A STUDY TO EVALUATE VARIABLE NUMBER OF TANDEM REPEAT DNA POLYMORPHISMS IN DISPUTED PATERNITY TESTING

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DEDICATED TO

MY HUSBAND, PAUL

AND

OUR CHILDREN, ROBERT AND BRONWYN

I declare that this thesis represents my own work. It is being submitted for the Masters Diploma in Medical Technology, to the Cape Technikon, Cape Town. It has not been submitted before for any diploma or examination at any other Technikon or tertiary institution. The work was carried out at the Provincial Laboratory for Tissue Immunology, Cape Town. The opinions and conclusions drawn are my own and not necessarily those of the Cape Technikon.

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LIST OF ABBREVIATIONS

AMP-FLP	amplified fragment length polymorphism
bp	base pairs
cDNA	complementary DNA
Ci	Curie
°C	degrees Celcius
dpm	disintegrations per minute
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
g	measure of centrifugal force
gm	grams
HLA	human leukocyte antigen
HVR	hypervariable region
kb	kilobase
ml	millilitre
mm	millimetre
M	molar
mM	millimolar
ng	nanogram
OD	optical density
OLB	oligomer labelling buffer
PCR	polymerase chain reaction
PEX	power of exclusion
PI	paternity index
pg	picogram
RFLP	restriction enzyme fragment length polymorphism
RNA	ribonucleic acid

s.a.	specific activity
SDS	sodium dodecyl sulphate
μg	microgram
μl	microlitre
V	volts
VNTR	variable number of tandem repeat
W	probability of paternity (Wahrscheinlikheit)

SUMMARY

The use of genetic marker testing to resolve cases of disputed paternity, is well established. The number and range of systems used depends on the expertise of the laboratory, and for this reason various laboratories offer different systems. Standard testing includes tests in the following genetic marker systems: human leukocyte antigen (tissue) typing; red cell blood groups; and red cell enzyme and serum protein testing. The Provincial Laboratory for Tissue Immunology currently offers a range of 16 genetic marker systems capable of excluding >99% of falsely accused men.

Following the discovery DNA polymorphisms, particularly VNTR DNA polymorphisms, and the commercial availability of VNTR DNA probes, PLTI decided to offer this service to our clients. This study was the initial phase in the establishment of a VNTR DNA typing laboratory and covered the determination of inter-and intra-gel accuracy and precision, selection of restriction enzyme/probe combination, and evaluation and comparison of the results of 100 disputed paternity cases tested using both standard and VNTR DNA typing.

Of the 100 cases tested, in 33 cases, the putative father was excluded using standard testing. These exclusions were confirmed using VNTR DNA typing, and, furthermore,

an additional two exclusions of paternity were shown using only VNTR DNA typing. In another two cases of disputed paternity, the exclusions obtained using standard tests required further confirmation. VNTR DNA typing convincingly excluded both falsely accused putative fathers.

The VNTR DNA typing laboratory now functions as an integral part of the disputed paternity service. Due to the cost and time involved in VNTR DNA typing it is reserved at this stage for: those cases which require further confirmation of the results of standard testing; when the probability of paternity is low (<99.7%); or when a specific request is made.

OPSOMMING

Toetse vir die oorerwing van genetiese faktore om gevalle van betwyfelde vaderskap te ondersoek, is welbekend. Die getal en reeks sisteme wat gebruik word, hang af van die deskundigheid van die laboratorium, en om dié rede bied elke laboratorium verskillende sisteme aan. Standaard toetse sluit in toeste in die volgende genetiese sisteme: weefseltipering; rooiselbloedgroepe, rooiselensieme en serum proteïene. Die Provinsiaale Laboratorium vir Weefsel Immunologie bied 'n reeks van 16 genetiese sisteme aan wat >99% van mans wat van vaderskap valsbeskuldig is, kan uitsluit.

Na die ontedekking van DNA polimorfismes, veral VNTR DNA polimorfismes, en die kommersiële bekombaarheid van VNTR DNA peile, het PLTI besluit om hierdie diens aan ons kliënte te bied. Hierdie studie was die eerste fase om 'n VNTR DNA tipering laboratorium te stig. Hiermee was bepaal: die akkuraatheid en presisie van inter- en intra-gelontleding, keuse van restriksie-ensiem/peil kombinasie en die evaluasie en vergelyking van die uitslae van 100 gevalle van betwiste vaderskap getoets met standaard en VNTR DNA tipering.

In 33 van die 100 gevalle wat getoets was, was die vermeende vader uitgesluit van vaderskap met standaard toetse. Hierdie uitsluitings van vaderskap was bevestig

met VNTR DNA tipering, verder was 'n addisionele twee mans uitgesluit van vaderskap slegs op VNTR DNA tipering.

Die VNTR DNA tipering laboratorium is nou 'n integrale deel van die betwyfelde vaderskap diens. Gegewe die koste en tyd nodig om VNTR DNA tipering uit te voer word dit op hierdie stadium alleenlik gebruik vir: gevalle waar die uitslag van standaard toetse verdere bevestiging nodig het; waar die waarskynlikheid van vaderskap laag is (<99.7%); of wanneer dit spesifiek aangevra word.

The individuality of human blood will someday be comparable to the individuality of fingerprints.

Karl Landsteiner, 1900.

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CHAPTER ONE

1.0 INTRODUCTION

The use of polymorphic genetic marker systems is an accepted procedure in disputed paternity investigations. The primary objective of testing is to ensure that a man falsely accused of paternity is excluded. The various laboratories offering this service use different sets of marker systems which can be broadly categorised into three groups:

1. Human Leukocyte Antigen (HLA) typing,
2. Red cell blood groups, and
3. Red cell enzyme and serum protein polymorphisms.

The Provincial Laboratory for Tissue Immunology (PLTI) has been offering disputed paternity investigations since 1972, and to date has examined more than 3 000 cases. The range of genetic marker systems used is constantly evaluated: less informative systems are dropped in favour of those which give more information and new techniques are introduced to provide additional information. Currently, the laboratory offers a range of 16 genetic marker systems capable of excluding 99% of falsely accused men.

The discovery of DNA polymorphisms was to revolutionise genetic marker analysis. One class of DNA polymorphism, the insertion/deletion variable number of tandem repetitive (VNTR) DNA polymorphism, has been found to be

a valuable addition to the range of genetic marker systems used in disputed paternity investigations. It was proposed that it would be possible not only to exclude all falsely accused men, but that positive proof of paternity could be obtained. VNTR DNA polymorphism is determined using Restriction Fragment Length Polymorphism (RFLP) analysis, and may be detected at multiple loci simultaneously throughout the genome (multilocus VNTR DNA typing) or at specific sites on a single chromosome (single-locus VNTR DNA typing).

While the discovery of individual-specific DNA typings using probes which detect polymorphisms at multiple loci was hailed as a major breakthrough (Jeffreys *et al*, 1985a; 1985b), this approach presented many problems due to the complex nature of the autoradiograph result which required expert interpretation as well as there being inadequate laboratory controls. Single-locus VNTRs are simple to use and interpret and have found wide acceptance for disputed paternity investigations. Although single locus VNTR probes do not have the sensitivity of multi-locus probes, this aspect can be overcome by using a carefully selected range of probes.

Following the commercial availability of VNTR DNA typing probes, PLTI decided to offer this technology to our clients. This study documents the initial phase of establishing a laboratory which would become an integral part of the disputed paternity testing service.

OBJECTIVES OF THE STUDY

1. Determination of the inter- and intra-assay precision and accuracy,

2. selection of the restriction enzyme/probe combination, and

3. evaluation and comparison of the results of 100 cases of disputed paternity, using both standard and VNTR DNA testing.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 A REVIEW OF GENETIC MARKERS IN DISPUTED PATERNITY TESTING

The earliest recorded parentage dispute was resolved by King Solomon, who threatened to cut the disputed infant in half, and easily determined the child's natural mother (Old Testament, I Kings). It was the discovery in 1900 by Karl Landsteiner of the existence of the ABO blood group that paved the way for a more scientific basis upon which to discriminate between individuals (Landsteiner, 1900).

While Landsteiner was credited with the discovery of blood grouping, it was Epstein and Ottenberg who suggested that the ABO blood groups were inherited characteristics (Epstein and Ottenberg, 1908). This was confirmed by von Dungern and Hirzfield, who postulated that the blood groups were inherited in accordance with Mendelian Laws (Von Dungern and Hirzfield, 1910). However, their theory of two pairs of allelic genes (Aa and Bb) was later to be disproved. Bernstein evolved the presently accepted theory of multiple allelic genes using population genetics (Bernstein, 1924).

As additional blood group systems were reported (MN: Landsteiner and Levine, 1927; Rhesus: Landsteiner and Weiner, 1940; Kell: Coombs *et al*, 1946, Levine *et al*, 1949; and Duffy: Cutbush and Mollison, 1950) and found to be inherited in a Mendelian fashion, so the range of genetic marker systems which could be applied in paternity testing, widened.

The discovery of plasma protein polymorphisms was a further advance in improving the range of genetic marker systems which could be used for paternity testing. Smithies demonstrated that the serum protein, haptoglobin, exhibited polymorphism using starch gel electrophoresis (Smithies, 1955). Numerous plasma proteins have since been identified, with the more polymorphic systems such as Alpha 1 Antitrypsin (Hirshfield, 1959) and Vitamin D Binding Protein (Constans *et al*, 1978), being added to the repertoire of genetic markers offered by laboratories undertaking disputed paternity testing.

The discovery of polymorphic forms of red cell enzymes such as erythrocyte acid phosphatase (ACP), which was the first to be identified, was another significant advance in the application of genetic markers in paternity testing. While the existence of ACP had been noted as early as 1943 (Behrendt, 1943), several heritable forms were first reported by Hopkinson (Hopkinson *et al*, 1963).

The discovery of the human leucocyte antigen (HLA) system (Dausset and Nenna, 1953; Lalezari and Spaet, 1959) with its high degree of polymorphism, set new standards for paternity testing. The use of HLA typing was greatly facilitated by the introduction of the microlymphocytotoxic technique, (Terasaki *et al*, 1974), and it soon became clear that a power of exclusion (PEX) >90% was possible using HLA alone. When used in conjunction with other markers, PEX values of 99% were achieved (Dykes, 1982; Du Toit *et al*, 1989).

The discovery of DNA restriction fragment length polymorphisms (RFLPs) and specifically of the insertion/deletion polymorphisms such as variable number of tandem repeat DNA polymorphisms (VNTRs), (Wyman and White, 1980), revolutionised the field of human genetic analysis. The discriminating power of VNTR DNA polymorphisms, either using a single probe to detect polymorphism at multiple loci scattered throughout the genome (multilocus probes), or using a number of probes which detect polymorphism at a single site on a single chromosome (single locus probes), was found to be superior to any previously applied range of conventional genetic marker systems, (Balazs *et al*, 1986; Baird *et al*, 1986; Balazs *et al*, 1989).

DNA polymorphisms have thus become an important tool in medico-legal investigations such as disputed paternity testing.

2.2 DEMONSTRATION OF GENETIC VARIATION USING VARIABLE NUMBER OF TANDEM REPEAT DNA POLYMORPHISMS

Genetic variation between humans at the DNA level may be demonstrated by restriction enzyme digestion of DNA, electrophoresis and Southern Blotting followed by detection, in the membrane-bound DNA, of specific sequences by hybridization to a cDNA or genomic probe (Southern, 1975). The procedure is referred to as restriction fragment length polymorphism (RFLP) analysis (Figure 2.1). RFLP analysis is dependant upon the ability of a particular restriction enzyme to cut, or restrict, the DNA at a specific sequence, and the specificity with which a DNA probe hybridizes to a complementary sequence in the vast population of fragment lengths created by the restriction enzyme (Kessler and Holtke, 1986; Roberts, 1987). Restriction enzymes characteristically recognise palindromic sequences of 4-6 base pairs of double stranded (ds) DNA.

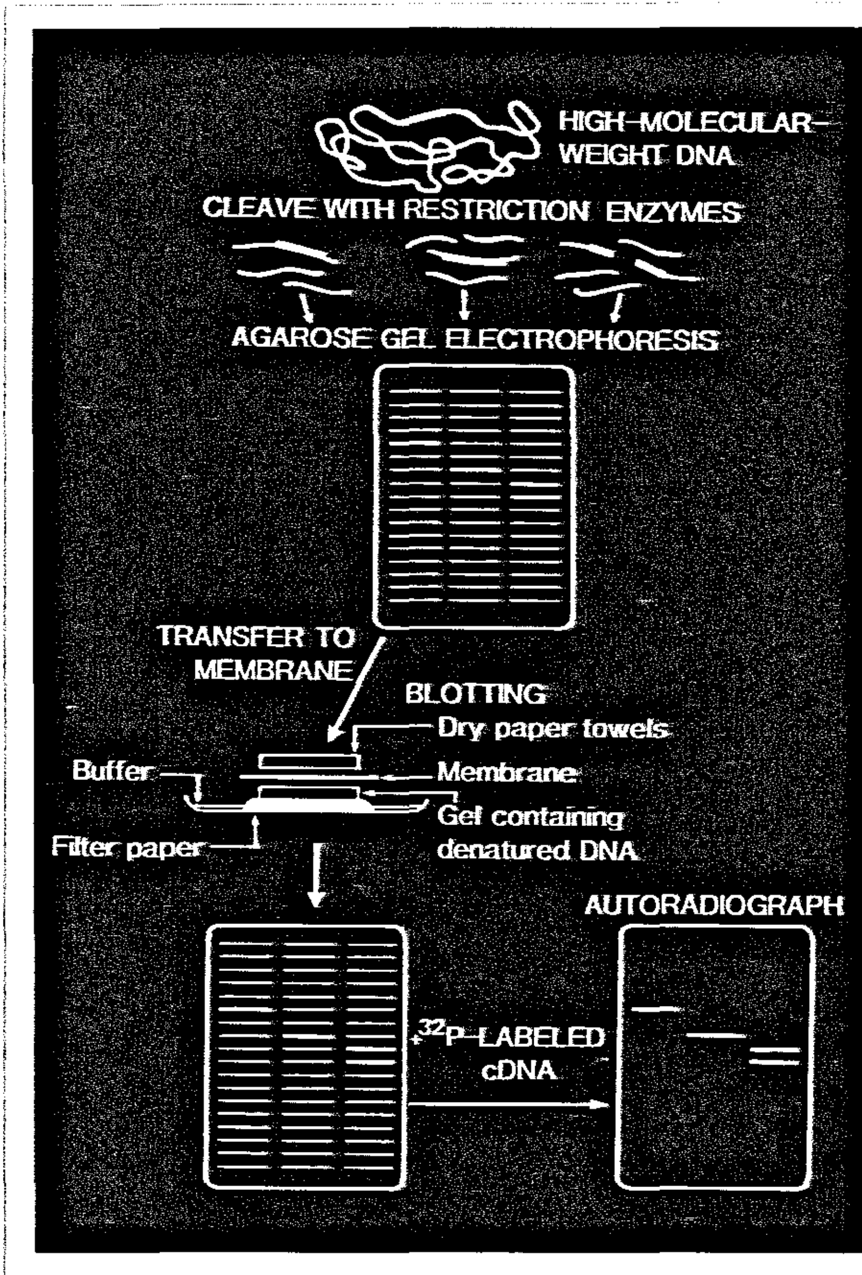


Figure 2.1

Restriction Fragment Length Polymorphism analysis: illustration of major steps involved.

RFLPs occur for one of two reasons (Cooper and Schmidke, 1985). They may occur because of a point mutation in the palindromic sequences which the restriction enzyme recognises. This results in the generation of a polymorphic fragment due to a change (loss/gain) in the restriction enzyme recognition site. A second mechanism by which RFLPs arise, is in the alteration of the length of the DNA between the restriction sites as a result of the insertion or deletion of a stretch of DNA, with the generation of polymorphic fragments i.e. insertion/deletion polymorphism.

An understanding of this second mechanism dates back to 1980, when Wyman and White discovered the first highly polymorphic DNA marker, pAW101, in humans (Wyman and White, 1980). By using a randomly cloned DNA sequence as a probe, polymorphic fragments could be demonstrated in the DNA isolated from random individuals. The basis for the polymorphism was suggested to be the result of DNA rearrangement rather than base-pair substitution. Polymorphism at such loci could be revealed by several restriction enzymes, whereas polymorphism arising from a single base change could be detected using only one restriction enzyme.

Botstein suggested that a large number of DNA sequence variations existed in the human population, which could be detected using RFLP analysis (Botstein *et al*, 1980). These

loci could define arbitrary genetic sites, not necessarily associated with any specific genes, but located within non-coding DNA. Construction of a linkage map of the human genome using these polymorphisms, could then be possible and could be employed in genetic linkage analyses. It was shown that these insertion/deletion polymorphisms consisted of repetitive sequences, characterised by head-to tail tandem repetition of lengths of DNA of a core sequence. The length of a repeat unit was typically between 10 and 64 base pairs (bp) long. The mechanism leading to frequent variation in the number of tandem repeats was unknown, but may have been due to slippage during DNA replication. These arrays might exist at multiple dispersed loci, at multiple sites either on the same chromosome or on different chromosomes (Jeffreys *et al*, 1985a; 1985b), or at a single locus, at a single site on a single chromosome (Nakamura *et al*, 1987a; Wong *et al*, 1987).

The existence of such repeated sequences, which comprise 20-30% of the human genome (Marx *et al*, 1976), was first established by the observation that a fraction of the genome re-annealed very rapidly after denaturation due to the presence of multiple copy sequences of DNA (Britten and Kohne, 1968). Centrifugation of DNA in cesium chloride gradients revealed the existence of DNA fractions which were slightly more bouyant than the main DNA band (Jelinek and Schmid, 1982). This fraction was termed satellite DNA, and was found to contain highly repeated sequences (Jeffreys *et*

al, 1985a). Gel electrophoresis and ethidium bromide staining of restricted genomic DNA, revealed the presence of distinct banding patterns superimposed upon a smear of DNA fragments, arising from repeated sequences.

Tandem repetitive DNA polymorphisms may be revealed by restriction enzymes capable of yielding DNA fragment lengths that contain variable numbers of the repeated species (Nakamura *et al*, 1987a). An enzyme which cuts the DNA externally to the block of tandem repeats and not within the tandem repeat, will reveal any variation that may exist in the number of repeats, and hence a variation in fragment size. Polymorphisms arising from this mechanism have been referred to as variable number of tandem repeats (VNTRs) and have been shown to be a powerful tool in forensic and other medico-legal investigations such as disputed paternity testing. In a similar manner to the analysis of protein polymorphisms, it is the number and frequency of alleles which is of prime importance. The greater the allelic heterozygosity, the greater is the possibility that a genetic marker system will demonstrate inter-individual variation.

Of the probes which have been reported to detect VNTR DNA RFLPs, many detect simple diallelic systems. Some, however, reveal single loci that are highly polymorphic, e.g. 3' alpha globin (Higgs *et al*, 1981), YNH24 (Nakamura *et al*, 1987a), with heterozygosities exceeding 90%, and make

these probes extremely useful in genetic linkage mapping as well as for identifying individuals.

The study of Nakamura and co-workers, using a series of oligonucleotides, showed that many VNTR loci existed within the human genome. Individual VNTR single locus probes (SLPs) were cloned by probing a genomic library with a core sequence. The core sequence, associated with a unique flanking sequence, could then be used as a single-locus probe, detecting alleles at a single chromosomal locus. Probes which revealed heterozygosities >90% (e.g. YNH24 and EFD52) were targeted for further evaluation in individualisation testing (Nakamura *et al*, 1987a).

Single-locus VNTR DNA probes have been technically simple to use in conventional RFLP analysis, and result in a one or two band pattern per individual (Baird *et al*, 1986; Allen *et al*, 1989; Chimera *et al*, 1989; Yokoi *et al*, 1990). In identifying only two alleles per individual, the assignment of genotypes was straightforward. Fragments could be accurately sized using commercially available molecular weight markers, and by incorporating an internal control such as HeLa or K562 cells in every gel, it was possible to continuously monitor inter-assay performance.
The simple autoradiograph patterns thus generated do not require a high level of expertise in order to interprete results, and can therefore be subjected to scrutiny outside of the laboratory, e.g in a court of law. Furthermore,

single locus VNTRs for disputed parentage testing have been selected to not be associated with any hypervariable sequences so that the attendant mutation rate is negligible. For these reasons, single-locus VNTR DNA polymorphisms have been chosen for use in disputed paternity investigations.

NOTE: Multilocus VNTR DNA probes have been found to be unsuitable for paternity testing due to the extreme skill required for the interpretation of the complex multiband autoradiographs. The level of acceptance in court of the results of multilocus VNTR DNA typing has been poor (Barinaga, 1989; Lander, 1989), and the technique has since been shown to be best suited to demonstration of monozygosity in twins, and forensic applications of identity, i.e. matching an individual to DNA isolated from an evidentiary sample.

2.3 SINGLE-LOCUS VNTR DNA PROBES SELECTED FOR THIS STUDY

2.3.1 ALPHA GLOBIN 3'HVR (D16S85)

The alpha-globin 3'HVR was isolated by Higgs and co-workers (Higgs *et al*, 1981). It comprises a tandem repeat array of 17 base pairs. The core nucleotide sequence is 5'-GNGGGG[n]ACAG-3. The DNA probe was supplied comprising a 4kb fragment of the alpha-globin 3'HVR locus, containing approximately 220 copies of the variable repeat and <200 base pairs of flanking sequence. The vast number of alleles and the high degree of heterozygosity (>90%) observed, reflect that there is considerable variation in the number of times that this sequence is repeated between different alleles (Lazarou, 1987). The alpha-globin 3'HVR has been localized to 16p12pter (Reeders *et al*, 1985).

2.3.2 MUCIN HVR (1q21)

The mucin gene HVR was isolated from a lambda-gtll cDNA library by Gendler and co-workers in a study of tumour-associated mucin glycoproteins expressed by human mammary epithelium (Gendler *et al* 1987,1988). It was later shown to be located on chromosome 1 within the region 1q21-24 (Swallow *et al*, 1987). The polymorphic region of the locus comprises a tandem array of 60 base pairs, with a very GC rich repeat unit. The heterozygosity has been calculated at

>85%. The DNA probe was provided as a 0.4kb fragment in the plasmid pMUC7.

2.3.3 HA-RAS HVR (11p15-5)

The Ha-Ras gene was isolated by Capon (Capon *et al*, 1983). The hypervariable region at the 3' end of the gene comprises a tandem repeat of a 28 base pair consensus sequence. The chromosomal assignment is 11p15-5, and the heterozygosity rate is approximately 54% (Krontiris *et al*, 1985). The DNA probe was supplied as a 2kb fragment containing approximately 30 copies of the VNTR and 1.2kb of flanking sequence.

2.3.4 MR24/1 (DXYS14)

This probe has been assigned to the pseudoautosomal region at the telomeres of the X and Y chromosomes (Cooke *et al*, 1985). The frequency of heterozygosity has been calculated at >85%. The sequence of the probe, which was supplied as a 2.9kb fragment in the plasmid pMR24/1, was not known. This probe also identified a number of other DNA fragments (usually two) in some individuals.

2.3.5 YNH24 (D2S44)

The YNH24 DNA probe originated from the polymorphic clone pYNH24 isolated by Nakamura and co-workers (Nakamura *et al*, 1987a). It was isolated as a unique cosmid from a total human cosmid library screened with the HBV-2 oligonucleotide (GGAGTTGGGGGAGGAG), and has been assigned to chromosome 2 by multipoint linkage analysis (Nakamura *et al*, 1987b). The probe was supplied as a 2kb insert, containing a 31 base pair repeat consensus sequence.

2.3.6 CMM101 (D14S13)

The polymorphic clone pCMM101 was originally isolated as a unique cosmid from a total human library screened with the myoglobin-2 oligonucleotide GGAGGCTGGAGGAG (Nakamura *et al*, 1987a). Isolated and mapped to chromosome 14, (Nakamura *et al*, 1988a), this clone revealed a VNTR polymorphism that contained a 15 base pair repeat consensus sequence.

2.3.7 TBQ7 (D10S28)

Bragg and co-workers isolated and mapped the clone pTBQ7 from a library of the somatic hybrid cell line 762-8A which included the human chromosomes 10 and Y (Bragg *et al*, 1988). The probe was supplied as a purified insert, isolated from the cloning vector pUC 18. The insert size was 1.9kb with the core repeat sequence 33 base pairs in length. pTBQ7 has been mapped to chromosome 10p, by multipoint linkage analysis.

2.3.8 EFD52 (D17S26)

The sequence was isolated as a unique cosmid from a total human cosmid library screened with the HBV-4 oligonucleotide (GAGAGGGGTGTAGAG) (Nakamura *et al*, 1987a). The probe EFD52 was supplied as a purified insert, the 6.4 kb insert being released by Eco RI - Hind III double-digest. EFD52 has been mapped to chromosome 17 (Nakamura *et al*, 1988b).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SUBJECTS

This study was carried out on 100 consecutive disputed paternity cases tested at PLTI. There was no prior knowledge of the outcome of the standard testing, the only criteria for inclusion were:

1. that DNA had been successfully isolated; and
2. that the restriction endonuclease digestion, Southern transfer and hybridization steps had been carried out satisfactorily.

It was decided to use 100 cases as this study was the initial step in establishing a VNTR DNA typing service for routine medico-legal investigations. The extreme polymorphism of DNA typing systems, would require a much larger sample size for accurate evaluation.

Each case consisted of the biological mother, child and alleged father. The cases were referred by either a maintenance officer, lawyer, private pathologist or medical practitioner, and were handled by appointment only. The policy of the laboratory was that all parties in a given case be present simultaneously.

A strict identification protocol was observed:

1. The mother and putative father were required to

produce identity documents.

2. Polaroid photographs were taken, and signed by the mother and putative father.
3. The mother and putative father were required to identify each other.

Consent for the venesection was obtained; the mother gave consent to venesection of the child. Blood samples were drawn by a registered nurse, specially trained in infant phlebotomy.

The mother and putative father were required to certify that the blood samples were their own and that the samples were taken in each other's presence.

The various samples were dispatched to the appropriate laboratory and a chain-of-custody record maintained. Each laboratory was required to verify the labelling of the specimens and sign for receipt therof.

(See Appendix A for examples of forms which are required to be completed for a disputed paternity case at PLTI.)

3.2 METHODS

3.2.1 STANDARD TESTING

At PLTI, "Standard Testing" referred to those tests which were used routinely for all cases of disputed paternity which presented for investigation. The laboratory offers tests for a number of genetic marker systems: HLA typing, red cell blood groups, red cell enzyme and serum protein polymorphisms, and the methodology employed at PLTI has been published elsewhere (Du Toit *et al*, 1989; Du Toit, 1993). Table 3.1 lists those systems which were in use at the time of this study.

All cases of disputed paternity were tested for antigens of the HLA system in the first instance; if an exclusion of paternity was clearly demonstrated, no further tests were performed. If no exclusion could be shown using HLA testing, red cell blood group, red cell enzyme and serum protein testing were carried out.

In all cases where no exclusion of paternity was found, a paternity index (PI) and probability of paternity (W) were calculated (See 3.2.12 and 3.2.13).

TABLE 3.1

Genetic Marker Systems used at PLTI - Standard Testing

HLA LOCI	A, B, C
Red Cell Blood Groups	ABO, MNS, RH, KEL, FY
Red Cell Enzymes	ACP, ESD, PGM1, CAII, GLO
Serum Proteins	HP, DBP, BF, TF, C3

3.2.2 EXTRACTION OF DNA

Venous blood (5ml) taken into vacutainer tubes containing EDTA as anticoagulant, was used for the isolation of high molecular weight DNA. The white blood cell (WBC) count was determined immediately after collection and the appropriate volume of whole blood, to contain the required number of WBC, was calculated as follows. From a standard curve plotting WBC count versus DNA concentration, it was estimated that $6x10^6$ WBC yielded 10ug DNA, which was sufficient for a single VNTR DNA analysis to be performed in duplicate. The sample was aliquoted and stored at -70°C for up to 6 months.

The following method was used to extract DNA from frozen whole blood (Miller *et al*, 1988):

1. An aliquot of frozen whole blood was thawed at room temperature for twenty minutes.
2. The sample was centrifuged at 2 500g for 15 minutes at 4°C to pellet the white blood cells.
3. The cell pellet was washed 3 to 4 times with Red Cell Lysis Buffer [RCLB] (See appendix B) until clear of red cell contamination.
4. To the pellet the following were added:
   450µl White Cell Lysis Buffer [WCLB] (See appendix B)
   50µl 10% Sodium Dodecyl Sulphate [SDS]
   15µl Proteinase K (Sigma P-0390), (20mg/ml)

5. The samples were incubated at 56°C for 2 hours, with mixing by inversion every 30 minutes, until the white cell pellet was digested.
6. Saturated NaCl, 225µl, was added, and the sample was inverted vigorously by hand, followed by centrifugation at 2 500g for 15 minutes.
7. The supernatant was transferred to a labelled Eppendorf tube (1,5ml) and two volumes of ice cold absolute ethanol was added to precipitate the DNA. The samples were gently mixed until the white DNA precipitate was observed.
8. After centrifugation (2 500g, 15 minutes), the supernatant was carefully decanted and the DNA precipitate was washed three times with ice cold 70% ethanol (1ml per wash).
9. After the final wash, the samples were dried briefly under vacuum in a Speed Vac Concentrator (Savant, Farmingdale, New York, USA) and redissolved in 100µl sterile distilled water at 4°C overnight.

3.2.3 AGAROSE GEL ELECTROPHORESIS OF EXTRACTED DNA

The integrity of DNA was determined by agarose gel electrophoresis in the presence of ethidium bromide. Ethidium bromide has been found to act by intercalating between stacked DNA bases. The UV irradiation absorbed by DNA and transmitted to the dye, has been shown to be emitted as orange-red fluorescence.

1. Using a Hoefer Mini-sub apparatus (Hoefer Scientific Instruments, San Francisco) a 1% agarose gel was prepared by boiling 0.3gm agarose (Sigma Type II) in 30ml 1xTris, EDTA, Acetate Buffer [TEA] (See appendix B), to which was added ethidium bromide (final concentration 0.5μg/ml).
2. 1ul aliquots of sample were examined using 200ng of intact lambda DNA as a reference.
3. The DNA was electrophoresed at 4V/cm for 45 minutes. After completion of electrophoresis the gel (Figure 3.1) was inspected on a UV Transilluminator (UV Products, Gabriel, California, USA).
4. Intact DNA was observed as a single band of high molecular weight.
5. Degraded DNA samples, characterised by a smear on the gel, were discarded.

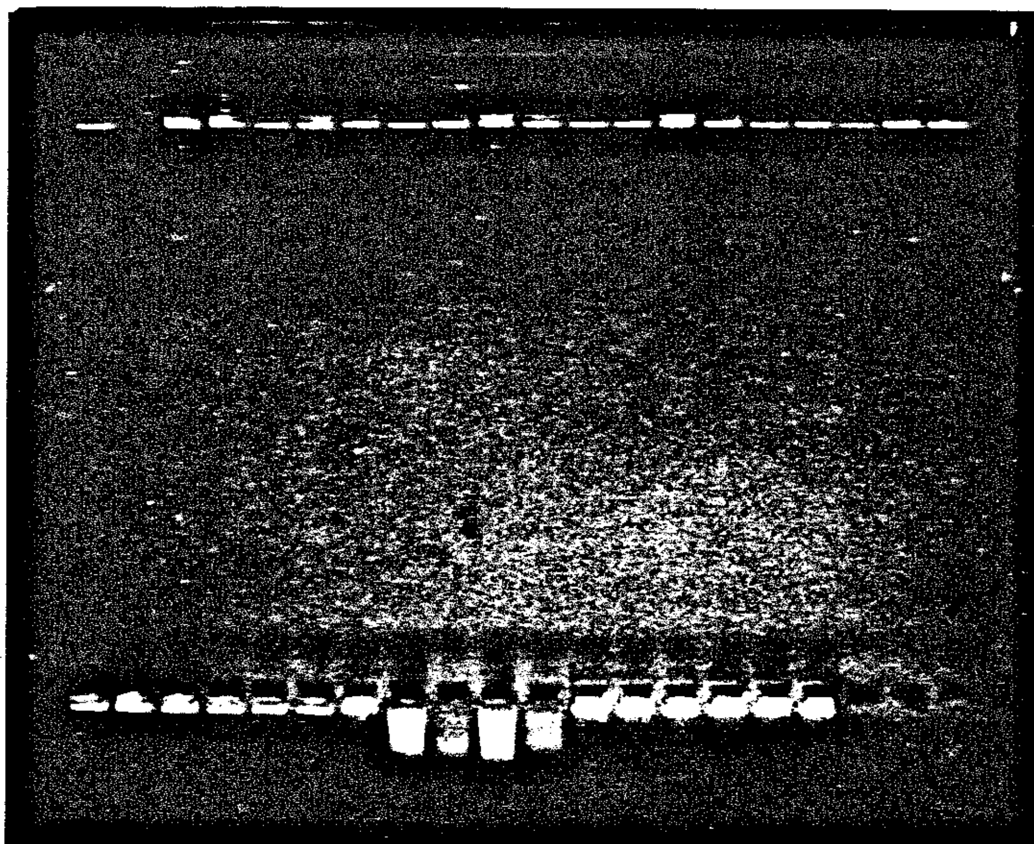


Figure 3.1

Agarose gel electrophoresis (in the presence of ethidium bromide) of DNA prior to restriction enzyme digestion showing from left: top row lane 1 - intact lambda DNA (control), bottom row lanes 8-11 - degraded DNA, other lanes - intact DNA suitable for analysis.

3.2.4 PLASMID PREPARARATION

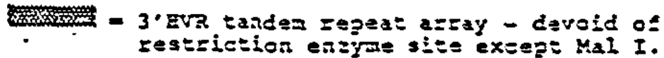
The probes 3′ alpha globin, Mucin, HaRas and MR24/1, were originally available from as part of a DNA profiling kit (DNA profiling kit, RPN 90, Amersham, UK). This kit was discontinued during the course of this study and, since only one of the probes (MR24/1) had been found to have an unacceptably high measurement error with the restriction enzyme chosen, Pvu II, it was decided to obtain DNA for amplification from the Human Genome Mapping Project (HGMP Resource Centre, United Kingdom). DNA was supplied as follows:

Plasmid	Vector
pα3′HVR.64	pSP64
pEJ6.6	pBR322
pMUC10	pUC8

Plasmid maps are shown in Figures 3.2, 3.3 and 3.4 respectively. The purified plasmid DNA obtained was transformed into competent *E.Coli* strain HB101 cells.

pα3'HVR.64

4.0kb Hinf I fragment from pSEA.I filled in and blunt-end ligated into Hinc II site of pSP64.



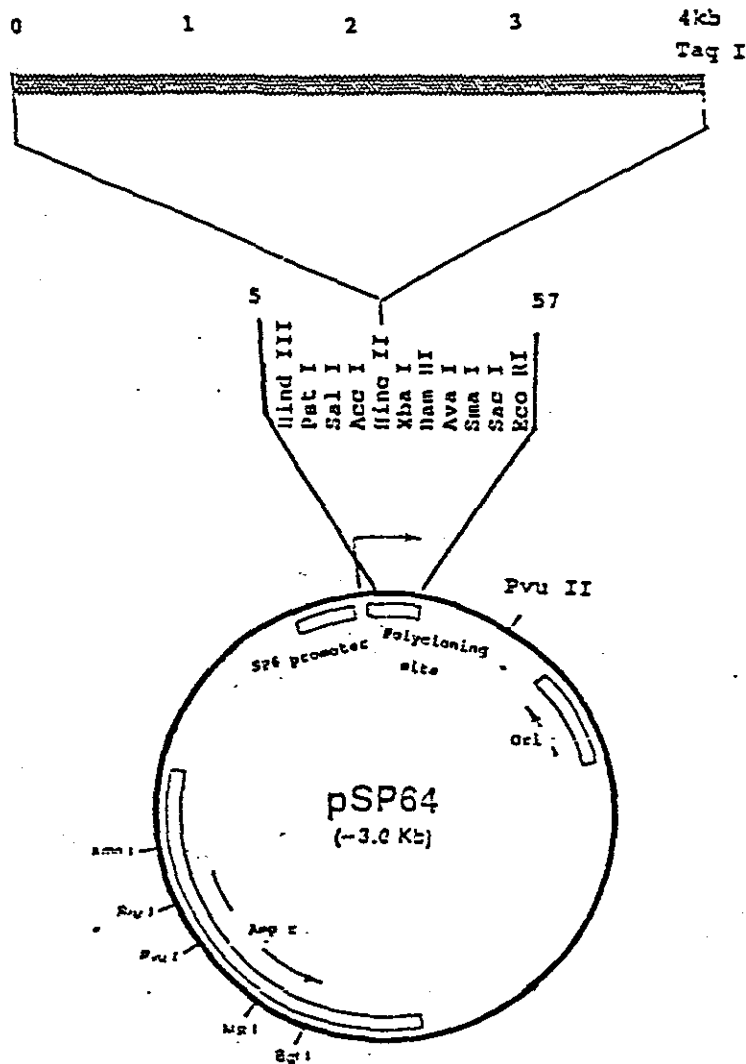


Figure 3.2

Map supplied by the Human Genome Mapping Project for the plasmid pα3'HVR.64 (3'α-globin HVR probe). The vector is SP64.

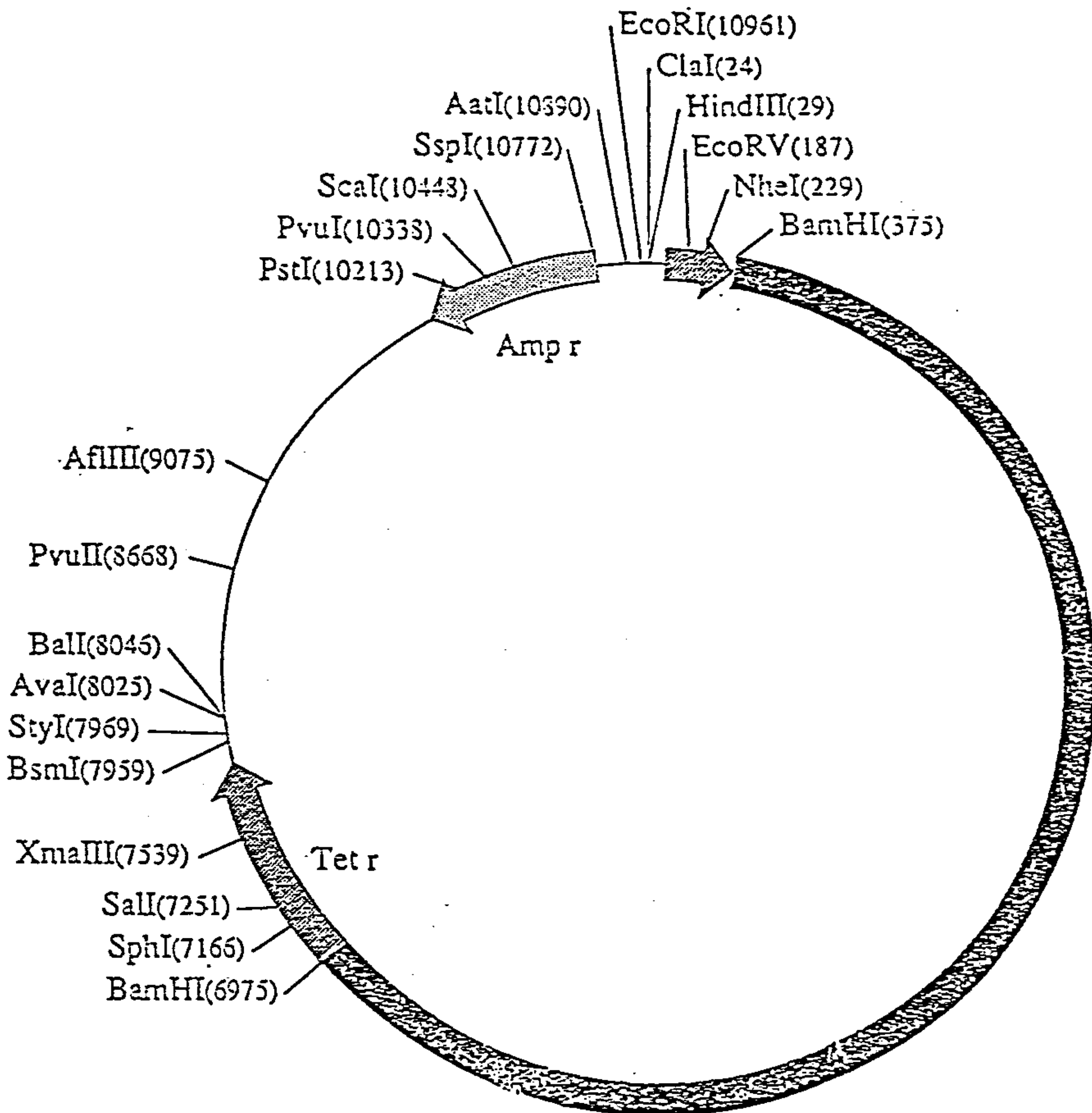


Figure 3.3

Map supplied by the Human Genome Mapping Project for the plasmid pEJ6.6 (Ha Ras HVR probe). The vector is pBR322.

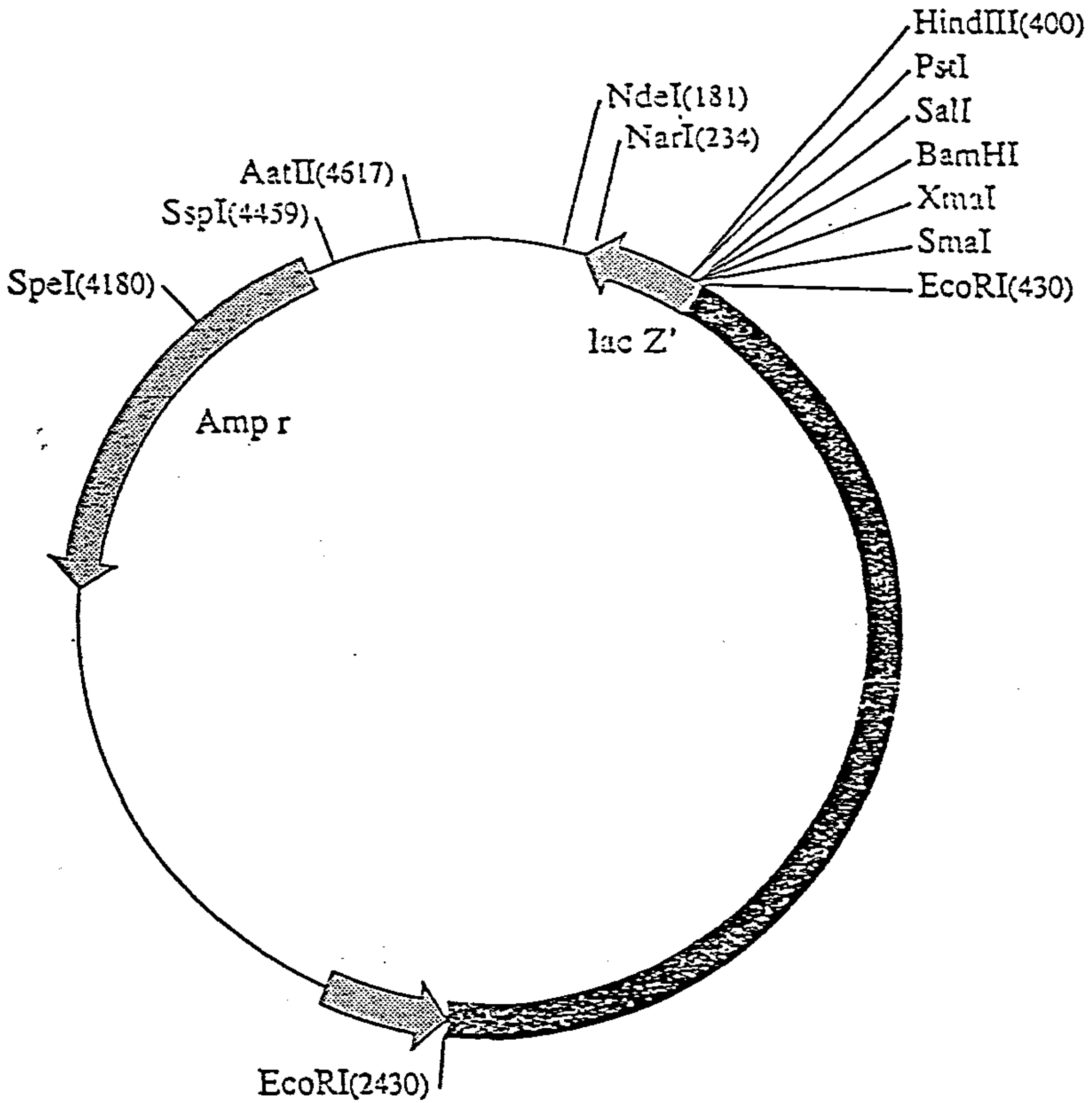


Figure 3.4

Map supplied by the Human Genome Mapping Project for the plasmid pMUC10 (Mucin HVR probe). The vector is pUC8.

3.2.4.1 TRANSFORMATION OF PLASMID DNA

The following technique was used for the transformation of plasmid DNA (Sambrook, 1989).

1. Bacterial cells were grown to the early log phase (OD 600=0.3-0.6) in Luria Broth [LB] (See appendix B) medium, then pelleted by centrifugation at 1 000g for 10minutes at 4°C.
2. The cells were resuspended in 1/10th volume of transformation and storage buffer [TSB] (See appendix B) at 4°C, and incubated on ice for approximately 10 minutes.
3. An aliquot of cells, 100μl, was pipetted into cold polypropylene tubes and mixed with 100pg of plasmid DNA.
4. The cells were left on ice for 5-30 minutes.
5. TSB with 20mM glucose (0.9ml) was added and the cells incubated at 37°C with shaking for 60minutes.
6. The cells were then plated onto antibiotic-containing agar plates for selection of transformants.
7. A plate was prepared without antibiotic as a control.

3.2.4.2 PLASMID MINIPREPS

This procedure was performed to select a suitable colony for large-scale amplification.

1. Culture plates were prepared using LB and 15% Bacto-agar with the addition of appropriate antibiotic.
2. The transformation mix, 50μl, was streaked onto the agar plates and cultured overnight at 37°C.
3. Individual colonies were picked and used to inoculate starter cultures (1 colony/10ml LB) which were incubated overnight in a rotary incubator.

The following technique, a modified procedure from Sambrook, was used to identify cultures suitable for further amplification (Sambrook *et al*, 1989).

1. A 1.5ml aliqout of the starter culture was transferred to a 1.5ml Eppendorf tube and centrifuged at 1 000g, while the remaining culture was stored at 4°C.
2. The culture medium was aspirated from the Eppendorf tube, leaving the bacterial pellet as dry as possible.

3. The pellet was resuspended in 100μl of an ice-cold solution of 50mM glucose, 10mM EDTA and 25mM Tris HCl (pH 8.0) containing lysozyme (8mg/ml) without vortexing as this action causes shearing of the DNA and inactivation of the large lysozyme molecules.
4. The tube was allowed to stand for 5 minutes at room temperature.
5. Fifty μl of an ice-cold solution containing 0.2M NaOH and 1% SDS was added.
6. The top of the tube was closed and the contents mixed by inverting the tube rapidly two or three times. The tube was allowed to stand on ice for 5 minutes.
7. Then 150μl of an ice-cold solution of potassium acetate was added. (Potassium acetate was prepared as follows: to 60ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 28.5ml of H_2O were added, resulting in a solution which was 3M with respect to potassium and 5M with respect to acetate.)
8. The cap of the tube was closed, vortexed in an inverted position for approximately 10sec. and left to stand on ice for 5 minutes.
9. After centrifugation at 2 500g for 5 minutes, the supernatant was transferred to a fresh Eppendorf tube.
10. An equal volume (approximately 450μl) of phenol/chloroform was added, the sample mixed by vortexing, and centrifuged.

11. The supernatant was transferred to a clean tube and two volumes of absolute ethanol were added, mixed by vortexing and allowed to stand at room temperature for 2 minutes.
12. The tube was centrifuged at 2 500g for 5 minutes to pellet the DNA, and the supernatant carefully decanted and discarded
13. The tube was inverted on a paper towel and excess fluid allowed to drain away.
14. The precipitated DNA was washed twice in 70% ethanol.
15. The pellet was dried briefly in a Speed-Vac, to remove any residual ethanol.
16. The sample was dissolved in 50μl TE containing DNAase-free pancreatic RNAase (20mg/ml), and allowed to dissolve overnight at 4°C.
17. An aliquot was then used for digestion with the appropriate restriction enzyme.
18. The fragments were analysed by gel electrophoresis to confirm the presence of the desired insert, and the culture most suitable for further amplification was selected for large scale plasmid amplification.

3.2.4.3 LARGE SCALE PLASMID AMPLIFICATION

Following selection of a suitable colony a large-scale amplification was set up.

1. The selected starter culture was used to inoculate 250ml LB culture flasks, with the addition of appropriate antibiotic.
2. The flasks were incubated overnight at 37°C in a rotary incubator.
3. The bacterial cells were harvested by centrifugation at 1 500g for 30 minutes at 4°C.
4. The resultant pellet was resuspended in 7ml 25% sucrose, 50mM Tris HCl, pH 8.0, with the addition of 100μl RNAse A to a final concentration of 100μg/ml.
5. Freshly prepared lysozyme (1.6ml of a 25mg/ml solution) was added and the solution gently mixed on ice for 5 minutes.
6. To this 1.3ml lytic mix (2% Triton X-100, 6mM EDTA, and 50mM Tris HCl, pH 8.0) was added and incubation on ice continued for a further 20 minutes. During this period the cells lysed to form a viscous mass.
7. The bacterial cell debris, carrying with it bacterial DNA, was pelleted by centrifugation at 1 500g for 30 minutes at 4°C.
8. The clear supernatant was decanted and further purified by ion exchange chromatography using the Qiagen plasmid purification kit (Diagen).

9. The DNA was precipitated from the supernatant with 0.7 volumes of isopropanol at -20°C overnight.
10. The plasmid DNA was recovered by centrifugation at 4°C at 1 500g for 10 minutes, washed twice in 70% ethanol, and dried under vacuum.
11. The resultant pellet was dissolved in 0.5-2ml Tris EDTA [TE] (See appendix B).
12. The plasmid DNA was then either linearised, or the insert excised from the vector using the recommended restriction enzyme.

3.2.4.4 ISOLATION OF INSERT

The following technique was used for the recovery of the insert from the vector.

1. The plasmid DNA was digested with the appropriate restriction enzyme for 2 hours.
2. The digest was monitored after 1 hour by gel electrophoresis, in the presence of ethidium bromide, of a 10μl sample (1% Agarose in 1xTEA, 100V, 20minutes). An aliquot of undigested DNA, and 200ng of a Hind III digest of lamda DNA were included as a control.
3. When digestion was considered complete, the reaction was stopped by the addition of 5μl 0.04M EDTA.
4. The presence of the insert was confirmed by electrophoresis of digested plasmid DNA, along with

undigested plasmid DNA and an electrophoresis marker (lamda DNA previously digested with Hind III) as a reference.

5. Following confirmation of successful digestion a 1% agarose gel was prepared, using an adapted well former which, in addition to forming the normal wells required for the electrophoresis marker, cast a single well 5-7cm long, which was used to load the total digest.
6. Electrophoresis was carried out until the insert could be clearly visualized as separated from the vector and excision from the gel was possible with minimum contamination by vector.
7. The gel was photographed and the insert band carefully excised using a scalpel blade (Figure 3.5).
8. Two circles of Whatman 3MM chromatography paper were cut to fit the diameter of the barrel of a 5ml syringe and carefully tamped into place using the plunger of the syringe.
9. The agarose containing the insert was placed in the syringe which was then left overnight at -70°C. Freezing of the agarose resulted in the destruction of the gel matrix.
10. After thawing at room temperature, the liquid containing the probe DNA was gently extruded through the filter paper.

11. The concentration of the DNA was determined without dilution using a spectrophotometer (Beckman DU65, Beckman Instruments, Fullarton, California, USA). It was convenient for probes to be stored at a concentration of 5-10ng/μl.

The insert to be used as a probe was further tested in a hybridization step using a control membrane. This membrane was prepared using Pvu II digests of 10 random individuals for which the fragment sizes of the probe in question had previously been established.

No problems were encountered during the amplification and hybridization control for the probes 3'α-globin and Mucin. For the probe HaRas, although amplification was unproblematic, in the hybridization step an additional non-polymorphic band was observed in every sample. This was eventually eliminated by adjusting the stringency of the final washing step.

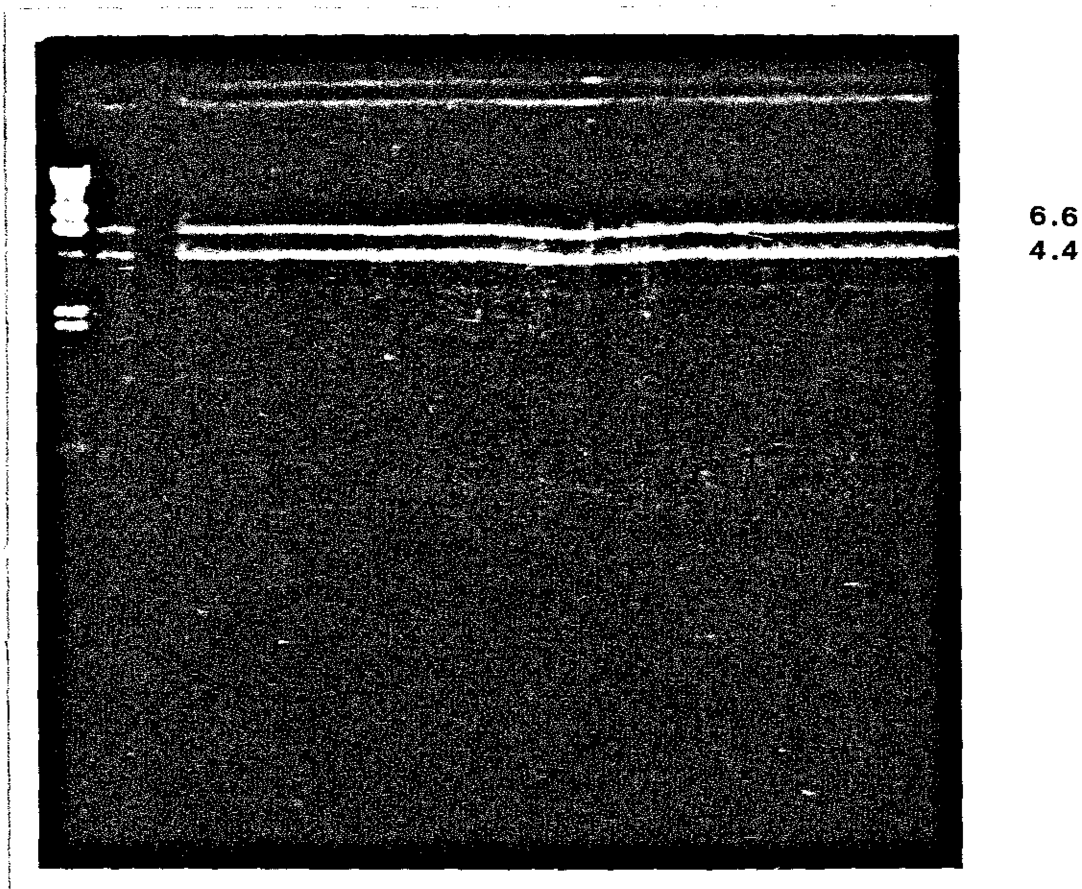


Figure 3.5

Agarose gel electrophoresis of amplified probe DNA, pEJ6.6, showing the vector (4.4kb fragment) and insert (6.6kb fragment) after release of the insert using the restriction enzyme Bam HI. From left: lane 1, electrophoresis marker (Hind III digest of lambda DNA); lane 2, a single well loaded with restricted, amplified product; lane 3, a large well containing the bulk product for excision of the insert.

3.2.5 RESTRICTION ENDONUCLEASE DIGESTION

Restriction enzymes cleave DNA at specific sites. In selecting a restriction enzyme for VNTR DNA analysis, it was important to choose an enzyme with recognition sites which flanked the repeat units, since cleavage within the repeat sequence would result in the production of small, unresolvable fragments. Furthermore for the chosen range of single-locus VNTR DNA probes, the selected restriction enzyme should produce fragments neither too large (>10kb) nor too small (<0.5kb) for routine analysis. Following a series of trials, (See 4.2 and 5.2), the restriction enzyme Pvu II was selected for routine VNTR DNA typing.

The digestion of DNA was performed as directed by the manufacturer (Boehringer Mannheim, Germany) at a concentration of 5 units restriction enzyme/μg DNA, with the addition of spermidine to a final concentration of 2mM. The restriction enzyme was added in two stages and samples were incubated at 37°C for 16 hours.

3.2.6 ELECTROPHORESIS

The DNA fragments generated by restriction enzyme cleavage were separated by sub-marine agarose gel electrophoresis.

1. A 0.8% agarose gel containing ethidium bromide was prepared in 1xTEA and cast in a Hoefer Simple Sub or similar apparatus. A 30 well gel was prepared which was sufficient for the analysis of 5-6 families, and allowed for the inclusion of an electrophoresis marker, analytical markers as well as the allelic control.
2. A Hind III digest of lamda DNA (4μg/lane) was used as an electrophoresis marker.
3. The analytical marker (5ng/lane) was a mixture of restriction enzyme digests of lamda and ϕX174 bacteriophage DNAs supplied by Promega (Promega Cat No DG1931).
4. The allelic control was a Pvu II digest of the erythroleukaemic K562 cell line.
5. The sample DNAs were divided to allow for loading in duplicate, and 1x loading buffer [LB] (See appendix B) added.

6. The electrophoresis tanks were prepared with fresh 1xTEA buffer which was recirculated to prevent formation of a ion gradient due to the length of the run.
7. Immediately prior to loading, the samples were heated at 65°C for 5 minutes.
8. The gels were run at 2V/cm for approximately 16 hours, until the 2.0kb fragment of the electrophoresis marker had migrated 140mm from the origin. Under these conditions the fragments generated by the selected restriction enzyme, and detected by the range of single locus VNTR DNA probes used, fell within the linear range of the molecular weight marker.
9. At the completion of electrophoresis, the gel was photographed under UV transillumination and excess gel trimmed away (Figure 3.6).

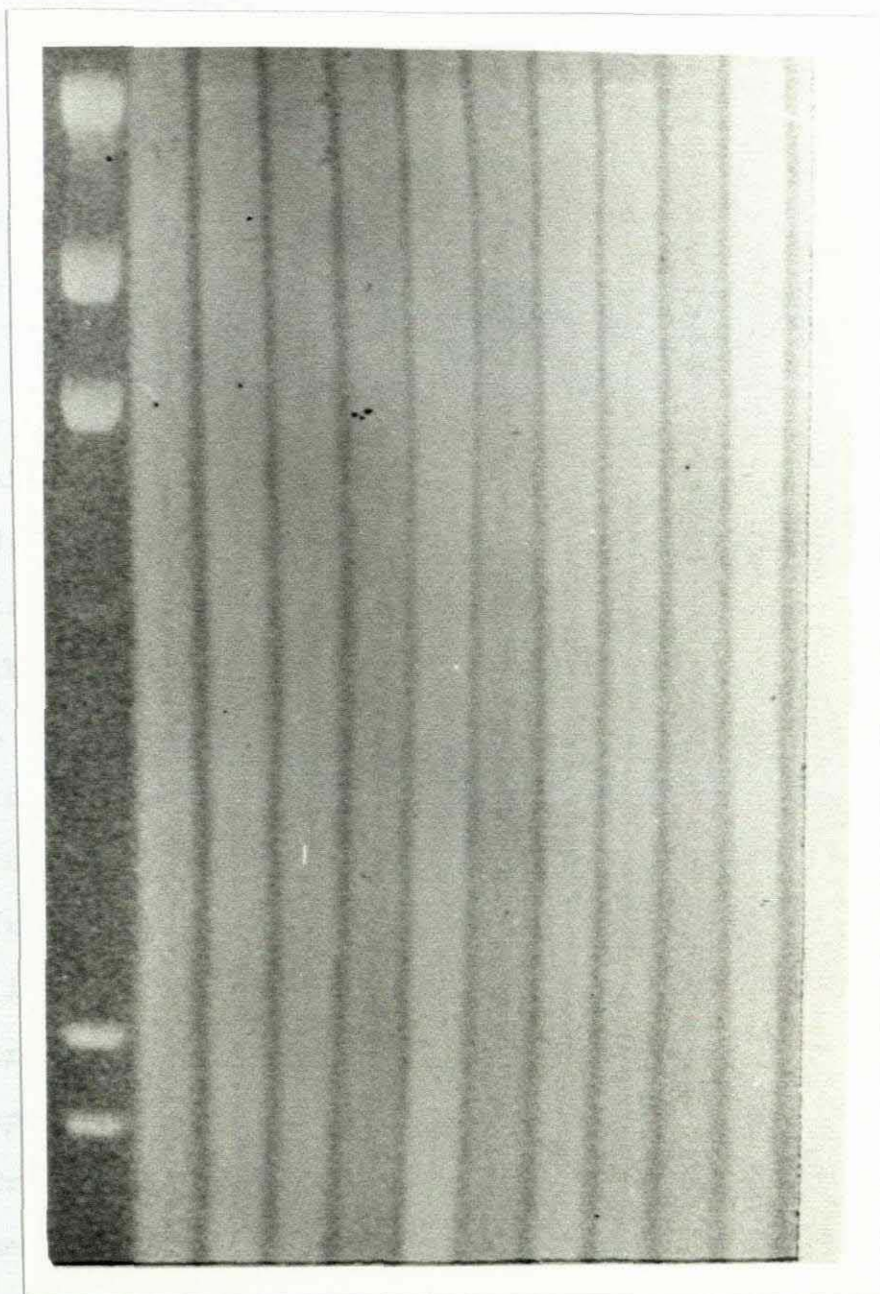


Figure 3.6

Ethidium bromide staining of high molecular weight DNA, digested with a restriction enzyme, subjected to agarose gel electrophoresis, and viewed under UV light.

3.2.7 SOUTHERN TRANSFER

Transfer of the separated DNA fragments was carried out essentially as described by Southern (Southern, 1975).

1. After electrophoresis, the gel was depurinated in 0.25M HCl for 10 minutes, to allow for more efficient transfer of larger fragments.
2. The gel was equilibrated in 0.4M NaOH for 30 minutes.
3. A nylon membrane, Hybond N^+ (Amersham), was used and the transfer buffer was 0.4M NaOH. This eliminated the need for post-transfer fixing of the DNA fragments by UV irradiation, since DNA was covalently linked to the Hybond N^+ membrane under alkaline transfer conditions. A diagram of the transfer apparatus is shown in Figure 3.7.
4. Transfer was carried out overnight, after which the membrane was rinsed in 2xSSPE (See appendix B), sealed in a plastic filing sleeve, and stored at 4°C until required for hybridization.

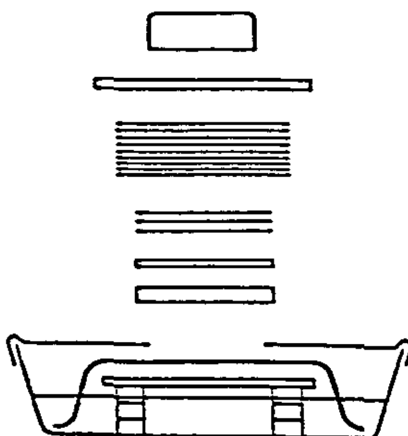


Figure 3.7

A typical Southern Blotting apparatus.

1. A pyrex dish is filled with blotting buffer (0.4M NaOH) to a depth of 3-4cm. A platform is made of 4 small glass bottles and a glass plate. The glass plate is covered with a wick (Whatman 3MM chromatography paper), the wick flooded with blotting buffer, and a pipette rolled over the surface to remove any trapped air bubbles.

2. The gel is placed on the platform, avoiding air bubbles. Strips of blank x-ray film are inserted around the edge of the gel, again avoiding air bubbles, to prevent blotting buffer being short-circuited past the membrane directly to the paper towels.

3. A sheet of membrane (Hybond N^+, Amersham), cut to the size of the gel is placed on the gel. The membrane is given an identifying mark, and the corner adjacent to lane No. 1 is cut off to assist orientation. Air bubbles beneath the membrane are again rolled out using a pipette.

4. Two sheets of 3MM paper (Whatman, chromatography) are wetted in transfer buffer, placed over the membrane, and rolled to remove air bubbles.

5. A stack of paper towel, approximately 10cm high is placed on top of the 3MM paper.

6. A glass plate is placed on top of the stack of paper towel, levelled using a spirit level, and a 1kg weight placed on top.

7. Blotting proceeds for 4-16 hours, after which the tranfer apparatus is carefully dismantled, and the membrane rinsed in 2xSSPE for 30 minutes. The membrane is stored at 4°C in a sealed plastic sleeve. It is not necessary to fix alkali blotted DNA.

3.2.8 PROBE LABELLING

The purified DNA inserts were biosynthetically labelled, using the random primer method, with ^{32}PdCTP, as developed by Feinberg and Vogelstein (Feinberg & Vogelstein, 1983). Labelling was carried out in the presence of random hexanucleotides and cloned DNA polymerase ("Klenow" fragment). The unincorporated nucleotides were eliminated by centrifugation through a Sephadex G-50 column. The percentage incorporation and the specific activity were routinely calculated. The normal percentage incorporation was 50-70% and the specific activity was usually $2x10^9$dpm/μg DNA.
A typical labelling reaction contained:

1. 25-50ng VNTR DNA probe,
2. 0.5ng analytical marker,
3. 1 x oligomer labelling buffer (OLB),
4. 10μl 32PdCTP (specific activity 3000Ci/mM)
5. 5-10U DNA polymerase, "Klenow", (Amersham)
6. Spermidine (Final concentration 2mM)

See Appendix B for details of preparation of OLB. Note: OLB was stored at -20°C, and was not adversely affected by repeated freezing and thawing.

3.2.9 HYBRIDIZATION

The pre-hybridization and hybridization steps were carried out using a Hybaid Incubator (Hybaid Ltd, Middlesex, UK) which allowed for the simultaneous testing of up to 10 membranes per hybridization tube. The membranes were prehybridized and hybridized in the following solution:

50% Formamide,
5xSSPE
7% Sodium Dodecyl Sulphate
6% Polyethylne Glycol 6000
Salmon sperm DNA 200μg/ml

(This solution was made up in bulk, dispensed into plastic screw-cap tubes, and stored at -20°C until required.)

The membranes were each wetted with approximately 5ml solution and layered on each other, finally rolled up using a 25ml pipette and carefully inserted into the hybridization tube. The membranes were pre-hybridized for 4-24 hours in 30ml hybridization solution at 42°C. The probe was denatured by boiling for 5 minutes, after which it was placed on ice for 5 minutes to prevent re-annealing of single DNA strands. The volume of the hybridization solution was reduced to 10ml and the probe added to the hybridization tube. It was not necessary to

remove the membranes from the tube for this step. Hybridization proceeded for 24 hours at 42°C. The membranes were then washed twice at room temperature in 2xSSC (See appendix B), 0.1% SDS for 15 minutes, followed by 2 stringency washes at 65°C during which the salt concentration of buffers was decreased.

For the probes 3'alpha globin HVR, Mucin HVR and HaRas HVR, the stringency washes were performed for 30 minutes using 0.5xSSC, 0.1%SDS.

Stringency washes for the probes YNH24, CMM101 and TBQ7 were carried out for 5, 15 and 20 minutes respectively, using 0.1xSSC, 0.1% SDS.

Throughout the washing procedure, the membranes were monitored using a Geiger counter, as a guide to the efficiency of the washes. Whenever the count was unacceptably high following the final stringency wash, this step was repeated. The membranes were then sealed in a plastic filing sleeve, or similar plastic cover, and excess moisture was carefully wiped from the plastic cover.

3.2.10 AUTORADIOGRAPHY

The membranes were autoradiographed using Fuji RX Medical X-ray film, between two intensifying screens. Autoradiography was carried out at -70°C. While it was possible for most films to be developed after overnight exposure, the approximate exposure time was judged by the strength of the Geiger counter signal, and it was occasionally necessary to expose films for up to 7 days. The films were developed manually, using Polycon A tropically stable developer, and fixed with Super Amfix, with added hardener. All films were washed for 20 minutes in running water before drying. If the exposure time was not adequate, the membrane and cassette were carefully dried, and re-exposed for a further period.

Examples of autoradiograph patterns are shown in Figures 3.8 and 3.9 illustrating both non-excluded and excluded cases of disputed paternity. Figure 3.10 gives an example of inverse homozygosity, which cannot be used as a basis for exclusion of paternity using RFLP analysis, as there may be very small fragments present in the child and putative father which could not be detected.

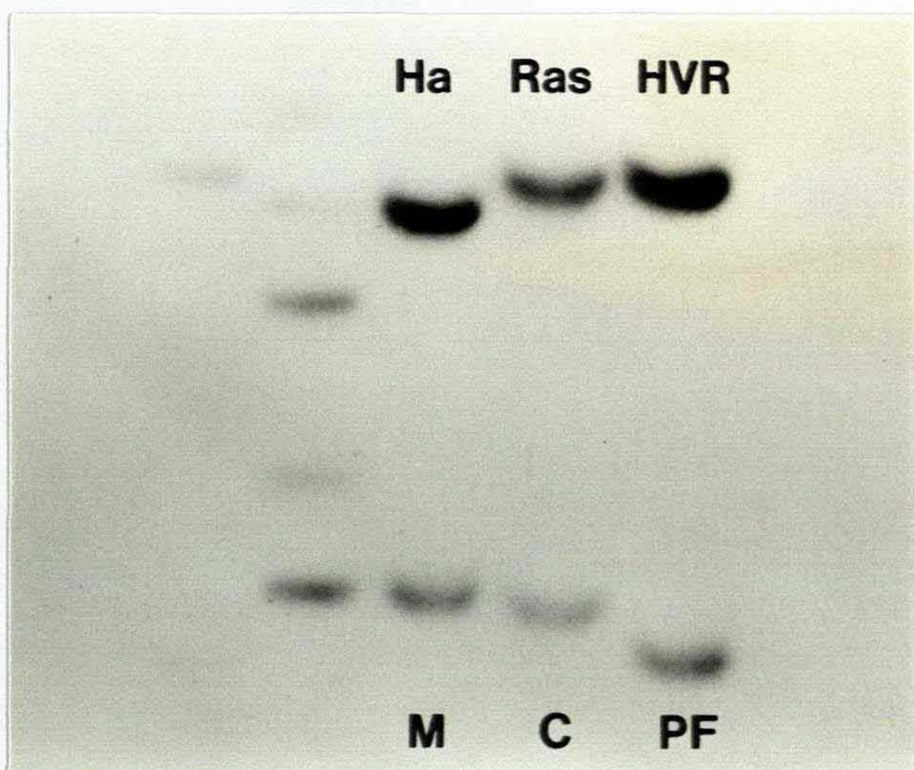


Figure 3.8

Disputed paternity case investigated using VNTR DNA analysis - paternity not excluded. The restriction enzyme Pvu II, and the probe Ha Ras HVR were used. The samples are: lane 1, allelic control (K562 cells); lane 2, analytical marker; lane 3, mother; lane 4, child; lane 5, putative father.

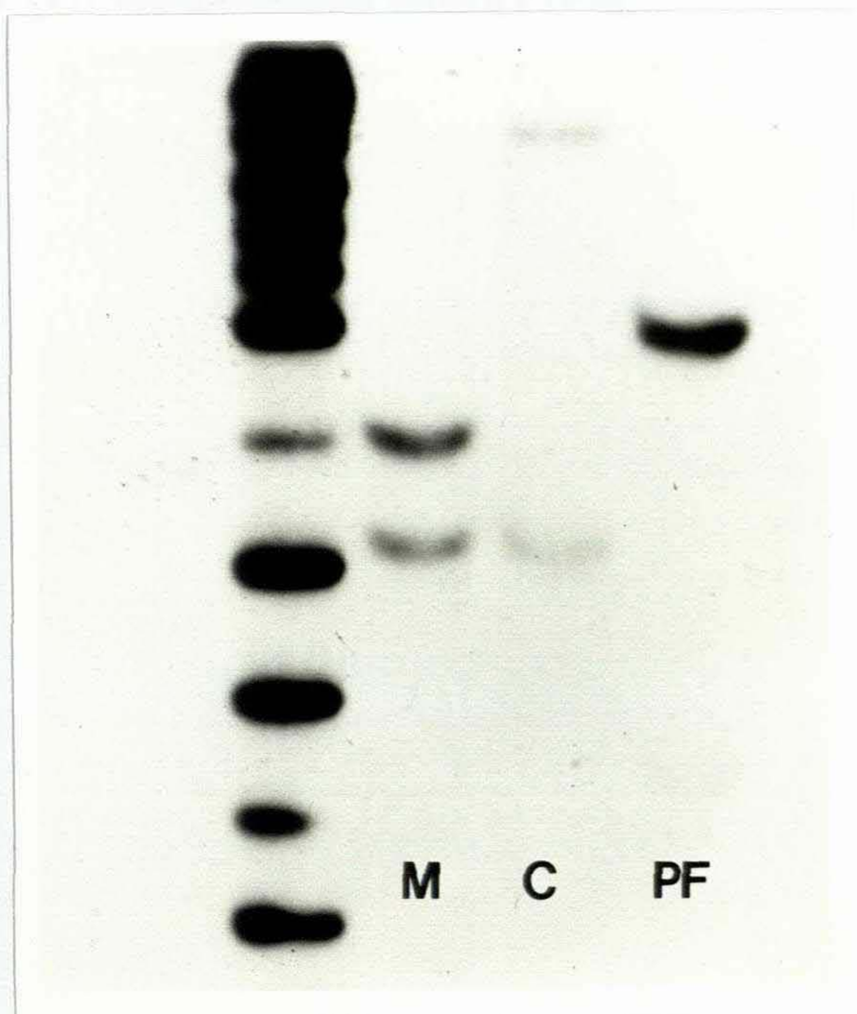


Figure 3.9

Disputed paternity case investigated using VNTR DNA analysis - paternity excluded. The restriction enzyme Pvu II, and the probe Mucin HVR were used. The samples are: lane 1, allelic control (K562 cells); lane 2, analytical marker; lane 3, mother; lane 4, child; lane 5, putative father.

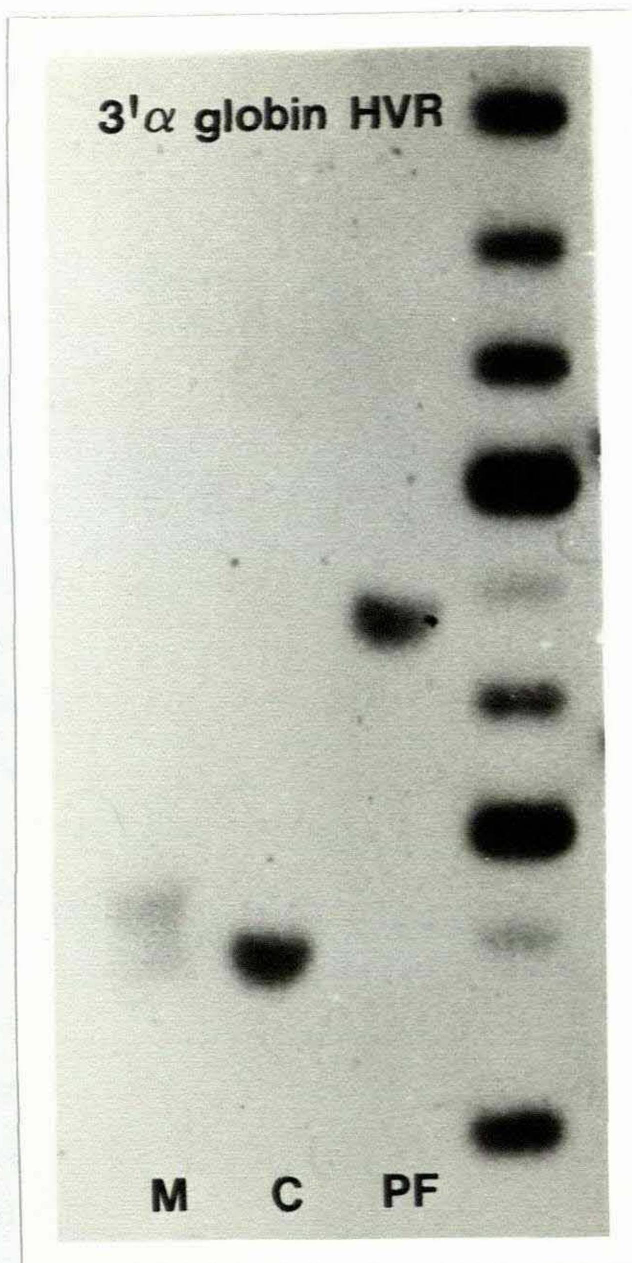


Figure 3.10

Disputed paternity case investigated using VNTR DNA analysis - and example of inverse homozygosity. The restriction enzyme Pvu II, and the probe 3'α-globin HVR were used. The samples are: lane 1, mother; lane 2, child; lane 3, putative father.

Exclusion of paternity is not possible on the basis of this result, as there may be undetected fragments present in both child and putative father.

3.2.11 FRAGMENT SIZING

Fragments were sized according to the principles established by Elder and Southern, (Elder & Southern, 1983). The molecular weight (MW) of double-stranded DNA fragments was shown to be inversely proportional to the mobility of the fragments through agarose gels, provided electrophoresis was performed at low voltage. Sizing of unknown fragments depended on electrophoresis of known standards which spanned the range of unknowns to be measured. A curve was constructed by plotting the sizes (kb) of known fragments versus the mobility of the same fragments. The size (kb) of the unknown fragments was read from this curve. Measurement was initially performed manually. The development of a computer assisted video-densitometer to interpret autoradiograph patterns eliminated most of the manual steps in the sizing of fragments and generation of the database.

3.2.12 CALCULATION OF THE PATERNITY INDEX

The paternity index (PI) was proposed as a means of expressing the genetic odds in favour of paternity, given the phenotypic combination of Mother-Child-Alleged Father (Gurtler, 1956). In calculating the PI, we are testing two alternate hypotheses: first, that the alleged father is the biological father, and the second that the alleged father is not the father. In other words, the likelihood of the alleged father versus a random man to be the father of the child.

$$PI = X/Y$$

Where X represents the likelihood that the accused man is the biological father and Y represents the likelihood that a random man, of the same ethnic background of the accused man, is the biological father. It is expressed as a numerical value. At the time of Gurtler's publication, a ratio of at least 20:1 (corresponding to a W of 95.24) was considered to afford `significant guidance'. Currently a value of 400:1 (corresponding to a W of 99.75) would indicate that paternity was `practically proven'.

3.2.13 CALCULATION OF THE PROBABILITY OF PATERNITY

Based on Bayes' Theorum, Essen-Moller published a method of calculating the probability of paternity, or Wahrscheinlikheit (W). It is the posterior probability, based on calculated allele frequencies and includes a prior probability of 0.5. It is the probability, after genetic testing, that the Alleged or Putative Father is the Biological Father. The Hummel modification of the Essen-Moller Equation is most often used (Hummel, 1984):

$$W = X/(X + Y)$$

Where X is the probability of the alleged father being the biological father, and Y is the probability of a random man, of the same ethnic background as the alleged father, being the biological father.

3.2.14 ESTIMATION OF THE POWER OF EXCLUSION

The power of exclusion (PEX) of a genetic marker system refers to the ability of that system, used alone, to exclude a man who has been falsely accused of paternity. Calculation of the power of exclusion is dependent on the actual phenotypes of the Mother and Child, and the ethnic backgrounds of the Mother and Alleged Father.

The PEX may be calculated when satisfactory gene frequency tables are available. When, however, the database is too small to be considered a representative sample, alternate methods can be used which compare well with the calculated figure. The first method involves simple observation, over a period of time, of the number of times the system has demonstrated an exclusion of paternity.

The second method involves manipulation of the data to construct false trios. In this method, only the cases where no exclusion of paternity could be demonstrated were used. The putative father of Case No 1 is allocated to Case No 2 and so on and the final putative father is allocated to Case No1. All the putative fathers which were previously not excluded will now be allocated to incorrect mother/child pairs. The number of times the incorrectly assigned putative father is shown to be

excluded would be taken as a measure of the PEX of the genetic marker system.

Method 2 was used to assess the PEX of three of the single locus VNTR DNA probes used in this study: YNH24, CMM101 and TBQ7, as the gene frequency database for the local population was not adequate to permit a calculated PEX.

CHAPTER FOUR

4.0 RESULTS

4.1 INTER- AND INTRA-ASSAY PRECISION AND ACCURACY

Inter- and intra-assay precision were determined by repetitive analysis of volunteer DNA using eight commercially available VNTR DNA probes. In addition, the accuracy of band sizing techniques was gauged by repetitive analysis of DNA from the HeLa cell line.

4.1.1 INTER-ASSAY PRECISION

Inter-assay precision was calculated using the results obtained from eight random DNA samples, digested with the restriction enzyme Pvu II, electrophoresed on ten gels, transferred to membranes, and hybridized sequentially with eight VNTR DNA probes. For each of the eight individuals two bands were observed, giving a total of 16 bands for analysis.

The inter-gel precision was reported as a coefficient of variation ($CV = S \times 100/x$) calculated from the means and standard deviations for each of the sixteen bands. If the CV was >2%, the probe was rejected. Two probes, MR24/1 and EFD52, detected bands which were >12kb with CV's of 2.42 and 3.72% respectively. This was due to difficulty in accurately sizing large molecular weight fragments. For the other six

probes, CV's ranged from 0.33 to 1.07%. The mean fragment size values, obtained from 10 repeated gel runs for each probe, are shown in TABLE 4.1.

TABLE 4.1

Inter-assay precision

PROBE	MEAN ALLELE SIZE* (kb)	CV(%)
3'alpha	2.06	0.38
	5.06	1.02
Mucin	3.47	0.36
	5.92	0.92
HaRas	2.73	0.36
	4.18	0.93
MR24/1	3.65	0.57
	15.70	2.42
YNH24	2.85	0.33
	5.31	1.07
CMM101	3.93	0.45
	5.97	1.02
TBQ7	3.18	0.39
	5.76	1.01
EFD52	6.40	0.77
	12.70	3.72

* The mean allele size, in kilobase, is given for 16 bands (2 bands reported per individual) from 10 repeated gel runs for each probe.

4.1.2 INTRA-ASSAY PRECISION

Intra-assay precision was calculated from the results obtained from DNA isolated from 3 individuals, digested with Pvu II each loaded in twenty wells of an electrophoresis gel, interspersed with molecular weight size markers, followed by hybridization with the MUCIN HVR probe. This was repeated three times for 3 individuals for a total of 9 gel runs, resulting in a total of 60 readings per allele, since the three individuals selected were heterozygous.

The results of the intra-assay precision are illustrated in FIGURE 4.1. From the values shown in TABLE 4.2 it can be seen that there was minimal deviation of band migration across a single gel. The CV's ranged from 0.51 to 0.92% for 20 measurements per allele, and the CV's for 60 repeated measurements per allele were all <0.6%.

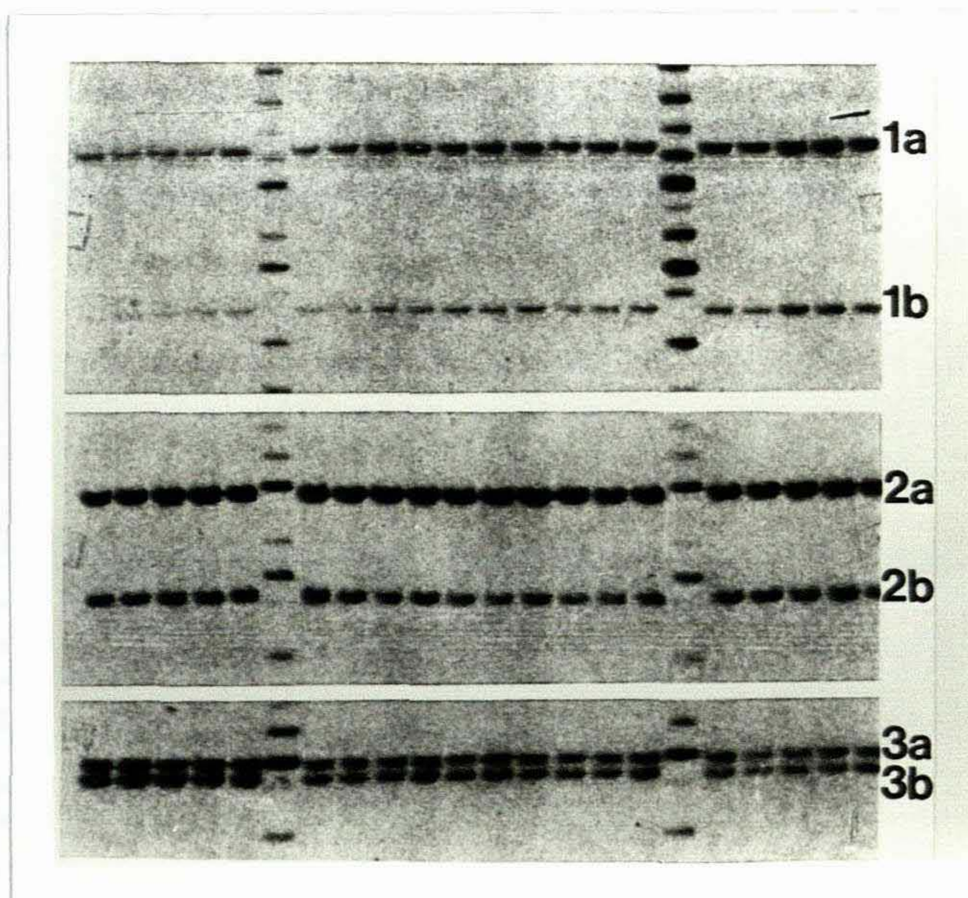


Figure 4.1

Intra-assay precision. Three volunteer DNA samples were digested with the restriction enzyme Pvu II, blotted to individual membranes and hybridized with the Mucin HVR probe.

4.1.3 INTER-ASSAY ACCURACY

A Pvu II digest of the HeLa cell line was used for the inter-gel accuracy assay. The bands detected with each of the probes and compared to the reported fragment sizes were between 1.1 and 7.9% of the reported fragment sizes. (Table 4.3)

TABLE 4.3

Inter-assay accuracy[§]

PROBE[*]	MEAN[#]	S.D.	REPORTED[◆]	DIFFERENCE(%)[△]
3'alpha	8.90	0.08	9.00	1.1
3'alpha	3.41	0.03	3.20	6.3
Mucin	5.81	0.03	6.00	3.2
Mucin	3.79	0.02	3.50	7.9
HaRas	2.94	0.02	2.80	4.9
MR24/1	9.63	0.08	10.00	3.8

§ Band sizes detected for a Pvu II digest of the HeLa cell line were compared to band sizes reported for four probes.

* Probes listed twice detected heterozygous loci.

\# The means were calculated from the average value for 10 repeated measurements.

◆ Band sizes reported by probe supplier (Amersham) to 100 base pairs.

△ Size differences reported as a percentage of the average band size.

4.1.4 CALCULATION OF OPERATOR ERROR

The readings obtained by two independent observers (160 repeated measurements) were used to calculate operator error, expressed as the standard deviation calculated from the difference in the two independent readings. Values ranged from 0.53% to 0.79% indicating close agreement between the two readers. The results of this exercise are shown in Table 4.4.

TABLE 4.4

Calculation of operator error

PROBE	S.D. FROM REPEATED MEASUREMENTS *
3'alpha	0.60
Mucin	0.60
HaRas	0.53
MR24/1	0.72
YNH24	0.61
CMM101	0.57
TBQ7	0.66
EFD52	0.79

* The standard deviation was calculated from 160 repeated measurements per probe and expressed as a percentage of the mean band size.

4.2 SELECTION OF RESTRICTION ENZYME/PROBE COMBINATION.

In order eo select the most suitable restriction enzyme and VNTR DNA probe combination, DNA samples from 8 unrelated individuals were digested using the five restriction enzymes: Eco RI, Hind III, Hae III, Pvu II and Taq I, followed by sequential hybridization with a selection of VNTR DNA probes. The object of this exercise was to select a single restriction enzyme, for hybridization with as many probes as possible, which would result in fragments in the 1 to 10kb range. The results obtained are shown in TABLE 4.5. The autoradiograph shown in FIGURE 4.2 clearly indicates that choice of restriction enzyme is crucial to success in VNTR DNA typing

Using the enzyme Taq I, the hybridization pattern was shown to be very similar to that obtained using Pvu II, however, the overall fragment sizes were smaller. This could possibly present problems with detection of smaller fragments, although sizing would be more accurate.

TABLE 4.5

Size range for Probe/Enzyme combinations[*]

PROBE	Eco RI	Hind III	Hae III	Pvu II
3'alpha	8.27-15.8	19.4-23.1	0.84 2.57	2.02-5.09
Mucin	26.6-30.1	7.16-9.69	NPD[#]	3.43-5.95
HaRas	25.6-29.5	<30.0	NPD[#]	1.80-4.80
MR24/1	10.1-15.8	7.52-11.9	1.71-5.76	3.89-15.7
YNH24	11.8-16.2	12.7-14.2	1.35-3.91	2.79-5.31
CMM101	4.53-9.41	3.52-8.42	1.54-4.86	3.92-6.04
TBQ7	12.2-14.8	9.41-12.5	1.62-4.72	3.21-6.31
EFD52	9.61-17.6	9.45-16.8	NPD[#]	6.31-12.6

* Size ranges were obtained for DNA from 8 unrelated individuals.

\# NPD = No polymorphism detected after 24 hour autoradiograph exposure

For the restriction enzyme Hae III and the probes Mucin, HaRas and EFD52, no hybridization appeared to have taken place. Two possible explanations exist for this phenomenon: the restriction enzyme used may be cutting within the repeat region, or the repeat region may be relatively short, with very little flanking DNA between the repeat region and the restriction site, and therefore contained within fragments <500base pairs, which would not be detected under standard conditions.

For the probes MR24/1 and EFD52, some of the fragment sizes generated were >10kb, and the CV for these probes had been shown to be unacceptably high (See 4.1.1). These probes were therefore not included in the final panel of probes.

As a result of this exercise, the restriction enzyme Pvu II was selected for use with the VNTR DNA probes 3'alpha globin, Mucin, HaRas, YNH24, CMM101 and TBQ7. The fragments generated by Pvu II for the probes selected could be accurately sized as they fell within the linear range of the molecular weight standards i.e. between 1.5 and 9kb. Furthermore, Pvu II was found to be suitable for use with DNA isolated using the salt extraction technique.

Due to problems related to commercial availability of the probes 3'alpha globin, Mucin and HaRas, the bulk of the study was carried out using YNH24, CMM101 and TBQ7.

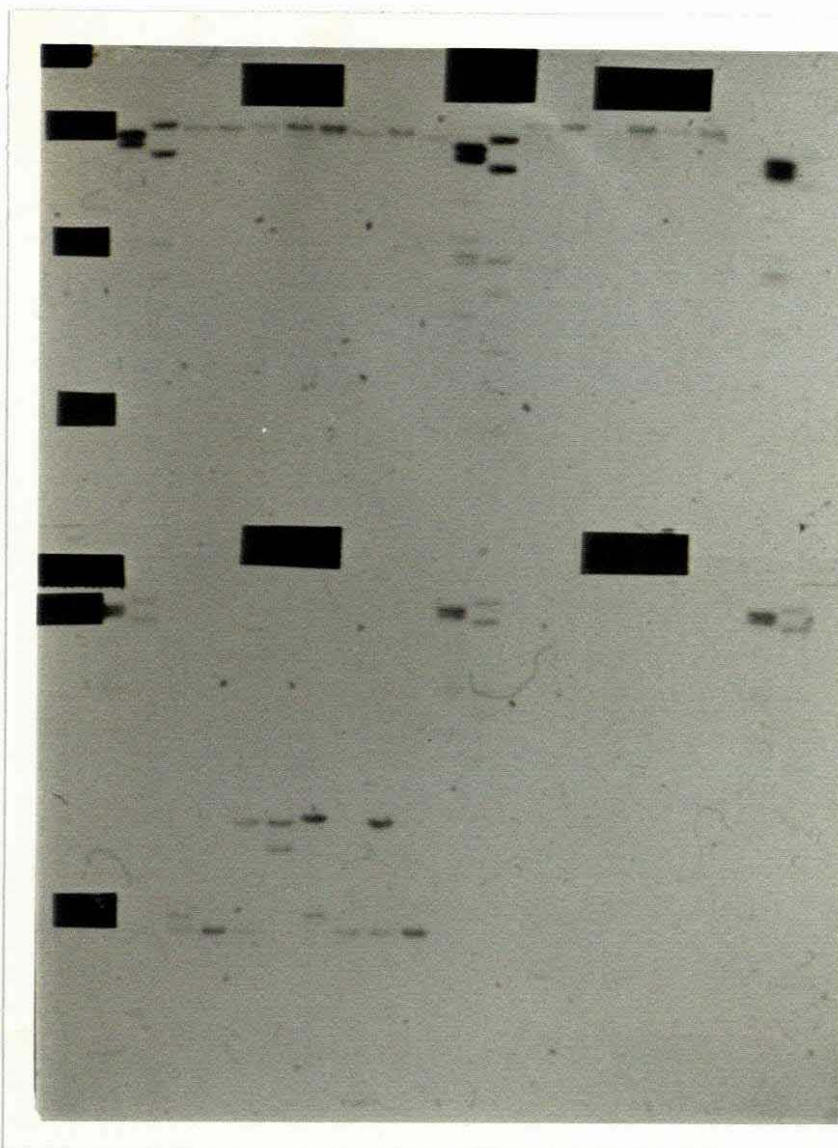


Figure 4.2

Autoradiograph patterns derived from 4 restriction enzymes - Eco RI, Hind III, Pvu II and Hae III - and hybridized with the probe Ha Ras.

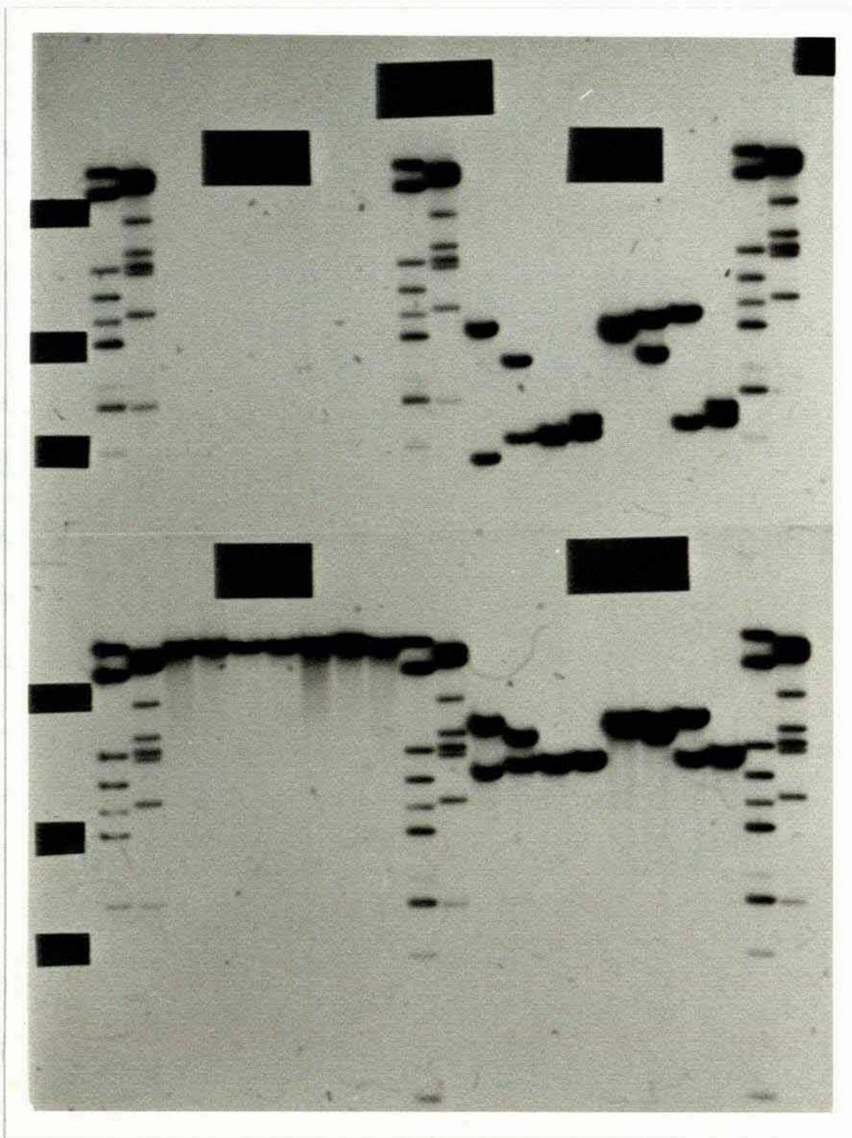


Figure 4.3

Autoradiograph patterns derived from 4 restriction enzymes - Hae III, Pvu II, Eco RI and Hind III - and hybridized with the Mucin HVR probe.

4.4 COMPARISON OF STANDARD AND VNTR DNA TESTING

This part of the study was carried out using the probes YNH24, CMM101 and TBQ7, which were available throughout the study: where additional information was required, one or more of the probes 3'alpha globin, Mucin or HaRas was used. The results obtained with standard testing were compared to those obtained for VNTR DNA typing. The laboratory had previously established that exclusion of paternity would be based on the finding of a single direct exclusion, i.e. a genetic marker present in the child which had not been inherited from either parent, or two indirect exclusions i.e. where neither of the genetic markers present in a putative father had been inherited by the child. However, using VNTR DNA testing, only direct exclusions were acceptable since indirect exclusions could be based on an technical difficulty in detecting very small fragments in a child or putative father.

The results obtained from comparing both standard and DNA testing are summarised in Tables 4.6, 4.7 and 4.8.

Table 4.6 lists those cases which were not excluded using standard tests, showing the probability of paternity (W) values. W values were routinely calculated for all cases where no exclusion could be demonstrated using the full range of standard tests.

TABLE 4.6

Paternity cases analysed using standard and VNTR DNA polymorphisms. (These cases were NOT EXCLUDED using Standard Tests)

PROBABILITY of PATERNITY (W) STANDARD TESTS*	RESULTS OF VNTR DNA TESTING	
	NOT EXCLUDED#	EXCLUDED§
>99.00	51	0
95.00 - 98.99	13	1
90.00 - 94.99	1	0
<90.00	0	1

* W values obtained from the results of standard testing using 18 genetic marker systems.

Number of cases not excluded using both standard and VNTR DNA testing.

§ Number of cases not excluded using standard testing but excluded using VNTR DNA testing.

In two cases, one with a W of 98.98 and one with a W of 86.82, exclusions were shown using only VNTR DNA testing (Schlaphoff, in preparation). The results of these two cases are summarised in Table 4.7.

TABLE 4.7

Cases excluded by only VNTR DNA testing

	PI	W	Probe# TBQ7	CMM101	YNH24
Case No. 1	6.58	86.82	Excluded	Excluded	Excluded
Case No. 2	97.13	98.98	Excluded	Excluded	Not Excluded

* W value obtained using the results of standard testing with 18 genetic marker systems.

\# Results obtained using three VNTR DNA probes.

Table 4.8 lists those cases which were excluded by both standard and VNTR DNA testing. All the exclusions observed using standard tests were confirmed using VNTR DNA testing. In most instances exclusions were observed using more than two VNTR DNA probes, and in only one cases could an exclusion be demonstrated with only one VNTR DNA probe. The case numbers used in the table are not the actual numbers assigned by PLTI to the individual cases. If only one exclusion was observed using the three VNTR DNA probes YNH24, TBQ7 and CMM101, testing continued until a second exclusion of paternity was found.

TABLE 4.8

Paternity cases analysed using standard and VNTR DNA polymorphisms. (These cases were EXCLUDED using Standard Tests)

CASE No.	EXCLUSIONS OBSERVED USING VNTR DNA PROBES:			
	YNH24*	CMM101#	TBQ7§	OTHER♦
1	+	-	-	MUCIN
2	+	-	-	MUCIN
3	+	+	+	
4	+	+	+	
5	-	+	+	
6	+	+	+	
7	+	+	+	
8	+	-	+	
9	+	+	-	
10	+	-	+	
11	+	-	+	
12	-	+	+	
13	+	+	+	
14	-	+	+	
15	-	+	+	
16	+	+	+	
17	-	+	-	
18	+	-	+	
19	+	+	+	
20	+	+	+	
21	-	+	+	
22	+	-	-	MUCIN
23	+	+	+	
24	+	+	+	
25	+	+	+	
26	+	+	+	
27	+	+	+	
28	-	+	-	3'ALPHA
29	+	+	-	
30	+	+	+	
31	-	+	+	
32	+	+	+	
33	+	-	-	3'ALPHA

+ = Exclusion
- = No Exclusion

* Results obtained using the probe YNH24

Results obtained using the probe TBQ7

§ Results obtained using the probe CMM101

♦ Additional exclusions demonstrated using other VNTR DNA probes.

In two cases, although standard tests indicated that paternity could be excluded, the exclusions found were not satisfactory. In Case No.1, an exclusion was based on a discrepancy in the Duffy (Fy) blood group system; in Case No.2, a rare haplotype - MS^u - in the MNSs blood group system would have had to be postulated to explain paternity. VNTR DNA typing convincigly excluded these two accused men from paternity. These findings have been published (Schlaphoff *et al*, 1993), and the results of VNTR DNA typing are summarised in Tables 4.9 (Case No. 1) and 4.10 (Case No. 2).

For Case No.1, an exclusion of paternity was shown with three of the probes used. For Case No.2, exclusion of paternity was demonstrated with all three probes used. The range of probes used for these two cases was different, as those used to test Case No.1 were no longer commercially available when Case No.2 was investigated.

TABLE 4.9

Allele sizes of bands observed using VNTR DNA PROBES: Case No.1. (Exclusion of paternity based only on discrepant Duffy blood group typing*)

	Fragment sizes (kb)				
Probe	Presumed Mother	Child	Putative Father	Result	
3'α-globin	1.97	1.97	2.28		
		1.97	2.12	3.24	Exclude
HaRas	3.94#	3.88#	3.71	Not	
	5.88#	5.75#	5.75	Excluded	
Mucin	4.29#	4.23#	3.04		
	3.92	3.56	3.04	Excluded	
MR24/1	8.68	8.68	6.58		
	10.03	19.64	11.77	Excluded	

* Mother Fy(a-b+), Child Fy (a+b+), Putative Father Fy (a-b-)

\# Differences in fragment size of <2% were not significant

TABLE 4.10

Allele sizes of bands observed using VNTR DNA PROBES: Case No.2. (Paternity explained by postulation of a rare haplotype in the MNSs blood group system*, and subsequently shown to be excluded using VNTR DNA testing)

	Fragment sizes (kb)			
Probe	Presumed Mother	Child	Putative Father	Result
YNH24	4.51#	4.53#	3.07	
	4.04	5.71	3.16	Excluded
CMM101	5.16	5.16	4.07	
	5.00	5.68	6.16	Excluded
TBQ7	4.25#	4.28#	3.22	
	4.65	3.61	4.69	Excluded

* Mother Ns, Child MNs, Putative Father MNS; postulated haplotypes: Child MS^u, Putative Father MS^u, NS.

Differences in fragment size of <2% were not significant

4.5 ESTIMATION OF POWER OF EXCLUSION (PEX) OF VNTR DNA PROBES.

This part of the study was carried out using the probes YNH24, CMM101 and TBQ7.

The Power of Exclusion (PEX) of any system may be calculated when adequate gene frequency tables are available. (See 3.2.14). However, the number of individuals tested for VNTR DNA polymorphisms during this study did not constitute a representative sample which would allow this type analysis to be carried out with sufficient accuracy. It was, therefore, decided to test the PEX of the probes by physical manipulation of the data, using only cases where no exclusion could be demonstrated using both standard and VNTR DNA testing. The putative father in case number one was assigned to case number two, until each putative father had been incorrectly assigned to a mother/child combination. The cases were then recalculated and the number of times the incorrectly assigned father could be shown to be excluded, was counted. A total of 60 cases could be analysed.

The results are shown in Table 4.11. For the probes YNH24 and CMM101, a band match could be shown in 3 cases (95%), and for the probe TBQ7, a band match was shown in 5 cases (92%). When the probes were analysed in combination, an exclusion of

paternity could be declared in all 60 cases (100%); in those cases where a band match could be shown for one of the probes, an exclusion could be demonstrated using the other two probes.

Table 4.11

Band match rate in false trios using VNTR DNA probes:

Probe	Band Match Observed	No Band Match Observed	Total Cases	% Excluded
YNH24	3*	57	60	95%
CMM101	3	57	60	95%
TBQ7	5	55	60	92%

* Number of cases

CHAPTER FIVE

5.0 DISCUSSION

5.1 INTER- AND INTRA-ASSAY PRECISION AND ACCURACY

A certain degree of variability is inherent to the complex, multistep VNTR DNA assay. Factors which may affect the precision and accuracy of the result include

* measurement error,
* poor separation of large fragments,
* weak visualisation of small fragments,
* variation caused by uneven heating of gels, and
* differences in ionic strength of buffers.

These differences may occur between gels (inter-assay error) or within gels (intra-assay error), and have a direct effect on the sizing of bands (fragments).

Because of this variability, determination of band sizing error is one of the most important parameters to be established in VNTR DNA typing (Baird *et al*, 1986; Gjertson *et al*, 1988). The level of band sizing error is then used to determine whether the bands from one sample can be matched to those of another sample. In single locus VNTR DNA typing for disputed paternity investigations, we are

concerned with determining whether the non-maternal band of a child matches either of the fragments present in a putative father, i.e. whether an exclusion of paternity can be demonstrated.

The effect of band sizing error is illustrated in Figure 5.1. For convenience, each of the fragments is numbered: the maternal fragments are 1 and 3, the child's fragments are 1 and 5, and the putative fathers fragments are 2 and 4. The non-maternal band in the child is number 5. Visually, an exclusion of paternity is observed. However, if an allowance is made for a band sizing error of 2% , the error areas of the two fragments overlap, and it is then not possible to exclude paternity.

Figure 5.1: Effect of band sizing error

	Mother	Child	Putative Father
Fragment 1	———	———	
Fragment2			———
Fragment 3	———		
Fragment 4			* ———
Fragment 5		———	

* The shaded area indicates the effect of allowing for a 2% band sizing error; because the two areas overlap, these fragments would be declared a match, although visual inspection appears to exclude paternity.

One of the recommended methods for establishing the level of band sizing error is by repetitive analysis of volunteer DNA samples using commercially available probes (Budowle *et al*, 1991). In this way, inter-assay and intra-assay precision and accuracy can be determined.

Inter-assay precision measures the degree of variability between gels. As was shown in Table 4.1, inter-assay precision was acceptable for probes 3'alpha globin, Mucin, HaRas, YNH24, TBQ7 and CMM101. For the probes MR24/1 and

EFD52, inter-assay precision was not acceptable and these probes were rejected.

Intra-assay precision measures the degree of variability across a single electrophoresis gel. From the results shown in Table 4.2, intra-assay precision was within acceptable limits. In all cases the CV was <0.6%.

Inter-assay accuracy is a measure of the variability with which fragment sizes are assigned between gels. The results shown in Table 4.3 show that inter-assay accuracy varied considerably, 1.1% to 7.9%. This difference, although large, may be due to differences in sizing techniques. In VNTR DNA testing applied to paternity testing, inter-gel accuracy is less important than precision. Testing is primarily aimed at determining whether fragments match or mismatch, therefore good precision is essential.

After carrying out this exercise, PLTI has established a conservative band sizing error of 2% (Reavis *et al*, 1992), which compared well with that used by other laboratories (Baird *et al*, 1986; Gjertson *et al*, 1990).

Operator error is a measure of the degree of variability between two independent observers in manual measurement of autoradiograph patterns. As can be seen in Table 4.4, there was good agreement between operators. However, when using an automated system, it is not necessary to determine

operator error, since determination of fragment positions relative to the molecular weight markers is done automatically.

5.2 SELECTION OF RESTRICTION ENZYME/PROBE COMBINATION

The use of a single restriction enzyme in combination with a set of probes is a simple and cost-efficient method of single locus VNTR DNA typing; a single membrane is produced which is then tested sequentially with a series of probes. (Major cost factors in RFLP typing systems are the Proteinase K used to digest DNA, the agarose used for the electrophoresis gel, and the membrane used in the transfer step.)

It is also essential that the fragments generated should fall within the linear range of the molecular weight markers, i.e. from 1 - 10kb, since outside of these limits, resolution is poor and sizing inaccurate. It is also considered necessary to have a panel of 4-6 probes to achieve a PEX which equals or exceeds standard genetic marker systems.

In order to select a single restriction enzyme compatible for use with the probes supplied in the Amersham and Promega DNA typing kits, a panel of volunteer DNA samples was digested with a small number of readily available

restriction enzymes, and used in a hybridization step. The following restriction enzymes were used:

* Eco RI, Hind III and Taq I: these had been used in this laboratory for a previous RFLP study;
* Hae III: recommended for use with the probes YNH24, TBQ7, CMM101 and EFD52, supplied by Promega; and
* Pvu II: compatible with the four probes initially supplied by Amersham: 3'alpha globin, Mucin, HaRas and MR24/1.

The restriction enzymes Eco RI and Hind III were found to generate fragments >10kb for most of the probes (Table 4.5), with only the probe CMM101 resulting in fragments within the prescribed limits. These two restriction enzymes were therefore considered unsuitable.

The restriction enzyme Hae III was found to generate smaller fragments (0.84 -5.76kb), and, surpisingly, for three of the probes - HaRas, Mucin and EFD52 - no hybridization appeared to have taken place. By lengthening the autoradiograph exposure period, small indistinct bands were detected (<0.5kb), a finding which had also been reported by Allen and co-workers (Allen *et al*, 1990). In addition to the problems of indistinct bands and low signal, small fragments

may electrophorese off the gel, resulting in a heterozygous individual being considered homozygous. This factor precludes the use of inverse homozygosity, termed an indirect exclusion, as a basis for paternity exclusion using any VNTR DNA typing sytem, regardless of the range of fragment sizes observed. Hae III was therefore considered unsuitable for use with the range of probes selected for this study.

For the restriction enzyme Taq I, RFLP patterns similar to those produced by Pvu II were generated. However, the fragments were smaller than those of Pvu II, and it was considered that this could potentially present problems with resolution and visualization.

Other than for the probe EFD52, the restriction enzyme Pvu II, used in conjunction with the probes mentioned, resulted in fragments which fell within the linear range (1 - 10kb) of the molecular weight marker, with good resolution and 24-48 hour autoradiograph times. Although the fragment sizes generated by Pvu II and detected by the probe MR24/1 were within the 1-10kb limit in this exercise, this probe and EFD52 were found to have a high CV in inter-assay precision tests. For these reasons the probes EFD52 and MR24/1 were not considered for further use.

From the results of this exercise it was decided to use the restriction enzyme Pvu II and the probes 3'alpha globin, Mucin, HaRas, YNH24, TBQ7 and CMM101.

Although Pvu II is still used by this laboratory, it may become necessary in the future to change to a restriction enzyme that is more universally used. At the time that this study was begun, there was very little consensus regarding the use of restriction enzymes or probes. However, an important aspect of quality assurance in VNTR DNA typing, is comparison of data with other centres, which is one of the recommendations of the DNA Commission of the International Society for Forensic Haemogenetics (See Appendix C). Such comparisons can only be made if a common restriction enzyme is used by participating laboratories. Furthermore, continued use of the probes supplied by the Promega Corporation may require a switch to the restriction enzyme Hae III, and the replacement of the probes Mucin and HaRas. Hae III is also stable up to 42°C, with very low levels of partial digestion making it a robust enzyme, suitable for a routine environment.
Another restriction enzyme which may become more widely used is Hinf I, recommended for use with the Jeffreys single locus probes (MS1, MS31, MS8, MS43A, g3: Cambridge Research Biologicals), and which is also compatible with the probes YNH24, and 3'alpha globin (Henke, Dusseldorf, Personal Communication).

5.3 COMPARISON OF STANDARD AND VNTR DNA TESTING

Prior to the discovery of DNA polymorphisms, laboratories which undertook disputed paternity testing offered tests for a range of genetic marker systems: HLA antigens (tissue typing), red cell blood groups, and red cell enzyme and serum protein polymorphisms. These systems comprised the "standard" or conventional tests. Currently, PLTI offers a range of 16 genetic marker systems capable of excluding 99% of falsely accused men.

Following the commercial availability of probes to test for DNA polymorphisms, particularly VNTR DNA loci, and the vigorous promotion of these genetic markers, both in scientific and lay literature, as the ultimate test system for individualization, PLTI embarked on establishing a laboratory to offer this service to our clients.
This study served as the initial phase of the introduction of VNTR DNA typing, and was undertaken to evaluate the usefulness of this new technology compared to standard tests.
A comparison of the results of both standard and VNTR DNA testing was carried out on 100 consecutive cases of disputed paternity. VNTR DNA typing was carried out without prior knowledge of the outcome of the standard tests. The results of both sets of tests were not compared until all the typings were completed.

Of the 100 cases of disputed paternity which were studied, 33% demonstrated an exclusion of paternity. Each of these exclusions was confirmed using single locus VNTR DNA testing. With only one exception, the exclusion could be shown using two or more probes. In order to allow for the possibility that an exclusion of paternity shown by VNTR DNA typing may be due to a mutation, it was decided that an exclusion be shown with two probes.

In addition, two cases which had not previously been excluded using standard tests, were clearly excluded using VNTR DNA testing, giving a total of 35 excluded cases. For both these cases the probability of paternity (W) was considered low, 98.98% and 86.82 respectively, indicating that according to this laboratory's criteria, further testing was necessary.

Also included were two cases where the results of standard testing indicated an exclusion of paternity, but would require further confirmation. In one case, an exclusion was based on a single discrepancy in the Duffy (FY) blood group system. The mother was Fy (a-b+), the child Fy (a+b+) and the putative father Fy (a-b-). Since this was the first exclusion of paternity based only on this system in this laboratory's experience of >3 000 cases, it was decided that further confirmation of the exclusion was necessary. In the second case, to explain paternity the haplotype MS^u in the

MNS blood groups system, would have had to be postulated in the putative father. Since the occurrence of this haplotype in SA Blacks, the ethnic group of the putative father, was 0.0025 (May and Du Toit, 1989), it was decided to carry out VNTR DNA typing. Both these falsely accused fathers were excluded using VNTR DNA typing (Schlaphoff *et al*, 1993).

These results indicate that single locus VNTR DNA typing for disputed paternity testing has many advantages. By careful selection of the range of probes, the system has the potential to be more informative, i.e. more exclusions shown using VNTR DNA typing, than using standard tests. It could, therefore, replace standard genetic marker systems. Furthermore, DNA typing has the advantage of requiring a much smaller blood volume, usually <2ml, a factor of prime importance when sampling babies. This, in turn permits testing to be carried out on infants younger than 6 months, the lower age limit for withdrawal of the blood volume required for standard testing and the age at which all the red cell surface antigens can be reliably detected. Testing is then possible at birth or even in utero, if amniotic cells are cultured. In addition, VNTR DNA typing can also be successfully carried out using post-mortem samples.

At present this laboratory has reserved the use of VNTR DNA typing for the following situations:

1. those cases in which exclusion of paternity requires further confirmation,

2. where the calculated probability of paternity is <99.7%; or
3. when a specific request is made.

5.4 ESTIMATION OF POWER OF EXCLUSION (PEX) OF VNTR DNA PROBES.

The main purpose of genetic marker testing in disputed paternity is to exclude a man falsely accused of paternity. A measure of the ability of a set of genetic marker systems to exclude these falsely accused men is termed the power of exclusion (PEX). It has been recommended that laboratories offering these tests should be capable of excluding at least 95% of falsely accused men. However, factors such as the expertise of laboratories, differences in ethnic groups (populations) and gene frequencies has resulted in various laboratories offering different ranges of genetic marker systems. Currently PLTI offers a set of 16 genetic marker systems, with a PEX >99%. This may appear to contrast with the results presented in this study, where 2 men, not excluded using conventional systems were found to be excluded using VNTR DNA typing. However, the result may reflect a cluster effect, as the number of cases analysed in this study is small.

When DNA typing was first applied to individualization, using the Jeffreys multilocus probes (Jeffreys, 1985a,

Jeffreys 1985b), it was hailed a major breakthrough, and it was proposed that, due to the nature of the tests, it would be possible to positively link individuals to a crime scene or to each other. For example, in the case of State vs Pitchfork, an extensive screening programme was successful in tracking down double murderer and rapist (Wambaugh, 1989). The system was also used in immigration cases by the British Home Office, to prevent the unlawful entry into Britain of non-family members (Jeffreys, 1991).
The use of multilocus DNA typing was the subject of much debate following the Castro Case (Lander, 1989). The controversy resulted from lack of laboratory controls, and was also due to the high level of expertise required to interprete the complex banding pattern. This experience led to the promotion of single locus VNTR DNA probes, particularly in the United States.

Single locus VNTR DNA probes are technically simple to use, resulting in a much simpler banding pattern (at most two bands per individual), analagous to protein polymorphisms which are familiar and accepted by courts of law. However, the greater simplicity of single locus typing systems, with a correspondingly lower PEX, requires the use of a range of probes. For this reason the PEX for any given set of single locus VNTR DNA probes should be assessed, before the system is implemented for routine use.

As the sample used for this study is small, it was not possible to calculate the PEX (See 3.2.14). However, creation of false trios from available data, can give some indication of the usefulness of any genetic marker system. This exercise was conducted for the three probes which had been used throughout the study, YNH24, TBQ7 and CMM101. Only those cases which had not been excluded using both standard and VNTR DNA typing were used, and 60 of the 65 non-excluded cases could be analysed.

As was expected, while no individual probe excluded every falsely assigned putative father, a combination of the three probes excluded all of the falsely assigned putative father. This indicates a very good PEX (>99%) for this series of VNTR probes.

CHAPTER SIX

6.0 CONCLUSIONS AND DIRECTIONS FOR FUTURE STUDY

6.1 CONCLUSIONS

The discovery of DNA polymorphisms has revolutionised the field of genetic marker analysis. One class of DNA polymorphism which has had tremendous impact on disputed paternity testing is VNTR DNA polymorphisms, particularly single locus VNTRs.

Prior to the development and commercial availability of DNA probes, laboratories offering paternity testing relied on a combination of genetic marker systems: HLA typing, red cell blood grouping, and red cell enzyme and serum protein polymorphisms. At PLTI a total of 16 genetic marker systems is used routinely, capable of excluding >99% of falsely accused men.

This study was the initial phase of establishing a VNTR DNA typing service for disputed paternity testing. The results are preliminary, as the extreme polymorphism of this category of DNA typing system would require a much larger study for detailed analysis. As a result of this study, the initial essential parameters were established, namely:

* basic methodology,
* inter- and intra-assay precision and accuracy, and
* choice of restriction enzyme/probe combination.

In addition, 100 cases of disputed paternity were examined using both standard tests and VNTR DNA typing. The results of this part of the study showed:

* all exclusions shown using standard tests were confirmed using VNTR DNA typing,

* an additional 2 exclusions of paternity were shown using VNTR DNA testing, both having low W values;

* in two cases, where exclusion of paternity using standard tests required further confirmation, VNTR DNA testing convincingly excluded the two falsely accused men, and

* a powerful PEX for the probes YNH24, CMM101 and TBQ7 used together.

The laboratory now functions as an integral part of the paternity service offered at PLTI, which is the only

laboratory in Southern Africa capable of carrying out the complete range of tests for genetic marker analysis.

From these results it is clear that VNTR DNA polymorphisms for paternity testing are extremely useful; VNTR DNA typing has been termed the `fourth generation' genetic marker system (Walker and Crisan, 1991). Careful selection of the restriction enzyme/probe combination, can result in a test system with sensitivity above that observed using existing genetic marker systems.

VNTR DNA typing has many practical advantages, chief of which is that laboratory techniques are robust, and therefore well-suited to a routine environment. It is also simpler to establish a DNA typing laboratory than one which performs HLA typing, as equipment and reagents are all commercially available. Results are easily interpreted and, although sophisticated equipment has been developed to read autoradiographs, manual reading is acceptable if time-consuming. There are added advantage to clients, in that DNA-based polymorphisms require a much smaller blood sample, and the sensitivity of the system can be tailored to match and supercede that obtained using standard testing.

The chief disadvantage of VNTR DNA typing systems is the time necessary to carry out the tests. Laboratory tests for standard genetic marker systems are usually completed within 1-2 weeks, and the report can be issued within 2-3 weeks of

receipt of samples. VNTR DNA testing, being a complex multi-step assay, could take from 8-10 weeks, depending on the number of probes to be used and the results obtained in each sequential hybridization step. The system is also more expensive than standard tests, and realistic pricing would place this technology beyond the financial capability of most clients seeking disputed paternity testing. Another disadvantage is that the system at present relies on the use of radio-isotopes to label the DNA probes, which has definite disadvantages for workers, and problems with management and disposal of laboratory waste.

The disadvantages of isotopic labelling and longer time-to-result, will certainly be eliminated in the future. Non-isotopic labelling techniques have been developed and the Chemiluminescent system marketed by Promega (Geneprint Light) has many features which are similar to isotopic labelling e.g. production of a hard copy result (autoradiograph), and successful re-probing of membranes.

6.2 DIRECTIONS FOR FUTURE STUDY

In order to calculate the paternity index and probability of paternity using VNTR DNA typing, it is essential that the allele frequencies for the local population be determined. This exercise will also include the establishment of an important aspect in the use of VNTR DNA typing for matching of individuals, namely the binning procedure (Devlin and Risch 1992). These two aspects are currently being analysed by Dr J Rousseau and Mr S Reavis of PLTI.

An important advance in DNA typing methodology, is the development of the polymerase chain reaction (PCR) (Saiki *et al*, 1985). PLTI has used PCR techniques in the analysis of the DR and DQ regions of the major histocompatibility complex, as well as for medico-legal purposes using the DQA Forensic typing kit marketed by Cetus.

A report by Budowle and co-workers has utilised PCR-based methodology as a useful alternative to RFLP analysis of VNTR's, in a technique termed amplified fragment length polymorphisms (AMP-FLPs) (Budowle 1990, Budowle *et al*, 1992, Berg *et al*, 1992). A high resolution electrophoresis system is employed to analyse PCR products, followed by silver staining. Fragments are sized using an allelic ladder comprised of control samples, analagous to the system used

to determine red cell enzyme and serum protein typings. This technique has the potential to be faster and more reproducible than RFLP based VNTR DNA typing.

APPENDICES

APPENDIX A

FORMS USED AT PLTI: DISPUTED PATERNITY TESTING.

ROVINCIAL LABORATORY FOR TISSUE IMMUNOLOGY

Private Bag 4, OBSERVATORY 7935
Cape
Falmouth Road, OBSERVATORY 7925
Cape
Tel.: (021) 47-3080
Tel. Add: "Translab" Cape Town

Date : ______________

DISPUTED PATERNITY

I hereby declare that my name is ______________________________
(in full)

of ____
(please print address)

Race : ______________ Date of birth : _____/_____/_____
day month year

and furthermore, that I as mother and guardian of the child

______________________ race : ______________ sex : __________

date of birth : _____/_____/_____ give consent to the withdrawal of
day month year

a sample of blood from myself and my child for blood grouping and other tests.

Have you had a blood transfusion in the last four months? __________

If yes, state when ______________________

Has the child had a blood transfusion in the last four months? ______

If yes, state when ______________________

____
SIGNATURE

IDENTITY NO. : ____________________

TELEPHONE NO. : ____________________

PROVINCIAL LABORATORY FOR TISSUE IMMUNOLOGY

Private Bag 4, OBSERVATORY 7935
Cape
Falmouth Road, OBSERVATORY 7925
Cape
Tel.: (021) 47-3080
Tel. Add: "Translab" Cape Town

PUTATIVE FATHER

Date : ______________

DISPUTED PATERNITY

I hereby declare that my name is ______________________________
(in full)

of ______________________________
(please print address)

Race : ______________ Date of birth : _____/_____/_____
day month year

and give consent to the withdrawal of a sample of blood for blood grouping and other tests.

Have you had a blood transfusion in the last four months? __________

If yes, state when ______________________

____
SIGNATURE

IDENTITY NO. : ____________________

TELEPHONE NO. : ____________________

PROVINCIAL LABORATORY FOR TISSUE IMMUNOLOGY

Private Bag 4, OBSERVATORY 7935
Cape
Falmouth Road, OBSERVATORY 7925
Cape
Tel.: (021) 47-3080
Tel. Add: "Translab" Cape Town

MOTHER : I ______________________________
(name of mother)

hereby certify that the blood in these tubes is correctly identified and that the putative father is present.

SIGNATURE ________________________

PUTATIVE FATHER : I ______________________________
(name of putative father)

hereby certify that the blood in these tubes is correctly identified and that the complainant mother is present.

SIGNATURE ________________________

WITNESS : 1. ______________

2. ______________ DATE : __________

PROVINCIAL LABORATORY FOR TISSUE IMMUNOLOGY

Private Bag 4, OBSERVATORY 7935
Cape
Falmouth Road, OBSERVATORY 7925
Cape
Tel.: (021) 47-3080
Tel. Add: "Translab" Cape Town

Case No. : ______________

Fax No. (021) 448-61(

LOCAL

PHOTOGRAPHED by : ______________________

Signature : ______________ Date : ________

BLED by : ______________________

Signature : ______________ Date : ________

PUT INTO TUBES by : ______________________

Signature : ______________ Date : ________

Identified and
Received **DNA** : ______________________

Signature : ______________ Date : ________

Identified and
Received **HLA** : ______________________

Signature : ______________ Date : ________

Identified and
Received **RCE** : ______________________

Signature : ______________ Date : ________

Identified and
Received **RBC** : ______________________

Signature : ______________ Date : ________

PROVINCIAL LABORATORY FOR TISSUE IMMUNOLOGY

Private Bag 4, OBSERVATORY 7935
Cape
Falmouth Road, OBSERVATORY 7925
Cape
Tel.: (021) 47-3080
Tel. Add: "Translab" Cape Town

Case No.:______________

Fax No (021) 448-6107

PARCELS ARRIVED

RECEIVED on : TIME : ____________ DATE : _______________

OPENED by : _________ Signature: ___________ Date: ______

CONDITION of parcel :

no obvious tampering with parcel : ______

all specimens correctly labelled : ______

all forms correctly filled in : ______

Identified and received HLA : ____________ Signature__________ Date______

Identified and received RCE : ____________ Signature__________ Date______

Identified and received RBC : ____________ Signature__________ Date ______

APPENDIX B

STOCK SOLUTIONS

1. RED CELL LYSIS BUFFER [RCLB] - pH 7.5

5mM $MgCl_2$
10mM NaCl
10mM Tris/HCl

2. WHITE CELL LYSIS BUFFER [WCLB] - pH 7.5

75mM NaCl
25mM EDTA
10mM Tris/HCl

3. 1xTris EDTA Acetate BUFFER [TEA] - pH 8.0

40mM Tris
20mM Glacial Acetic Acid
2mM EDTA

4. 20x Saline Sodium Citrate [SSC] - pH7.0

3.0M NaCl
0.3M Na^3 citrate

5. 30x Saline Sodium Phosphate EDTA [SSPE] - pH7.7

4.5M NaCl
0.3M NaH2PO4
30mM EDTA

6. TRANSFORMATION and STORAGE BUFFER [TSB]

LB broth, pH 6.1 containing -
10% Polyethylene Glycol (PEG)
5% Dimethlylsulphoxide (DMSO)
20mM MgCl

7. LURIA BROTH [LB] - pH7.5

5g/l Bacto-yeast extract
10g/l Bacto-tryptone
10g/l NaCl
Autoclaved at 15psi for 15 minutes

8. Tris/EDTA [TE]

1mM EDTA
10mM Tris-HCl pH7.6

9. LOADING BUFFER [lb]- 5X STOCK

40% sucrose
50mM EDTA
0.5% SDS
50mM Tris-HCl pH7.6.

10. OLIGOMER LABELLING BUFFER [OLB]

Solution O - pH 8.0

0.125M $MgCl_2$
1.25mM Tris

Solution A

1ml Solution O
18μl 2 Mercaptoethanol
5μl dATP*
5μl dTTP*
5μl dGTP*

*Each previously dissolved at 0.1M in 3mM Tris pH7.0, 0.2mM EDTA.

Solution B

2M Hepes adjusted to pH6.6 with 4M NaOH.

Solution C

Hexadeoxynucleotides (Pharmacia No. 2166) suspended in 3mM Tris pH7.0, 0.2mM EDTA, at 90 OD units/ml.

Solutions A, B, and C were mixed in a ratio of 100:250:150.

APPENDIX C

RECOMMENDATIONS OF THE DNA COMMISSION OF THE INTERNATIONAL SOCIETY FOR FORENSIC HAEMOGENETICS.

1991 Report of the DNA Commission of the Society for Forensic Haemogenetics concerning the use of DNA polymorphisms.

"The time has come" the Walrus said "to speak of many things": Lewis Carroll.

1. Introduction

In 1989 the ISFH publiched its first recommendations concerning the application of DNA investigations to forensic science (Forensic Sci Int, 1989, 43: 109-11; Voxx Sang, 1989, 57:276-277; Biotech-Forum 1989 6: 111-112). It was obvious, even at the time of publishing, that these guidelines would need revision and updating because of the rapidly changing situation with regards to DNA technology. Since then the DNA Commission of the ISFH has met twice wiht the aim of improving the recommendations so that they encompass the new developments in this field. This report is concerned primarily with the detection of DNA polymorphisms by restriction fragment length polymor-phism (RFLP) analysis. It also contains general recommenda-tions applicable to all DNA polymorphism analysis.

2. Definitions of genetic systems and documentation

A genetic locus is defined by a segment of unique DNA sequence that occupies a specific position on a chromosome. Genetic polymorphism at the DNA level can generally be divided into two categories

a) polymorphism in sequence resulting from nucleotide base substitutions, and

b) polymorphism in sequence resulting from insertions or deletions of a nucleotide or nucleotides.

Among the most informative DNA polymorphisms for identification purposes are insertion/deletion polymorphisms containing variable number of tandem repeat (VNTR) sequences. These are conveniently detected by restriction fragment length polymorphism (RFLP) analysis.

2.1 Definition of systems and alleles:

2.1.1

a) A DNA typing systems is defined by the designation of a genetic locus and the information needed to detect allelic variation at that locus. RFLP systems are definded by the probe and restriction enzymes used for the typing. Systems based on gene amplification by the polymerase chain reaction (PCR) are defined by the sequence polymorphism.

DNA polymorphism detection systems can be divided operationally into two groups; single-locus systems (SLS) and multi-locus systems (MLS). With the latter, polymorphisms at multiple loci are detected simultaneously.

b) At VNTR loci "alleles" are defined by DNA fragments of discrete length, which are inherited in agreement with a formal genetic model. They are detected by means of conventional Southern Blot analysis or comparable methods. They are usually represented by one or two restriction fragments of a given size generated by the use of one enzyme (or two enzymes in double digestions) and detected with one probe. For PCR products the fragments can be stained directly.

c) VNTR "allele" designation should be preferentially in kilobase size, but other methods can be applied if proven to be more appropriate (e.g. Rf, molecular weight or allele number).

2.1.2 Multilocus systems:

Multilocus systems use probes that recognise genomic sequences which are normally distributed throughout the entire genome. Multi-locus typing gives a pattern of bands, the so-called

"DNA-fingerprint". Although the pattern of bands (restriction fragments) is generally determined, it is not possible to specify the genetic locus from which each band originates. Accordingly, the bands in a multi-locus pattern cannot be defined explicitly by a formal genetic model.

2.2 Typing methodology

It is generally recommended that any method used should be based on an established protocol.

2.2.1 General and developmental requirements:

a) Collaboration and exchange of data should be encouraged to establish the usefulness of a system and comparability of data.

b) DNA polymorphisms should be difned by family and population studies. At least 500 meioses and an adequate population sample should have been tested and published, before a polymorphism can be introduced into paternity testing.

c) The chromosomal location and linkage data to other polymorphisms used in paternity testing should be available. This information should be documented in the publications of the International Human Gene Mapping Workshop.

d) The description must include: DNA sequence data defining the locus (if available), proof of Mendelian inheritance, "allele" or haplotype frequencies, frequencies of mutations and/or recombinations and a check using a suitable statistical procedure that the population is not out of genetic equilibrium. For RFLP systems, the description must include: information on the probe and restriction enzyme and information on the size of constant and variable fragments.

2.2.2 Requirements for RFLP methodology and standardization:

a) Size markers with discrete fragments of known size should span and flank the entire range of the DNA system being tested. Bands which do not conform to this rule should be designated appropriately (see 3.2).

b) A human control DNA of known allele composition should be included on each gel. Commercially available cell lines (eg K562) or other control human DNA are considered to be suitable.

c) Intactness of the individual genomic DNA before restriction enzyme digestion and complete digestion of the DNA should be assured by appropriate control experiments. It is recognised that there are situations where this may not always be possible (e.g. stain work). Under these circumstances analysis can still be carried out but the results should be interpreted with caution.

3. Establishment of a population data base

3.1 Construction of data bases:

Each laboratory should construct its own data base for appropriate local populations. Such data bases should be composed of not less than 100 individuals.
The population sample should be representative of the relevant local population(s). Data bases for different local populations should not be merged until it can be demonstrated that it is statistically acceptable to do so. Raw data on RFLP fragment sizes should designate band sizes to at least 10bp resolution.

No particular method of fragment size measurement is recommended. However, for RFLP's automatic methods would be preferred to manual methods due to their accuracy and reproducibility but for systems with discrete alleles manual methods can be more convenient.

3.2 Fragment (band) size determination:

A good size marker with an adequate number of fragments is essential and should be placed at regular intervals to correct for possible inhomogeneity across the gel. Such a ladder should span and flank all the fragments to be measured. The number of bands and the spacing of bands cannot be precisely stated and must be left to the discretion of the operator depending on the system in question. If a fragment lies outside the standard ladder this cannot be assigned an accurate size, but should be coded as lying above or below a particular fragment size.

4. Recommendations for paternity testing

4.1 Single locus system:

4.1.1 Mutation:

a) The mutation rate for single-locus systems should be known.

b) Systems with high mutation rates should not be used routinely and require special considerations.

4.1.2 Matching criteria:

A match is considered to occur when genetic types cannot be distinguished.

a) Comparison of specimens in paternity cases can be made by (purely) visual comparison (side-to-side) or co-electrophoresis in the same gel. If the comparison is made from different gels a numerical evaluation is essential. A mathematical matching rule can be applied to confirm or refute a visual match.

b) The initial visual comparison of bands may be confirmed by numerical methods such Bayes, sliding or fixed windows as long as the prerequisites and limitations of each method are taken into account.

c) By definition 2 bands to be compared can be called a match if they fall within the limits of the match-window.

d) The significance of an inconclusive or borderline determination may be estimated by the Bayesian approach using standard error and standard deviation of the bands in combination with rehybridization using other probes.

4.1.3 Fragment frequency:

a) A discrete allele system can be unambiguouslyu resolved by comparison with suitable ladders of known fragment lengths. However suitable biostatistical methods can be used to define and check frequencies.

b) If the fragment distribution is quasi-continuous the frequency of a single fragment can be estimated according to prdefined criteria for matching, but will vary depending on the method used (i.e. floating or fixed bins). Under normal circumstances it is essential to estimate the frequency of a given fragment size. This requires the application of predefined criteria for a match (e.g. if matching window = ± 3 x sigma, the corresponding bin for frequency estimation must be at least the same or greater).

c) For small data bases with less than 200 individuals consideration should be given to the measurement errors of the frequency estimate.

e) Care must be taken that the population is truly representative to eliminate sampling error.

f) Bayesian approaches based only on observed fragment sizes may also be used.

g) It would be preferable to give the correct result concerning the fragment frequency. As this is not possible the best alternative is to give a conservative estimate. In the sliding window approach and its variations, the centre o;f the window is the point corresponding to the actual measured fragment size. In the fixed bin approach, the actual measured size of the fragment falls somewhere within the bin. Binning is based on rungs of molecular weight ladders, or on natural valleys in a fragment distribution curve.

h) Phenotype frequencies from several systems can be combined by multiplication unless it has been proved by appropriate statistical testing that there is disequilibrium between the single-locus systems in common use.

4.1.4 Compliance to Hardy-Weinberg equilibrium expectation:

a) Population data bases should be checked using suitable statistical procedures for deviations from the norm. However, potential artefacts exists which can influence the results of such tests so that apparent significant deviations may also be due to technical problems and should be investigated further.

b) Estimation of profile (band) freqeucies should be tested for dependence. If non-independence is demonstrated then frequency estimates incorportating 2 or more probes cannot be calculated by multiplication of genotype frequencies.(8U

Either haplotype frequencies should be quoted or a suitable statistical analysis used in which it has been demonstrated that it is not necessary to make the assumption of independence.

c) Obvious artefacts must be explained and must be adequately addressed and taken into account for the interpretation of results.

4.2 Multi-locus systems:

a) Mutation rates must be known, but it should be recognised that rates of 10^{-2} and higher can occur.

b) Band sizes and/or band patterns should be scored in an objective manner.

c) All questions of independence, allelism and linkage disequlibrium need to be addressed and used in a conservative way if included in biostatistical calculations.

d) Calculated probabilities on the statistical basis for paternity are at present still under discussion. Probabilities must be based on full genetic and biostatistical analysis as outlined in (b) otherwise only a verbal opinion on exlcusion or non-exlcusion should be given.

4.3 Conclusions:

Paternity testing with conventional techniques is a well established procedure for producing evidence in court cases, and can continue to be used either alone or in combinaition with DNA polymorphisms. Providing that a DNA system has been suitably and adequately scrutinised there is no reason why DNA should not be used alone.

5. Concerns for identity testing

5.1 Specific requirements for the application in criminal investigations:

In this section some specific requirements are listed with regard to the analysis of stains; however, it is stressed that many (but not all) of the requirements discussed previously are also relevant under this heading. The sections referring to family studies and to mutation rates are not applicable except in those cases where identity testing entails testing of family members such as in missing person cases.

5.2 Somatic stability:

The application of DNA analysis in criminal investigations is mainly concerned with the comparison of genetic types obtained from a reference blood sample with those obtained from an evidentiary body fluid or stain. The stain may be a deposit or blood, semen, vaginal fluid, saliva or even a smear of tissue. Also the analysis of hairs, in particular hair roots, may be undertaken. The system used should therefore be shown to be somatically stable: that is, tissue specific modifications to DNA (such as methylation) must be shown not to affect genetic typing determinations.

5.3 Band matching and statistical interpretation:

5.3.1 Genetic typing systems yielding discrete genetic types:

Typing results should be interpreted according to standards established for blood group and protein genetic markers.

5.3.2 RFLP typing systems:

In addition to the facotrs mentioned under paternity testing it is recognised that the reproducibility of any method may lead to the imprecise alignment of bands which are nevertheless considered to be a match (for definition see 4.1.2).

Furthermore in stain analysis inteference from substrates or degradation of the DNA can cause minor variations in band position again leading to some distortion. Such positional variations must be shown to be within the expected experimental variation and wherever possible the statistical assessment should be correspondingly adjusted. A record of the analysis, the associated results and the method of the statistical evaluation should be readily available for examination by a second independent analyst.

5.4 Analysis of semen-contaminated vaginal swabs:

When differential extraction of vaginal swabs is carried out the supernatent generally containing mainly female DNA may also be tested, as it can provide a useful internal control.

6. Quality assurance

6.1 Intra-lab quality assurance:

Quality assurance for the individual laboraotry is essentially covered under points 2.2, 3 and 4 of this report. For RFLP typing systems intra-assay measurement precision (i.e. inter- and intra-gel) within one laboratory should be evaluated and used as the basis of any statistical calculations, in determination of match window and allelic frequencies.

6.2 Minimum requirements for inter-lab comparisons:

if it is required to pool or compare data from two separate laboratories, the exchange of a relatively small number (e.g 20) of samples between laboratories allows some assessment of the ability to reproduce typing results. For RFLP systems samples spanning the full range of allele fragments are required in order to obtain information as to whether or not results correlate between two laboratories. An adequate number of samples needs to be exchanged between laboratories if databases are exchanged, and it is necessary to determine by experiment how much variation exists exists between laboratories over the whole range of fragment sizes.

6.3 Blind trials:

Laboratories should participate in appropriate inter-laboratory trials and aim to achieve consensus results. Each laboratory should operate in its sphere using its own database so long as the required standards have been obtained, but to compare results with other laboratories the recommended criteria should have been met.

The Executive Committee of the International Society for Forensic Haemogenetics (B. Brinkmann, R. Bütler, P. Lincoln, W.R. Mayr, U. Rossi) and coopted external experts (W. Bär, M Baur, B. Budowle, R. Fimmers, P. Gill, K. Hummel, J. Morris, S. Rand, Ch. Rittner, G. Sensebaugh).

Vox Sanguinis 1992;63:70-73

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GLOSSARY

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AGAROSE: The gelling fraction of agar that is extracted from seaweed and used in gel electrophoresis.

ALLELE: One of two or more alternate forms of a gene (or DNA region such as VNTR) that occupy a specific locus in a genome.

ANNEALING: The pairing of a mixture of two complementary single stranded nucleic acids to form double-stranded (duplex) nucleic acids.

AUTORADIOGRAPHY: The process of detecting radiolabeled molecules as a result of the image they produce on an X-ray film.

BAND: The image produced on an autoradiograph which corresponds to a specific DNA fragment which is the result of hybridization to a complementary DNA probe.

BASE: One of the four molecules - adenine (A), cytosine (C), guanine (G) and cytosine (C) - which make up DNA and whose linear sequence encode the genetic information. The bases on complementary strands are hydrogen bonded (A=T, G=C) to form a DNA duplex.

CHROMOSOME: A structure found within the cell nucleus consisting of a single duplex of DNA combined with RNA and proteins. Each chromosome consists of a specific linear sequence of genetic information. Humans have 23 pairs of chromosomes.

DEOXYRIBONUCLEIC ACID (DNA): A polymer of deoxyribonucleotides, each containing one of the bases: Adenine, Thymine, Cytosine or Guanine. DNA serves as the primary genetic material of all cells.

DNA TYPING: The characterization of an individual's genome by developing a unique DNA fragment (allele) pattern. (DNA fingerprinting; DNA profiling)

ELECTROPHORESIS: The process of separating charged molecules through a porous medium such as agarose, by the application of an electric field. DNA fragments are separated according to their length by agarose gel electrophoresis.

EXCLUSION: Positive elimination of an individual suspected of being a criminal or father by virtue of a non-matching genetic profile.

GENE: A sequence of nucleotides along a molecule of DNA which codes for specific genetic information. Genes may be transcribed into RNA and subsequently translated into polypeptides, or only transcribe into specific RNAs.

GENETIC MARKER: An inherited (genetic) characteristic that can be recognised in the phenotype.

GENOME: The sum total of all genetic matrial within a cell.

GENOTYPE: The genetic information at one or more loci.

HETEROZYGOUS: The presence of different alleles in an individual at corresponding homologous chromosome loci.

HOMOZYGOUS: The presence of identical alleles in an individual at corresponding homologous chromosome loci.

HYBRIDIZATION: The annealing of two complementary strands of nucleic acid to form a duplex molecule.

HYPERVARIABLE REGION (HVR): A chromosomal segment characterised by considerable variation in the number of tandem repeats at one or more loci.

KILOBASE (kb): A fragment of 1 000 bases (nucleotides) in a strand of DNA or RNA.

LOCUS: the position of a gene or chromosome segment on a chromosome. Alleles are located at identical loci on homologous chromosomes.

MINISATELLITE: Regions of DNA consisting of tandemly repeated short sequences of DNA.

MULTILOCUS PROBE (MLP): A probe that hybridizes to several loci.

NYLON MEMBRANE: A sheet of nylon to which DNA or RNA is bound during the transfer of DNA or RNA from an agarose gel during the Southern Blotting procedure.

OLIGONUCLEOTIDE: A polymer of nucleotides, usually <100, which are usually synthesised by an automated system, and are generally used as probes or primers.

POLYMORPHISM: The simultaneous occurrence of two or more allelic forms within a population.

PRIMER: A single stranded nucleic acid which upon hybridization with a complementary poriton of another single stranded molecule, acts as a template for initiation of polymerization mediated by an enzyme with DNA polymerase activity.

PROBE: A labeled single stranded fragment of DNA or RNA capable of being tagged with a tracer and which can by hybridized to a complementary target sequence.

PUTATIVE FATHER: A man who is contesting an accusation of paternity or wishes to establish it when a question of paternity exists.

RESTRICTION ENDONUCLEASE (ENZYME): An enzyme which recognises a palindromic DNA base sequence, usually 4-8bp, and cleaves at or near this sequence.

RESTRICTION FRAGMENT: A fragment of double stranded DNA that is generated following digestion with a restriction endonuclease.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP): A DNA polymorphism which is detected as different fragment lengths following digestion with a specific restriction endonuclease.

SINGLE LOCUS PROBE (SLP): A probe that hybridizes to a single locus.

SOUTHERN BLOT: The technique whereby DNA fragments separated by agarose gel electrophoresis are transferred to a solid support such as nylon. These fragments are generally detected by hybridization with labelled complementary DNA probes.

STRINGENCY: The buffer salt concentration and temperature used in the posthybridization DNA blot wash process. As these parameters are changed the degree of binding of probe to target DNA changes.

TANDEM REPEAT: The repeated end to end duplication of a core DNA sequence.

VARIABLE NUMBER OF TANDEM REPEATS (VNTR): Different numbers of tandem repeated core DNA sequences at a given locus. The variation in length of the alleles formed from the repeats provides the basis for unique individual identification.