UNIVERSITI SAINS MALAYSIA

Allele Frequencies of F13A01, FESFPS and vWA STRs in random Tamil population of Malaysia

Dissertation submitted in partial fulfilment for the Degree of Bachelor of Science (Health) in Forensic Science

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CERTIFICATE

This is to certify the dissertation entitled

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is the bonafide record of research work done by

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ABSTRACT

Short tandem repeat (STR) loci are characterized by high levels of length polymorphism within and among populations and they are ideal markers for forensic analysis of biospecimens. In this research project, allele frequencies for three STRs loci that are F13A01, FESFPS and vWA in random Tamil population were studied. Buccal swabs were collected from 100 unrelated individuals of ethnic Tamil population of Malaysia. STR profiling of the deoxyribonucleic acid (DNA) extracted from these samples were profiled for the three STRs using multiplex primer kit (Promega Corporation, USA).

Statistical analysis was also carried out to obtain some parameters and comparative studies were carried out with other populations. A database for random Tamil population will help in human identity testing in Malaysia. The genotype frequency distributions do not deviate from the Hardy-Weinberg expectations.

INTRODUCTION

Malaysia consists of multiracial population with dissimilarities of origin and it is impressive compared to other countries. There is very little research done on bio-medical and molecular genetic markers on various ethnic groups in Malaysia. The ethnic population structure for Malaysia is considered to be very complex. The main ethnic groups are Malay, Chinese and Indian. Establishment of a database based on genetic markers is essential for the application in personal identification (Lincoln and Thomson, 1998).

The genetic markers choosen for these study are withdrawn from the DNA, deoxyribonucleic acid molecule. It is an informative molecule that is present in almost every cell of the human body. In cells, DNA is tightly packed as chromosomes. The chromosomes are inside the nucleus of the cell. In humans the number of chromosomes are 46; 23 pairs- as each pair were inherited from the mother and father. The 46 chromosomes are same in all nucleated cells of an individual. One of the pair of chromosomes is determining the sex of a person.

Females carry 2 homologous X chromosomes and males carry one X and one Y chromosome. On the 23 pairs of chromosomes, there are approximately 6 billion bases. In human genome there are coding and non- coding DNA sequences. Approximately 95-97% of the human genome is non-coding. Much of these non- coding DNA exists as random bases, however approximately 25% of them are repetitive sequences (Gerbel and Saferstein, 1997). The original DNA loci examined in forensic science were minisatellites, which are large repeated DNA sequences.

Sir Alec Jeffreys (1985) first described these sequences and noticed that the DNA length varied from person to person. He was responsible for using this technique in forensic science for the first time. At the time he called the new technique as DNA fingerprinting, although the methods are now referred to as DNA profiling (Jeffreys, 1985). A number of problems with the application of DNA fingerprinting have been highlighted in the United States courts (Easteal et al, 1991; Lincoln and Thomas, 1998).

One particular concern in the application of genetic typing is the use of general population databases to determine DNA profile probabilities. It has already been established that there are racial differences in allele frequencies (Gerbel and Saferstein, 1997; Butler, 2001). This has led to the construction of databases for different racial groups. It has recently been proposed that minisatellite allele frequencies are different within sub-populations of a major population (Lander, 1989; Gerbel and Saferstein, 1997).

Population stratification would have important implications for forensic and paternity work (Butler, 2001). DNA profiling currently utilises another form of repetitive DNA called microsatellites. Microsatellites are much more smaller than minisatelittes. Minisatellites are known as variable number of tandem repeats (VNTRs) with core repeats of 9-80 bp, while microsatellites (short tandem repeats, STRs) contain 2-7 base pairs (bp) repeats (Easteal et al., 1991). STRs are abundant throughout the human genome (Tamarin, 2002).

Microsatellite loci are endowed with several properties that render them as desirable markers for fine scale genetic mapping (Edward et al., 1992), maternity or paternity determination (Hammond et al, 1994) and forensic analysis (Edward, et al.,

1992). The forensic DNA community has moved primarily towards tetranucleotide repeats, which may be amplified using the polymerase chain reaction (PCR) with greater fidelity than dinucleotide repeats (Lincoln and Thomson, 1998). The variety of alleles present in a population is such that a high degree of discrimination among individuals in the population may be obtained when multiple STR loci are examined (Werett et al, 1997).

Polymerase chain reaction (PCR)-based STRs has several advantages over conventional Southern blotting techniques of the larger variable number tandem repeats (VNTRs). Discrete alleles from STR systems may be obtained due to their smaller size, which puts them in the size range where DNA fragments differing by a single basepair in size may be differentiated. Compared to VNTR systems typed by Southern blot hybridisation, STR elements offer considerable advantages concerning sensitivity, precision and reproducibility of fragment size estimates and biomathematical evaluation.

However, to reach the discrimination power of highly informative RFLP systems, at least the two-fold number of STR loci has to be analysed (Gill et al, 1995). For the genetic markers such as VNTRs and STRs, it is desirable to collect allele or genotype data from relevant populations. This is for an estimation of the rarity of a genetic profile. Determination of discrete alleles allows results to be compared easily between laboratories without binning. In addition, smaller quantities of DNA, including degraded DNA, may be typed using STRs.

Thus, the quantity and integrity of the DNA sample is less of an issue with PCR-based typing methods than with conventional Restriction Fragment Length Polymorphism (RFLP) methods. Most laboratories are concentrating on DNA evidence

as the main form of biological evidence. The trend is now firmly established toward "PCR- STR" based technology and in particular to multiplexing (Werett et al., 1997). There are literally hundreds of STR systems, which have been mapped throughout the human genome (Werret et al., 1997).

The desirable features for STR systems include high heterozygosity, regular repeat unit, distinguishable alleles and robust amplification. There are several types of STRs that have been classified by Urquhart et al., (1994) which are simple consisting of 1 repeating sequence (FESFPS), simple with nonconsensus alleles (HUMTH01 and F13A01), compound consisting of 2 or more different repeat sequences (GABRB15), compound with nonconsensus alleles (vWA), complex repeats (D21S11) and hypervariable repeats (SE33) (Edwards et al., 1992).

In the present study, population database for three STR systems (F13A01, FESFPS and vWA) for Tamil population living in Malaysia was compiled. This multiplex was choosen because the STRs are validated STRs for forensic analysis and contain simple repeat motifs to aid interpretations, have high discriminating power, have high sensitivity across all of the loci and have low intensity stutter peaks and other artifact peaks. The allele distribution has been checked using X^2 test and the allele frequency confirm to Hardy- Weinberg expectations and is compared with other populations.

LITERATURE REVIEW

The important task of a forensic scientist is in identification and individualization of evidence materials. Forensic scientists have been using polymorphic genetic markers such as blood groups and serum proteins that are inherited in simple Mendelian way (Mendel, 1866; Peter, 1959). More information is available about these polymorphic genetic markers, which is the result of systematic evaluation and documentation of the antigens to characterize and individualize forensic human biological specimens (Kirk et al., 1962; Kostaszuk et al., 1974; Race and Sanger, 1975; Walter et al., 1975; Mourant et al., 1976; Schacter et al., 1977; Naidu et al., 1978; Chahal and Papiha, 1981; Sethuraman et al., 1982; Hadley et al., 1984; Geitvik et al., 1987; Lee, 1989; Yung et al., 1989; Tournamille et al., 1995; Lee, 1998) .

Forensic deoxyribonucleic acid (DNA) typing or DNA profiling (Jeffreys et al., 1985) has revolutionized the concept of fingerprints (Butler, 2001) which has entered as a routine analytical work in forensic science institutions, making an obsolete the use of blood groups and other polymorphic biochemical genetic markers. All living organisms are composed of cells (Hooke, 1665), which are the basic integrated units of biological activity. In humans or in any other higher organisms, the hereditary material (Avery, 1944) DNA (Watson and Crick, 1953) is contained in the cell nucleus as seen in the microscope by Walther Fleming (1882) and is known as chromosomes (Easteal et al, 1991).

Human cells have 46 chromosomes (23 pairs) (Tjio and Levan, 1956). A typical human cell has one pair of sex chromosomes and 22 pairs of non-sex chromosomes,

known as autosomes. The units of inheritance are genes. Human beings are determined as having 50,000 to 100,000 genes, which are contained in the chromosomal DNA in the cell nucleus. The science of forensic-DNA analysis, also known as forensic DNA typing was introduced more than a decade ago. DNA is a complex, double stranded molecule that is twisted into a helical form and is known as the "double helix" structure (Watson and Crick, 1953).

The structure of DNA resembles a spiral ladder in which the "sides" are made of sugar-phosphate molecules and the "center" is formed from pairs of chemicals known as bases or nucleotides (Chargaff, 1949). There are only four bases in DNA, which are adenine (A), thymine (T), guanine (G) and cytosine (C). The pairing of these bases is specific: A is always paired with T and C is always paired with G (Chargaff, 1949). The absolute specificity of base pairing also provides a mechanism through which "parent" DNA molecules can be copied to form identical "progeny" DNA molecules in the process of reproduction.

Marshall Nirenberg (1963) and Gobind Khorana (1966) cracked the genetic code. They demonstrated that each of 20 amino acids is coded by a sequence of three bases of the nucleotide. The mechanism is known as replication, which is an opposed process of duplication. This mechanism is possible because the two sides of the parent DNA molecule are complementary rather than identical. In the replication process, the parent DNA molecule "open like a zip" along its length, each side serving as a template for one of the new daughter molecules.

Although genes are composed of DNA and is contained in the chromosomes in the cell nucleus, only a small fraction of that DNA is actually used to form genes. That

is, most of the DNA in our chromosomes has no known function; the portion of such DNA may be more than 95% of the total complement in humans. The presence of so much DNA with no known genetic role in chromosomes is ironically, "flotsam and jetsam". These "junk DNA" is of special interest to the forensic scientist. It is also known that the non-coding DNA contains repeated base-pair sequences arranged in tandem that, while having no known function, are inherited by the individual from his or her parents just as the functional genes.

Coding DNA also contains such repeats, but less commonly than the non-coding DNA. These tandem repeats, in total, make up a molecular-DNA "fingerprint" (Jeffreys et al., 1985) which is believed to be unique for each individual. But, with possible exception in the case of identical twins because the number of repeated sequences can vary from person to person. These non-coding base-pair repeated sequences bear the complicated name variable number tandem repeats, abbreviated to VNTR (Nakamura et al., 1987). David Botstein (1978) discovered very important information on DNA polymorphism, called restriction fragment length polymorphisms (RFLPs).

RFLPs are found throughout the genome and are extremely valuable as genetic markers in human genetic studies (Jeffreys et al., 1985). The polymerase chain reaction (PCR) is an innovative technique for increasing the amount of a specific sequence of DNA in a sample (Mullis et al., 1980). Alec Jeffreys et al (1985) introduced DNA fingerprinting using minisatellite based on the RFLP technique as a method of identification. Many RFLP loci were found useful in paternity testing and for personal identification (Capon et al., 1983; Goodbuorn et al., 1983; Jeffreys et al., 1985).

Population database on many RFLP polymorphisms is also well documented in the literature (Baird et al. 1986; Balazset et al., 1989; Chimera et al., 1989; Kidd et al., 1991). Short tandem repeats (STRs) are similar to VNTRs described above, except that the repeated units are much shorter, ranging from 2 to 7 base pairs in length (Edwards et al., 1991). STRs are found widely distributed in the human genome and they are highly polymorphic and often detected using PCR technique (Erlich, 1991).

STR typing can also be done with degraded biospecimen and the amplification products are less than 400 base pairs (Nakamura et al., 1987). Published population database for many different populations are available (Polymeropoulous et al., 1992; Nellemann, 1994, Nagai et al., 1996, Garofano et al., 1998; Pu et al., 1998; Sinha et al., 1999; add 2000,2001 2002 2003). Published database on ethnic population groups in Malaysia on STRs is very few (Seah et al., 2003; Panneerchelvam et al., 2003). Hence in the present study allelic distribution for three validated STRs –viz F13A01, FESFPS and vWA for Tamil ethnic group was studied.

OBJECTIVES

The purpose of this research is to:

1. Understand the application of rules in genetics and the statistical tests.
2. Compile the distributions pattern of various alleles for F13A01, FESFPS and vWA STRs in random Tamil population of Malaysia for routine use in forensics.

MATERIALS AND METHODS

1.0 MATERIALS

Only sterile reagents and materials were used in this study. The short tandem repeat (STR) typing procedure was followed according to the guidelines of manufacturer of STR kits (GenePrint STR System Technical Manual D004, Promega, USA).

1.1 Sample source

Buccal swabs were collected from 100 unrelated ethnic Tamil individuals of Malaysia. Sterile cotton buds were used and each individual was asked to swipe the cotton buds inside their mouth at both cheeks for at least 10 seconds. The cotton buds were air dried and kept away from direct sunlight in an envelope labeled with subject's full name, age, sex and the state they are from.

1.2 Reagents

Digestion buffer (1M Tris HCl pH 7.5, 0.5M EDTA, 20% SDS, 5M NaCl),

proteinase K (20μg/μl) (Promega, USA), chloroform - isoamyl alcohol (24:1),

3M sodium acetate anhydrate (Fluka Garante), 2M sodium acetate, 70% ethanol, Tris-EDTA buffer, 10% NaOH. 0.5% acetic acid in ethanol, acetic acid, bind saline, Q421A, MSDS (Promega, USA), 10% ammonium persulphate, 40% acrylamide, 10X TBE buffer, 0.5X TBE buffer, distilled water, E- Pure, FFv Multiplex kit consisting of STR 10X buffer (500mM KCl, 100mM Tris-HCl (pH 9), 15mM $MgCl_2$, 1% Triton X-100, 2mM each dNTP), multiplex 10X FFv primer pair mix, Taq DNA polymerase (5u/μl), STR 2X Loading Solution (10mM NaOH, 95% formamide , 0.05% bromophenol blue ,

0.05% xylene cyanol FF) , FFv Allelic Ladder Mix (Promega , USA) , Silver staining solution, Fix/Stop solution, developer solution and Rain X (Blue Coral-Slick 50 , USA) .

1.3 Chemicals

Tris base (Promega, USA), concentrated HCL, Na_2EDTA (Promega, USA), NaOH (Merck, Germany), NaCl (Merck, Germany), sodium dodecyl sulphate (SDS) (Bio-Rad Lab.), chloroform (Merck, Germany), isoamyl alcohol (Merck, Germany), sodium acetate (Merck, Germany), glacial acetic acid (Merck, Germany), absolute ethanol (Merck, Germany), EDTA (Promega, USA), ammonium persulphate (Promega, USA), bisacrylamide (Promega, USA), boric acid (Promega, USA), urea (Promega, USA), silver nitrate (Promega, USA), 37% formaldehyde (Promega, USA), sodium thiosulphate (Promega, USA), sodium carbonate (Promega, USA), TEMED (N,N N'N' tetramethylethylene diamine)(Promega, USA), phenol (PIERCE, USA), ethidium bromide (Sigma, USA), Agarose (Promega, USA), and Orange-G dye (Sigma, USA).

1.4 Apparatus

Desiccators Nucerite (Nalgene/Syborn Corp), Vortex mixer EVM-6000 (ERLA), Spectrafuge 16M (National Labnet Co., USA), Gilson varying volume pipette (France) (1000µl, 200µl, 100µl, 20µl and 10µl), parafilm "M" (Pechiney Plastic packaging, Chicago), MJ Research PTC-200 Peltier Thermal Cycler (Gradient cycler) used for 0.2µl tubes, MJ Research PTC-100™ Peltier Thermal Cycler used for 0.5µl tubes, monopan balance sensitive for smaller weights (DRAGON 204 Mettler Toledo), Fisher & Pagkal N308 Freezer, Hitachi refrigerator, Mammart waterbath, SA 32 Electrophoresis apparatus

(GIBCO BRL Sequencing System) , High voltage powerpack EC 3000-90 (E-C Apparatus Co., USA), fumehood (Model : RICO), plastic trays for staining , stopwatch, Greiner bio-one filter tip 100, Microwave (Sanyo), Elite 300 Plus (Wealtec), Photo printer P91D UVP (Mitsubishi), Software on Chemi System, UVP (Bioimaging system), UV Transilluminator, EpiChemi Darkroom, UVP (Bioimaging system), pH Cyberscan 1000, Model RS232 Meter (Eutech Instruments, Singapore), Hot plate and magnetic stirrer, EMS-HP-7000 (ERLA), Disposable latex/ vinyl examination gloves, MF 22 (Fiocchetti, Italy), DuranFavorit WHL (Genristo Ltd, England), E-pure machine (Barnstead), Diamond aluminium foil (Renaults Consumer Products, USA), Sharps collector (Dispo-Med, Malaysia), 3M Comply™ Indicator Tape, C-fold Handtowels (Scott), Penguin Double Clips No.0222, B51 (China), Multipurpose assorted buds (Wang Zheng Co., Malaysia), Eve-Tape, 50ml Syringe W12875 (B-D, Singapore), Pharmacia Gel Electrophoresis Apparatus GNA-100 (Pharmacia Biotech, Sweden), short and long glass plates, Spacers, Combs, Beakers, Conical flask (Asahi Techno Glass), WTF Binder 7200 (Tuttlingen, Germany) and Autoclave: Steam Sterilizer Model MC-30LDP (ALP Co., Japan).

2.0 REAGENT PREPARATION AND METHODOLOGY

2.1 DNA Extraction Reagents

2.1.1 Reagent preparations

1M Tris HCl pH 7.5

121.1g Tris base is dissolved in 800ml deionised water and the pH is adjusted to 7.5 with concentrated HCl. Solution is made up to 1000ml and autoclaved.

0.5M EDTA

186.1g Na_2EDTA is dissolved in 800ml deionised water and the pH is adjusted to 8.0 with NaOH pellets. The solution is made up to 1000ml and autoclaved.

5M NaCl

292.2g NaCl is added to 800ml deionised water and made up to 1000ml.

20% SDS

An empty bottle is autoclaved. 100g Sodium dodecyl sulphate is added to 400ml deionised water. Solution is stirred with heat using magnetic stirrer. The solution was made up to 500ml and stored in an autoclave bottle.

Digestion buffer (Do not autoclave)

1ml of 1M Tris HCL pH7.5, 2ml of 0.5M EDTA, 10ml of 20% SDS, 1ml of 5M NaCl and 86ml of deionised water are mixed together. This solution should be autoclaved.

Proteinase K

20 mg of Proteinase K is mixed with 1ml of deionised water.

Chloroform - isoamyl alcohol (24:1)

240ml of chloroform is added to 10ml of isoamyl alcohol.

3M sodium acetate

102.025g of sodium acetate are added to 200ml of deionised water. The pH is adjusted to 5.2 with glacial acetic acid. Solution is made up to 250ml and autoclaved.

2M sodium acetate

16ml of 3M sodium acetate is dissolved in 8ml to deionised water.

70 % ethanol

350ml of absolute ethanol is mixed with 150ml deionised water.

TE buffer

10ml of 1M Tris HCL is mixed with 0.2ml 0.5M EDTA. 989.8ml of deionised water is added and autoclaved.

2.1.2 Extraction Method

The cotton swabs are cut into small pieces and placed into a 1.5ml microfuge tube. 500µl digestion buffer and 12µl proteinase K (20mg/ml) is added into the sample and incubated overnight at 56°C. 120µl buffered phenol is added and mixed vigorously using vortex. The samples are centrifuged at 10,000 rpm for 3 minutes. By using cut tips, the supernatant of the sample is transferred to a new 1.5 ml tube. To this tube, 1 volume (250µl) of buffered phenol and chloroform- isoamyl is added. The sample is mixed vigorously and again centrifuged at 10,000 rpm for 3 minutes.

Using cut tips, the supernatant is again transferred to a new tube. 1 volume of Chloroform – isoamyl alcohol (same volume as supernatant) is added to the sample and mixed vigorously. Samples were centrifuged at 10 000 rpm for 5 minutes. Supernatant is transferred to a new tube using cut tips. 500μl chilled ethanol and 50μl 2M sodium acetate are added to the sample. The tube is inverted slowly to mix the sample. The sample is then centrifuged for 10 minutes at 10000 rpm. The supernatant is discarded and 0.5 ml 70% ethanol is added.

The precipitated DNA pellet is dislodged by tapping the bottom of the tube and again centrifuged at 10 000 rpm for 3 minutes. Supernatant is discarded. The tubes were sealed with parafilm and the pellets are dried using vacuum pump. On drying, 50μl TE Buffer is added and kept overnight at 37°C. The samples are then stored in freezer at 20°C for further use.

2.2 Detection of presence of DNA using Agarose Gel Electrophoresis

2.2.1 Preparation of reagents

0.5x TBE Buffer

25ml 10x TBE Buffer is diluted in 475ml distilled water.

1% Agarose Gel

1g of agarose is added in 100ml 0.5x TBE Buffer.

2.2.2 Agarose Gel Electrophoresis Method

The 1% agarose solution is heated in the microwave oven for 3 minutes. The bottle cap is not closed tightly. Meanwhile, 10 well comb is positioned in the plate. The agarose TBE buffer is taken out and cooled under the pipe water by holding it with a cloth. For 100ml agarose TBE buffer, 10µl ethidium bromide is added. This solution is then mixed gently. The casting is then poured on the plate without any air bubble formation. The plate is left for 30 minutes. After the gel had polymerized, the comb is removed.

0.5x TBE Buffer is poured into the casting until it covers the gel and touches the electric connection. For the loading of the sample, 1µl of the orange G loading dye is added with 5µl sample. Then, first sample is added to the first well. The power pack is set to 60 minutes, V=100. The power pack is switched on. The movement of the dye downwards is observed to make sure that the power is not connected wrongly. The apparatus is stopped before the dye reaches the bottom of the gel. The results were observed under UV lighting. This is to determine the presence of the DNA from the extracted sample and their concentration.

2.3 Quantification of DNA

3µl extracted DNA from each sample is quantified using spectrophotometer. The optical density (OD) of each sample was done at A_{260} and the DNA samples are diluted appropriately.

2.4 PCR Amplification

The amplification of F13A01, FESFPS and vWA were performed according to the manufacturer's recommendations (Promega Corporation, Madison). The STR 10X Buffer and STR 10X FFv Primer pairs are thawed and kept in ice. The number of reactions to be set up is determined. 1 or 2 reactions are added to this number to compensate for pipetting errors. The required amount for each component of the PCR master mix is calculated as in the Table 1. The volume per sample is multiplied by the total number of reactions to obtain the final volume. The volume for each component is added to a sterile tube.

Table 1. PCR Master mix Components

PCR Master Mix Component	Volume per sample (µl)
Sterile water	17.35
STR 10X Buffer	2.50
FFv Multiplex 10X Primer Pair Mix	2.50
Taq DNA Polymerase (5u/µl)	0.15 (0.75u)
Total volume	22.50

22.5µl of PCR master mix is added to each tube and placed in ice. 2.5µl of sample is pipetted into the respective tubes containing 22.5 µl of the PCR master mix. The samples are then spun briefly to bring the contents to the bottom of the tube. The recommended PCR protocol is selected used to run the samples as in the Table 2.

Table 2. Amplification protocol for FFv multiplex

Initial denaturation	Cycling for first 10 cycles	Cycling for last 20 cycles	Extension Step	Hold step
96°C for 2 minutes	94°C, 1 minute 60C, 1 minute 70°C, 1.5 minute	90°C, 1 minute 60°C 1 minute 70°C, 1.5 minute	60°C for 30 minutes	4°C

The primers for the STRs that were used are as follows (Pena, et al., 1994)

VWA 3' GCC CTA GTG GAT GAT AAG AAT AAT CAG TAT GTG 5' (forward)

3' GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG 5' (reverse)

F13A01 3' GAG GTT GCA CTC CAG CCT TTG CAA 5' (forward)

3' TTC CTG AAT CAT CCC AGA GCC ACA 3' (reverse)

FESFPS 3' GCT GTT AAT TCA TGT AGG GAA GGC 5' (forward)

3' GTA GTC CCA GCT ACT TGG CTA CTC 5' (reverse)

The tubes are then placed in the thermal cycler for amplification. The thermal cycler is shown in Figure 1.

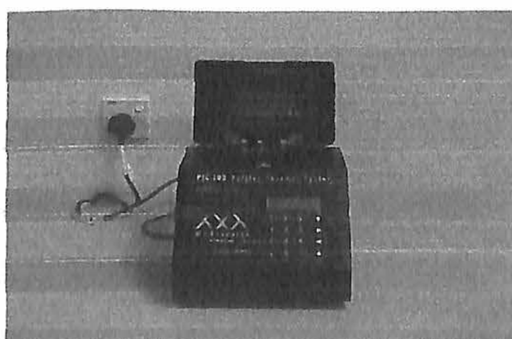

Figure 1. Thermal Cycler

2.5 Electrophoresis

2.5.1 Reagent Preparation

10 % NaOH

100g of NaOH pellets are dissolved in 1000ml of deionised water.

0.5 % acetic acid in ethanol

0.25ml acetic acid is added to 49.75ml absolute ethanol.

Bind silane

3µl bind silane (silver stain kit) is added to 1ml of 0.5% acetic acid in ethanol.

20 % ammonium persulphate

0.2g of ammonium persulphate (AP) is dissolved in 1ml deionised water. Solution must be freshly prepared.

40 % acrylamide : bisacrylamide(19:1)

190.0g of acrylamide and 10.0g of bisacrylamide are dissolved in 250ml of deionised water. Solution is made up to 500ml and kept chilled. Solution is stored in an amber bottle.

10X TBE buffer

107.8g of Tris base and 7.44g of Na_2EDTA are dissolved together in 800ml of deionised water. 46.0g boric acid is added slowly. The pH is adjusted to 8.3 by adding extra 9.0g of boric acid. Solution is made up to 1000ml and autoclaved.

0.5X TBE buffer

50 ml 10X TBE buffer is added to 950ml deionised water.

6% Acrylamide gel solution

25.2g of urea, 3.0ml of 10X TBE buffer, 9ml of 40% acrylamide and 29ml of deionised water are mixed together in a 100ml beaker. 40µl of TEMED and 400µl of 20% ammonium persulphate are added just before loading the sample into the well.

2.5.2 Electrophoresis methodology

A long and a short glass plates are used. Short plate is cleaned with 95% ethanol using tissue paper. 3ml of Rain-X water repellent is applied to the longer plate. The solution is spread all over the plate with a dry paper towel using circular motion. Fresh bind silane is prepared and is poured. The short plate is wiped with a tissue paper using circular motion. After 5 minutes, the short glass plate was wiped again with 95% ethanol for three to four times. Long plates are cleaned with 95% ethanol with tissue paper. 3ml of Rain-X water repellent is applied to the longer plate.

The long plate is wiped with deionised water. 0.4mm spacer is placed on each side of the long plate. Both the plates are assembled and sealed with sealing tape. A 6%

acrylamide gel solution is prepared and filtered using the 0.2 µ filter. The components of the acrylamide gel solution are shown in Table 3.

Table 3. Components of 6% acrylamide gel solution

Components	Quantity
Urea	25.2g
Deionised water	29.0 ml
10X TBE	3.0 ml
40% acrylamide (19:1)	9.0 ml
Total	60 ml

Then, 40µl TEMED (N, N N'N' tetramethylethylene diamine) and 400µl of 10% ammonium persulphate are added. Using a 50cc disposable syringe with 21-gauge needle, the acrylamide solution is poured between the glass plates.

The glass plate with the gel is positioned in a slant and the straight side of the sharktooth comb is inserted. The gel is left to polymerize for two hours. The sealing tapes are removed and the outside of the glass plates are cleaned. 0.5X TBE buffer is added to the bottom chamber of the electrophoresis apparatus. The glass plates are placed in the electrophoresis apparatus avoiding air bubbles, long plate facing out. The glass plates are secured by tightening the clamps. 0.5X TBE buffer is added to the upper buffer chamber.

Air bubbles on the top of the gel are removed and the apparatus is assembled for pre run for half an hour at 40 watts. Meanwhile, the PCR products are prepared for loading. 2.5µl of STR 2X loading solution and 2.5µl of amplified product are mixed in a tube. For each ladder lane, 2.5µl of STR 2X loading solution and 2.5µl of allelic ladder provided in the kits is mixed. The amplified samples and allelic ladder mix are denatured

at 95°C for 2 minutes. The samples are placed on ice immediately after denaturing. After the pre run, the buffer is flushed again with syringe.

The sharktooth comb is inserted into the gel. 5μl of the denatured samples and allelic ladder mix is loaded into the wells. The gel is run at 40 watts until the dye has reached almost the bottom of the plate. The plates are removed from the electrophoresis apparatus and the short plate, with the gel attached, is stained. A diagram of the electrophoresis apparatus is shown in Figure 2.

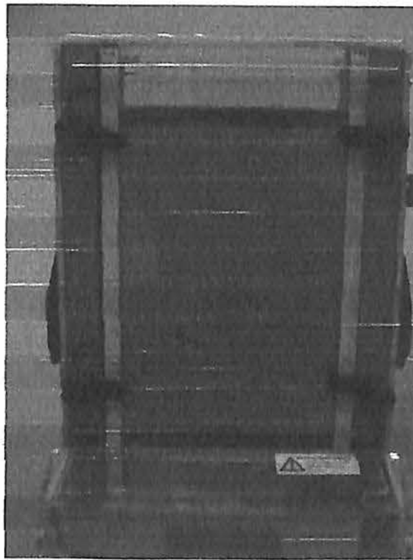

Figure 2. SA 32 sequencing electrophoresis (GIBCO BRL Sequencing system)

2.6 Silver Staining

2.6.1 Reagents preparation for staining

Silver nitrate solution

In a beaker, 600ml of deionised water is added. Then, 0.6g of silver nitrate and 0.9ml of 37% formaldehyde are added in and dissolved. The beaker with contents is wrapped with aluminium foil and stored at 4 °C.

Fix/stop solution

120ml of glacial acetic acid is added to 480 ml of deionised water.

Gel developer

0.6ml of 37% formaldehyde is added to 120µl of sodium thiosulphate and 18g of sodium carbonate. Solution is made up to 600ml using deionised water.

2.6.2 Silver Staining Methods

The gel bands were detected by silver staining method following the kits manufacturer guidelines (Promega, USA). The protocol is as follows; the shorter glass plate with gel is placed into the fix or stop solution for 20 minutes. Then, it is washed in deionised water for 2 minutes. The washing is repeated for another 2 minutes with fresh deionised water. The gel is stained for 30 minutes in the silver staining solution. A brief rinsing for 10 seconds is done with deionised water, immediately after the staining. The gel is then placed into the developer solution for 5 minutes or until the alleles and ladders are visible. The developed gel is fixed with the stop solution for 5 minutes. Finally, the gel is washed in deionised water for 2 minutes. The shorter plate is dried in upright position.