former (IQ range = 27 and 1.4 ng/μL, respectively) (Fig. 1). The method of quantification may have affected the DNA quantification values gained. Both laboratories using phenol–chloroform extraction followed by Quantifiler™

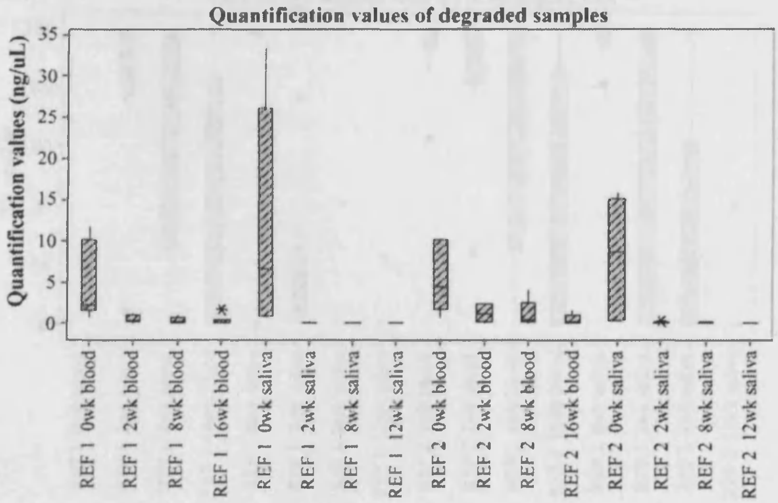

Fig. 1. Box–whisker plot showing the range of quantification values received for each reference individual for each sample type. Calculations are based on data from the six laboratories that submitted data.

Table 2
ANOVA results for percentage profile data for each laboratory for each sample type using each multiplex kit. Significant P-values are denoted in bold

Analysis of variance (ANOVA) tests	Degrees of freedom (DF)	Sum of squares (SS)	*F* ratio	Probability (*P*)
Multiplex	3	11705	2.49	0.061
Lab ID	7	96328	8.78	<0.001
Ref ID	1	11829	7.55	0.006
Sample type	1	177422	118.4	<0.001
Degradation time	3	280931	59.78	<0.001
Multiplex * lab ID	21	20122	0.61	0.909
Multiplex * ref ID	3	553	0.12	0.950
Multiplex * sample type	3	2293	0.51	0.676
Multiplex * degradation time	9	2949	0.21	0.993
Lab ID * ref ID	7	5841	0.53	0.809
Lab ID * sample type	7	15431	1.47	0.176
Ref ID * sample type	1	6126	4.09	0.044
Lab ID * degradation time	21	47521	1.44	0.098
Ref ID * degradation time	3	2867	0.61	0.609
Multiplex * lab ID * ref ID	21	3545	0.11	1.000
Multiplex * lab ID * degradation time	63	20937	0.21	1.000
Multiplex * ref ID * degradation time	9	2765	0.2	0.994
Lab ID * ref ID * degradation time	21	30254	0.92	0.566
Multiplex * lab ID * ref ID * degradation time	63	13408	0.14	1.000
Multiplex * lab ID * sample type	21	8114	0.26	1.000
Multiplex * ref ID * sample type	3	262	0.06	0.981
Lab ID * ref ID * sample type	7	14042	1.34	0.231
Multiplex * lab ID * ref ID * sample type	21	3571	0.11	1.000

quantification (labs 3 and 4) gave similar values, whereas quantification with qPCR (lab 5) and slot-blot (lab 8) produced much greater values (Table 1). However, all phenol–chloroform values (for control samples) were greater than those gained with Qiagen^{TM}, regardless of the quantification method.

3.2. Analysis of variance (ANOVA) calculations

ANOVA analysis on percentage profile data (Table 2) showed major significant differences as follows: (a) between different laboratories ($p < 0.001$); (b) between the two donating individuals (ref ID) ($p = 0.006$); (c) between the

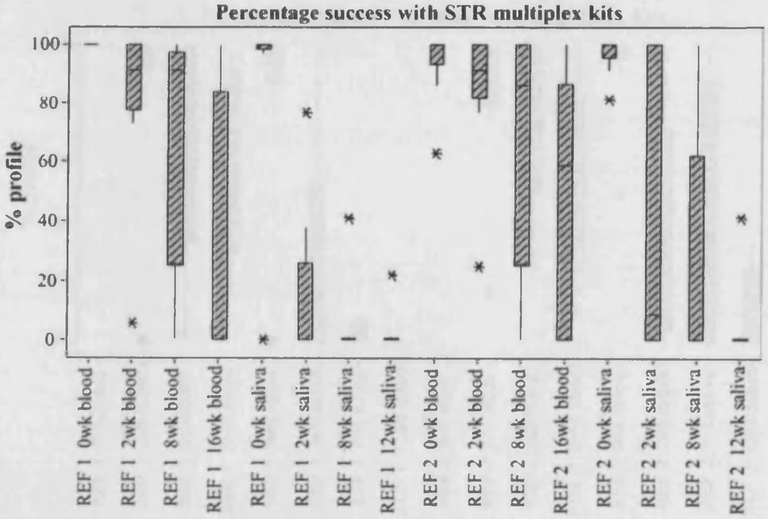

Fig. 2. Box–whisker plot showing the variation in percentage profiles per sample between the participating laboratories, using standard STR multiplex DNA profiling kits.

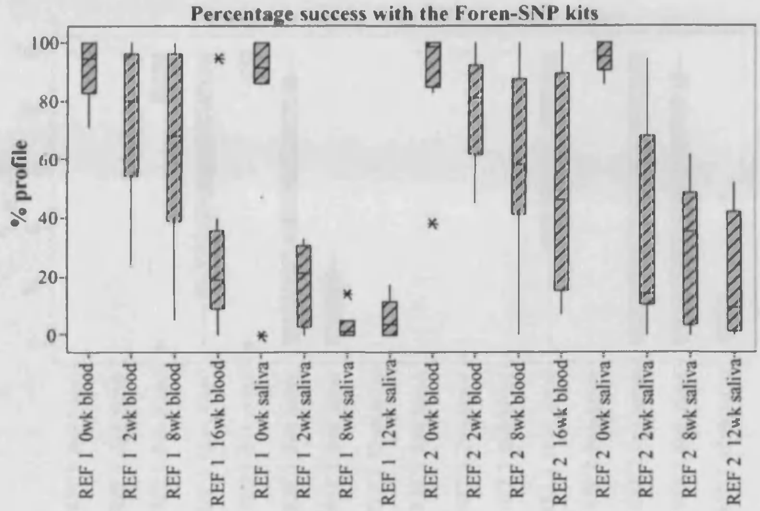

Fig. 3. Box–whisker plot showing the variation in percentage profiles per sample between the participating laboratories, using the Foren-SNP multiplex DNA profiling kit.

sample types—blood and saliva ($p < 0.001$); and (d) between different degradation times ($p < 0.001$). There was a smaller (borderline) significant two-way interaction between ref.ID and sample type ($p = 0.044$), i.e., there may be a significant difference between blood and saliva samples that are dependent on the reference individual. Otherwise, higher order interactions were not obvious in the data-set. Differences between the performance of the four multiplexes were almost significant using ANOVA ($p = 0.061$). Although there were differences between laboratories, these differences were consistent when averaged across different multiplexes, i.e., if a lab performed well with one multiplex then it would also perform well with another, and vice versa.

3.3. Intra-laboratory variation

Laboratories obtained full DNA profiles from control reference samples (time zero). As samples degraded, there was an increase in the amount of variation between the different laboratories in terms of percentage profile observed (Figs. 2–5). After several weeks, virtually all the DNA had degraded and no profile was obtained.

The most consistent multiplex across all laboratories was the mini-STR NC01 kit (Fig. 5). This multiplex consisted of three STR loci, D10S1248, D14S1434 and D22S1045, which are not available in commercial STR kits. The small number of loci present in the multiplex, compared to the 21

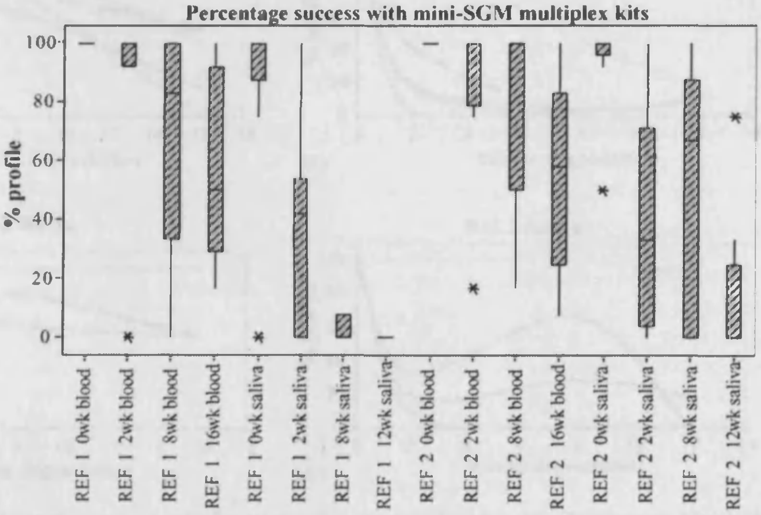

Fig. 4. Box–whisker plot showing the variation in percentage profiles per sample between the participating laboratories, using the mini-SGM DNA profiling kit.

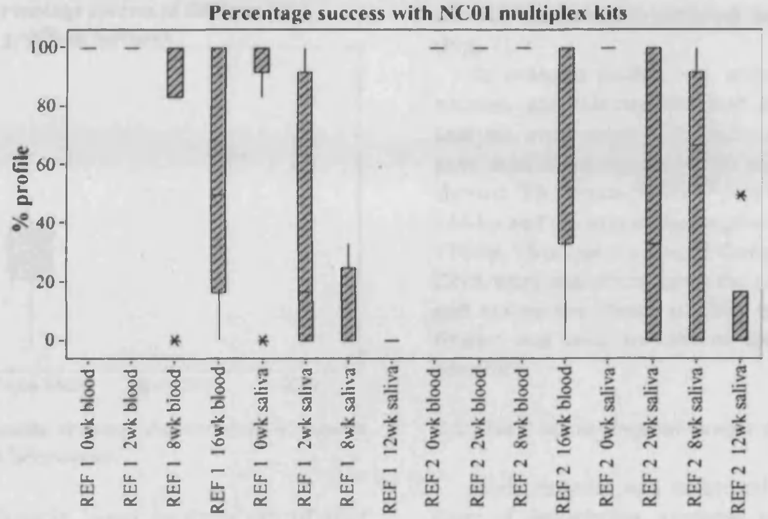

Fig. 5. Box–whisker plot showing the variation in percentage profiles per sample between the participating laboratories, using the NC01 mini-STR DNA profiling kit.

loci found in the Foren-SNPs^{TM} kit, eleven loci in SGM+ and seven loci in the mini-SGM multiplex, may have increased the robustness of the system.

In order to standardise the data and allow the different laboratories to be compared without bias, median polish analysis [36] was used. Median polish is similar to analysis of variance tests except that medians are used instead of means, thus adding robustness against the effect of outliers. The degradation time course of each sample averaged across all laboratories was compared and consequently, the performance of each multiplex—noting that the ANOVA showed no higher order interactions to complicate analysis. All four degradation profiles were quite different from each other (Fig. 6). As indicated by the ANOVA, saliva degraded faster than blood. It can be further generalised that the mini-STR systems performed better than SGM+. The SNP multiplex was inconsistent, but appeared to work better with the saliva compared to blood, possibly due to the presence of inhibitory factors in the blood samples. Interestingly, LCN SGM+ (34 amplification

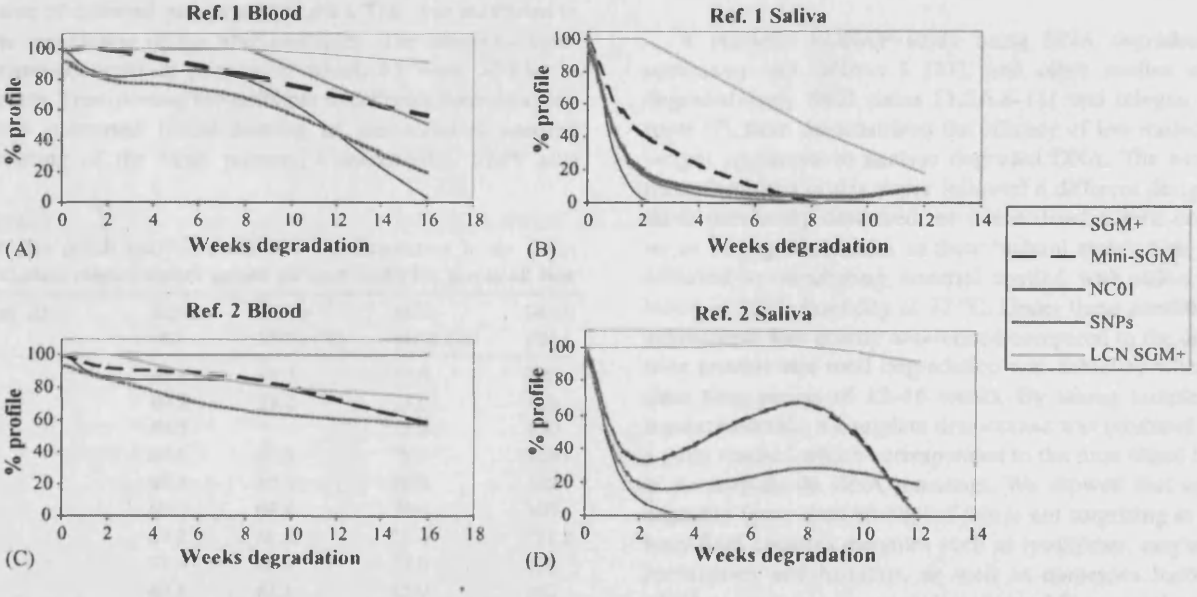

Fig. 6. Percentage profiles obtained across all labs for all samples and sample types. (A) Reference 1 blood. (B) Reference 1 saliva. (C) Reference 2 blood. (D) Reference 2 saliva. Values were calculated using median polish analysis to standardise the data obtained from all laboratories.

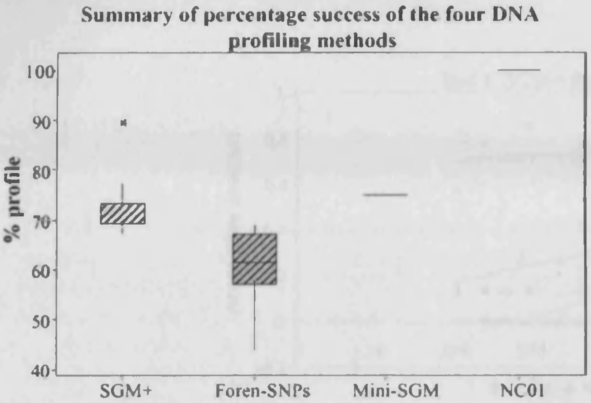

Fig. 7. Median polish results showing the variation in median percentage profiles across laboratories.

cycles) worked significantly better in three out of four samples (Fig. 6), compared to any other profiling method. Likewise, the single laboratory that reported LCN results with Powerplex® 16 achieved results that compared favourably to the overall results for the mini-STR multiplexes in this study.

3.4. Inter-laboratory variation

Median polish analysis (Fig. 7) was again used to provide a relative comparison of individual laboratory performance (averaged across all samples) (Table 3).

The analysis confirmed that the NC01 mini-STR multiplex kit performed the best, giving a median value of detected genotypes of 100% followed by mini-SGM (75%). The Foren-SNP™ multiplex gave the lowest median value of detected genotypes (61.6%). This was attributed to the complexity of the SNP multiplex. The multimix comprised 65 separate primers of which 63 were >35 bp in length. Transporting the multimix to different countries may have permitted freeze-thawing of the solution, causing shearing of the large primers. Consequently, SNPs also showed the greatest variation between the different labs (Fig. 7).

Table 3
Median polish analysis results for each laboratory in the study, including median values gained for each multiplex across all labs

Lab ID	SGM+ (%)	Foren-SNPs (%)	Mini-SGM (%)	NC01 (%)
1	69.5	69.3	75.0	100
2	69.5	58.2	75.0	100
3	69.5	*	75.0	100
4	69.5	43.5	75.0	100
5	89.5	60.0	89.6	100
6	69.5	64.4	75.0	100
7	67.2	56.5	35.4	91.5
8	77.4	68.3	75.0	100
9	69.5	63.1	75.0	100
Median across labs	69.5	61.6	75.0	100

To evaluate further, we ranked the SNPs in order of success, and selected the best ten for separate statistical analysis, irrespective of amplicon size. This modified system gave equivalent results to the miniSTR systems (data not shown). The Foren-SNPs™ loci ranged in size from 56 to 146 bp and the maximum amplicon size for mini-STRs was 170 bp. Thus, we concluded that good markers for degraded DNA were dependent upon the small size of the amplicon, and not on the choice of SNP or a mini-STR (unless the former was used to achieve the smallest amplicon size possible).

3.5. Total allele dropout across degradation periods

Allele dropout was measured for each sample at each stage of degradation, averaged across laboratories plotted against molecular weight (bp). Data from the two mini-STR systems (mini-SGM and NC01) were combined under a general heading of 'mini-STRs', with a maximum amplicon size of 170 bp. Linear regressions were plotted for reference 1 blood sample (Fig. 8a–c), confirming a general trend that lower molecular weight loci were more likely to stay intact. Allele dropout increased with increasing times of degradation for all three DNA profiling techniques. Foren-SNPs™ was the only multiplex to show allele dropout in control samples (time zero). Mini-STRs showed decreased allele dropout with the more degraded samples compared to SGM+.

4. Discussion

A previous EDNAP study using DNA degraded by sonication and DNAse I [37], and other studies using degraded body fluid stains [3,5,6,8–11] and telogen hair roots [7], have demonstrated the efficacy of low molecular weight amplicons to analyse degraded DNA. The experiment described in this paper followed a different design to those previously described, as it simulated a time-course series of degraded stains in their 'natural state'. This was achieved by incubating material spotted with saliva and blood in 100% humidity at 37 °C. Under these conditions, degradation was greatly accelerated compared to the dried-state process and total degradation was achieved within a short time period of 12–16 weeks. By taking samples at regular intervals, a complete time-course was produced and a point reached which corresponded to the time where little or no amplifiable DNA remained. We showed that saliva degraded faster than blood, but this is not surprising as this body fluid contains enzymes such as lysozymes, amylases, peroxidases and histatins, as well as numerous bacteria, which contribute micrococcal nuclease. Micrococcal nuclease is a non-specific endonuclease, that cuts adjacent to any base, with the rate of cleavage reported to be 30 times greater

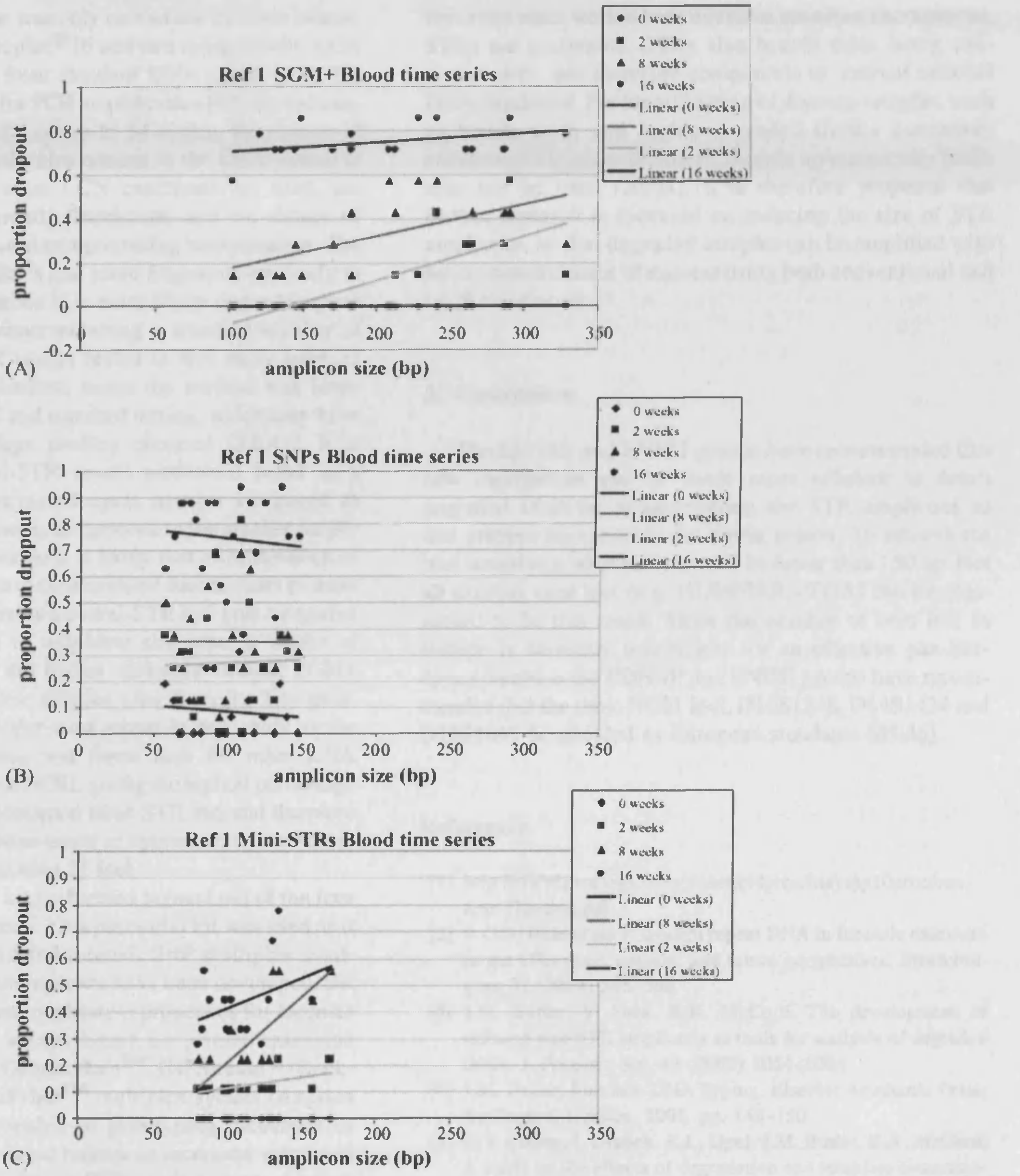

Fig. 8. Degradation plots for reference 1 blood investigated with SGM+, Foren-SNPs and mini-STR multiplexes. Graphs indicate the proportion of allele dropout compared to amplicon size. (A) SGM+ profiles. (B) SNP multiplex profiles. (C) mini-STR profiles. Mini-SGM & NC01 were combined for the mini-STR analysis.

at the 5′ side of A or T rather than G or C (fortunately most STR sequences tend to be GC-rich). Mammalian cells contain two additional DNAses that cleave non-specifically; DNAse I, which slightly favours purine–pyrimidine sequences [38] and DNAse II, an enzyme found in lysozomes associated with cell apoptosis [39].

Median polish analysis was carried out in order to standardise the data, allowing data sets from all laboratories to be compared regardless of variability in laboratory techniques, operator differences and sampling limitations [36]. Transformed data was analysed to investigate degradation rates, allele dropout and performance of the four assays used in this study. The artificially degraded samples gave similar results across all laboratories, showing the method produced samples with consistent levels of degradation across all sets.

The mini-STR assays tested gave the best results overall, when compared with standard SGM+ profiling and the Foren-SNPs^{TM} kit. Low copy number (LCN) DNA profiling proved to be the most successful method of amplification, although this technique was only carried out by three laboratories; one using Powerplex®16 and two using SGM+. LCN profiling only differs from standard DNA profiling by the number of cycles used for PCR amplification [40]. By increasing the number from 28 cycles to 34 cycles, the chance of amplifying the few molecules present in the DNA extract is improved. However, when LCN conditions are used, the allelic balance concurrently deteriorates and the chance of allele dropout is increased compromising interpretation. The advantage of mini-STRs is that more fragments are likely to survive degradation, hence it is more likely that a complete DNA profile will be observed using a standard number of cycles. The mini-STR assays tested in this study used 32 cycles in PCR amplification, hence the method was intermediate between LCN and standard testing, which may have increased the percentage profiles obtained [3,6,41]. It is possible that the mini-STR assays performed better as a consequence of an increased cycle number compared to standard profiling methods, as opposed to the smaller amplicon sizes targeted, however it is likely that a combination of both factors contributed to the increased success rates of these assays. Fig. 8 demonstrates the mini-STR loci give a negative regression in relation to amplicon size after 8 weeks of degradation, whereas the higher molecular weight SGM+ loci begin to show allele dropout after 2 weeks The mini-STR assays were also the most robust in this study as the number of loci targeted was lower than the other DNA profiling methods tested. NC01, giving the highest percentage profiles overall, only contained three STR loci and therefore would generally have been easier to optimise than the Foren-SNP™ multiplex containing 21 loci.

The Foren-SNP™ kit performed poorest out of the four assays tested in this study. This particular kit was used as it was the only fully validated forensic SNP multiplex available [19]. Other SNP multiplexes have been developed, but lack the quantitative and qualitative properties for forensic use [12,24,42]. SNP assays based on primer extension biochemistry, such as GenomeLab™ SNPStream® (Beckman Coulter) and SNaPshot™ multiplex system (Applied Biosystems™), are capable of genotyping thousands of SNPs in a single analysis but require an increased volume of either initial DNA template or PCR product, both of which are limited in crime scene samples. They also have the disadvantage of being multi-stage procedures, with sample tubes needing to be opened at various stages within the process. The Foren-SNPs™ kit allowed amplification of all 21 loci in a single tube reaction which were then analysed on an electrophoresis instrument. The potential certainly exists to further optimise SNP multiplex systems, as loci do benefit from being single base sites, therefore much smaller amplicons can be targeted [22,23,43]. The ability to obtain a result using SNPs would be beneficial, especially if the sample failed to give a profile using standard STR DNA profiling. However, the biallelic nature of SNPs makes it difficult to interpret mixtures and a well balanced assay would be required to make this feasible [22]. Consequently, for crime stain work where mixtures are often encountered, STRs are preferable. STRs also benefit from being consistent with, and therefore comparable to, current national DNA databases. For identification of discrete samples, such as bones, teeth and highly degraded tissues commonly encountered in mass-disasters, there is no reason why SNPs may not be used [20,44]. It is therefore proposed that further research is focussed on reducing the size of STR amplicons, so that degraded samples can be amplified with an increased chance of success using both conventional and LCN conditions.

5. Conclusions

The EDNAP and ENFSI groups have recommended that new multiplexes can be made more efficient to detect degraded DNA by re-engineering the STR amplicons so that primers lie closer to the repeat region. To achieve the best sensitivity, amplicons should be lower than 150 bp. Not all existing core loci (e.g. HUMFIBRA/FGA) can be engineered to be this small. Since the number of core loci in Europe is currently insufficient for an effective pan-European database the EDNAP and ENFSI groups have recommended that the three NC01 loci, D10S1248, D14S1434 and D22S1045 be adopted as European standards [45,46].

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