UNIVERSITI SAINS MALAYSIA

UNIVERSITI SAINS MALAYSIA

Allele frequencies of STRs (F13A01, FESFPS and vWA) in random Chinese population of Malaysia

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

Saravanakumar A/L Maniam

School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150 , Kubang Kerian ,
Kelantan , Malaysia

2004

CERTIFICATE

This is to certify that the dissertation entitled

"Allele frequencies of STRs (F13A01, FESFPS and vWA) in random

Chinese population of Malaysia"

is the bonafide record of research work done by

Mr. SARAVANAKUMAR A/L MANIAM

during the period <u>8 November 2003</u> to <u>26 January 2004</u>……

under my / our supervision.

Signature of Supervisor: ……………………………………………………

Name and address of Supervisor: S.Paneerchelvam,
Lecturer ,
School of Health Sciences ,
Universiti Sains Malaysia ,
16150 , Kubang Kerian , Kelantan.

Date: 04. 03. 2004 .

Signature of Co-Supervisor: ……………………………………………………

Name and address of Co- Supervisor: Prof. Dr Norazmi Mohd. Nor
Deputy Dean,
School of Health Sciences ,
Universiti Sains Malaysia ,
16150 , Kubang Kerian , Kelantan.

Date: 4/3/04

hesitation and to those who have contributed but their names were not mentioned here.

May the study done will benefit people who encounter it. Thanks again

TABLE OF CONTENTS

LIST OF TABLES, FIGURES, GRAPHS AND ILLUSTRATION

TABLES

FIGURE

ABSTRACT

Biochemical and molecular genetic markers have played a vital role in forensic analysis of bio-specimens and in kingships testing. However with the advent of DNA profiling technique positive identification has become a reality. The science of DNA profiling had revamped analytical procedures employed in the analysis. Short Tandem Repeats (STRs) DNA profiling is widely used in most of the laboratories for personal identification. A population database on the STR to be employed for analysis is a prerequisite. In the present study distribution of allele frequencies for three validated STRs – F13A01, FESFPS, vWA was compiled for the ethnic Chinese population of Malaysia. DNA samples form 100 random Chinese individual were processed using multiplex primer mix. There is no significant deviation from the Hardy-Weinberg equilibrium. The discrimination power of the three STRs is 0.9987.

INTRODUCTION

Throughout history, detectives have searched for the perfect method of catching law breakers, but criminals have the advantages of stealth. A crime can happen in the middle of the night or in an isolated area where no witnesses are present. It's the evidence left behind that is the true witness to their crime. According to Locard (1930) whenever an object comes in contact with another object, cross-transfer of evidence will take place. Biological evidence is the commonest physical evidence met in every crime scene. With the advancement of technologies relating to DNA profiling it is possible to associate a forensic bio-specimen to a particular individual with certainty because of the powerful discrimination among individuals and potential to identify criminal suspects from a minute amount of biological specimen.

The human genome contains approximately 3 billion nucleotides and exhibits variation ranging from 1 in every 100 to 1 in every 1000 nucleotides. Variation in the human genome is located in non-coding region which contain multiple repeat of certain nucleotide sequences (Wyman and White, 1980). The repeat sequence differs in numbers between individuals and thus gives rise to alleles of different length. This type of polymorphism is referred to as length polymorphism.

Individual identification by DNA analysis became a practical reality when Alec J. Jeffreys (1985) identified the hyper variable minisatellites or variable number of tandem repeat (VNTR) loci in the human genome. Several of VNTR regions can be detected simultaneously using a multi locus probe (MLP) based restricted fragment length polymorphism. (RFLP) (Jeffreys et al., 1986), which contains a core sequence common to many of the VNTR loci. The patterns revealed after Southern blot hybridization using

MLPs are termed individual specific "DNA fingerprints". The main probability that two randomly chosen, unrelated individuals posses the same DNA fingerprint has been calculated to be 3×10^{-11} when one multi-locus probe is used, thus DNA fingerprinting potentially allows an absolute identification of an individual. DNA fingerprinting by Restriction Fragment Length Polymorphisms (RFLP) analysis requires a relatively large and qualitative amount of DNA, which limit its usefulness in forensic casework. It is not possible to assign maternal or paternal alleles in RFLP analysis. The advent of single locus probe (SLP) profiling has over come this difficulty.

Then came another class of DNA polymorphisms called short tandem repeats (STR) typing also known as microsatellites analysis characterized by length variation in tandem arrays of simple repeat sequences of 2 to 6 base pairs. Short Tandem Repeats provide a rich source of polymorphic markers in the human genome. STR loci display several advantages that make them attractive genetic markers. STRs have high power of discrimination and rapid analysis when compared with the SLPs. Also STRs are short fragments and they can be analyzed three or more at a time, parallel with the development of a technique known as polymerase chain reaction (PCR) (Saiki et al., 1985 and Mullis et al., 1986). The PCR technology uses basic cellular chemistry and enzymes in a controlled "molecular copying process" to synthesize (amplify) exponential numbers or large quantity of certain segment or target sequence of a small amount (degraded DNA samples) of DNA. Therefore multiple STRs can be examined in the same DNA test, or multiplexed as long as the appropriate PCR primers for the region of interest are available.

Multiplexing is very valuable method in typing of sample mixtures and biological materials containing degraded DNA molecules. In this study, FFv triplex multiplex primer kit (Promega, USA) was used for STR profiling of F13A01, FESFPS and vWA STRs for ethnic Chinese population in Malaysia.

LITERATURE REVIEW

For a long time, forensic scientists have been using polymorphic genetic markers such as blood groups and serum proteins, which are inherited in simple Mendelian way (Peter , 1959) to establish identification and individualization of evidence to particular individuals or perpetrator. Karl Landsteiner (1901) (Race and Sanger, 1952; Peter , 1959) predicted that individuals can be placed into different groups on the basis of their blood types. Through this discovery, a range of genetic markers have been used to link bloodstains found at crime scene to suspects and victims. More studies has been conducted and crucial information is known about these polymorphic genetic markers, which is the result of systematic evaluation and documentation of these antigens to characterize and individualize forensic human biospecimen (Race and Sanger , 1952 ; Sanger et al. , 1952 ; Kirk et al. , 1962 ; Prokop and Uhlenbruk, 1965 ; Race and Sanger , 1975 ; Mourant et al. , 1976 ; Schacter et al. , 1977 ; Need et al. , 1977a ; Naidu et al. , 1978 ; Grunbaum et al. , 1981 ; Sethuraman et al. , 1982 ; Norrgard et al. , 1984 ; Salzano et al. , 1985 ; Swart et al. , 1985 ; Mohanraj et al. , 1986 ; Yung et al. , 1989 ; Lee , 1989; Chaudhuri et al. , 1993 and Tournamille et al. , 1995) . In the past the forensic bio-specimen analysis has been directed toward the detection of genetic variation expressed at the level of blood and soluble protein markers. Not long after that in mid 1980's Forensic DNA Fingerprinting were introduced in criminal investigations. Forensic DNA Fingerprinting or DNA typing or DNA profiling (Jeffreys et al., 1985) is a revolutionary in concept than fingerprints has entered in routine use in forensic science laboratories making obsolete the use of blood groups and other polymorphic biochemical genetic markers . This is because the genetic variation among individuals is higher at the

5

DNA level than at the protein level. The DNA typing is probably one of the most important advances in the forensic sciences in recent years.

All living organisms are composed of cells (Hooke, 1665, Peter, 1959), the basic integrated units of biological activity. This observation or finding was made possible through the invention of microscope by Galileo. Johann Gregor Mendel (1860) (Peter J A, 1959) was the first scientist to deduce clear and rational laws, which could explain the process of inheritance through observations about the inheritance of such characteristic as colour and shape in pea plants. He showed that these characteristics were controlled by hereditary factors, later known as gene (Sutton, 1902). Even though the concepts of inheritance were discovered but the basis material of inheritance was still unknown. A substance now known as nuclein (DNA) isolated from nucleus was discovered by Frederich Miesher in 1869 (Krawczak , and Schmidtke , 1998). Later DNA (Watson and Crick, 1953) was proved to carries genetic information which is the hereditary material.

In human cells, the hereditary material deoxyribonucleic acid or DNA is tightly wound up as chromosomes. Chromosomes are paired threadlike "packages" of long segments of DNA contained within the nucleus of each cell. In human the number of chromosomes are 46 (Tjio and Levan, 1956), being 23 pairs as each person inherits a chromosome from their mother and a chromosome from their father. In 22 pairs, both members are essentially identical. The 23rd pair also known as sex chromosomes is different. In females this pair has two like chromosomes called "X". In males it comprises one "X" and one "Y," two very dissimilar chromosomes. It is these chromosome differences, which determine sex. The maternal and paternal chromosomes of a pair are called homologous chromosomes (Peter, 1959). The chromosomes must contain a

centromere (middle of the band), and two telomeres at the end of the band. The display of the 46 chromosomes at mitosis is cell called the human karyotype (Peter, 1959). The function of the chromosomes is to carry genes. Gene is defined as one or more segments of DNA whose sequence encodes a particular RNA molecule (Paul, et .al 1990). The genes is composed of regions called exon (coding region) that codes for protein and intron(non-coding region) ,the intervening portions or connecter between two exon. Alleles are an alternative term for a gene or genetic locus. If alleles at a particular location (locus) in the genome are the same on both chromosomes of a pair, the situation is called homozygous. And if a small difference is present on a locus such that different alleles are present on each chromosome, the situation is called heterozygous.

Stretching out the DNA of single human cell can reach approximately 2 m in length (Krawczak and Schmidtke , 1998). Human genome consists of approximately 3 billion base pairs (bp) in which there are estimated to be 50,000 - 100,000 genes. DNA is a polynucleotide or also known as polymer made up of individual deoxyribonucleotide units. A single deoxyribonucleotide unit is composed of a sugar, a nitrogenous base, and a phosphate group (Figure 1a, 1b). The sugar and phosphate group are the backbone structure of DNA molecule whereas the bases are the genetic information carrier. The nitrogenous base can be divided to purine and pyrimidine (Chargaff, 1949). The purines are adenine (A) and guanine (G), and pyrimidines are thymine (T) and cytosine (C). The bases are attached to the doexyribose by N-glycosidic linkage between the C-1 carbon atom of the sugar, and the N-9 nitrogen atom of the purine or the N-1 nitrogen of the pyrimidine.

7

DNA is a complex double-chained molecule twisted into a helical form: the "double helix" structure (Watson and Crick, 1953). The pairing of the bases is specific: adenine is always paired with thymine and cytosine is always paired with guanine (Chargaff, 1949). The absolute specificity of base-pairing also provides a mechanism through which "parent" DNA molecules can be copied to form identical "daughter" DNA molecules in the process of reproduction. Marshall Nirenberg (1963), and Gobind Khorana (1966), lead teams that crack the genetic code and prove that each of 20 amino acids is coded by a sequence of three nucleotide base. The mechanism, known as replication (as opposed to duplication), is possible because the two sides of the parent DNA molecule are complementary but not identical. In the replication process, the parent DNA molecule "splits" into two and each side act as a template for one of the new (and identical) daughter molecules .

Although genes are composed of DNA and contained in the chromosomes in the nucleus of the cell, only a small fraction of that DNA is actually used to form genes that is to say 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 chromosomes also caries a large excess of interspersed DNA that does not any coding or critical information and usually called junk DNA to signify no usefulness. Some of this stretches contain many copies of particular DNA sequences known as repetitive regions and are called "satellite DNA" (Paul et al., 1990). These repeat regions can be a great deal of variability and is the key to some of the technique used in forensic science. Coding DNA also contains such repeats, but less commonly than non-coding DNA. These tandem repeats, in total, make up a molecular-DNA "fingerprint" (Jeffreys et al., 1985) that is

believed to be unique for each individual (with the possible exception of identical twins) because the number of repeated sequences can vary from person to person. These non-coding base-pair repeated sequences bear the name variable number tandem repeats, abbreviated to VNTR (Nakamura et al., 1987). David Botstein and co-workers, 1978, discover a very useful type of 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).

Alec Jeffreys et al., 1985, introduced DNA fingerprinting using minisatellite based RFLP technique as a method of identification. Many RFLP loci useful for use in paternity testing and for personal identification were widely documented (Capon et al., 1983; Goodbuorn et al., 1983; Jeffreys et al., 1985; Clark, 1987 and Delvin et al., 1990). Population data base on many RFLP polymorphism is also well documented in the literature (Baird et al. 1986; Long et al., 1986a; Long et al., 1986b; Balazset et al., 1989; Chimera et al., 1989; Deka, 1991 and Kidd et al., 1991). Short tandem repeats (STRs) consist of simple tandemly repeated sequences, 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 highly abundant where there are as many as half a million STR loci in the human genome, occurring on average every 6-10kb (Beamann and Weber, 1992) and often-detected using PCR (Mullis et al., 1987; Saiki et al., 1988; Sambrook et al., 1989; Innis et al., 1990 and Erlich, 1991). Although there is a large element of size variation, STRs span segments of DNA small enough to be amenable to PCR amplification and which can still be detected in highly degraded DNA (Hagelberg et al., 1991; Jeffreys et al, 1992). In the literature, population data base for many different

many different populations are available (Edwards et al.1991; Polymeropoulous et al.,1992; Budowle et al.,1994; Nellemann, 1994; Pfitzinger, 1995, Andres, 1996; Nagai et al 1996; Lorente et al., 1997; Garofano et al., 1998; Pu et al., 1998; Sinha et al.1999; Panneerchelvam et al., 2001; Filoglu et al., 2002; Hadi et al., 2002; Luiz et al., 2002; Morales ,2002; Lizandrina et al., 2003 and Miguel et al., 2003). Published data base on ethnic population groups in Malaysia on STRs is very few (Seah et al., 2003 and Panneerchelvam et al., 2003). Hence in the present study allelic distribution for three validated STRs –viz F13A01, FESFPS and vWA for Chinese ethnic group was studied.
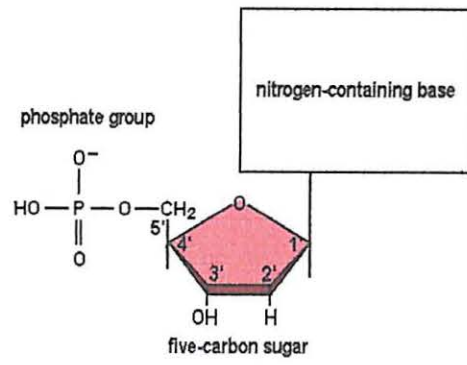
Figure .1a. Structure of deoxyribonucleotide
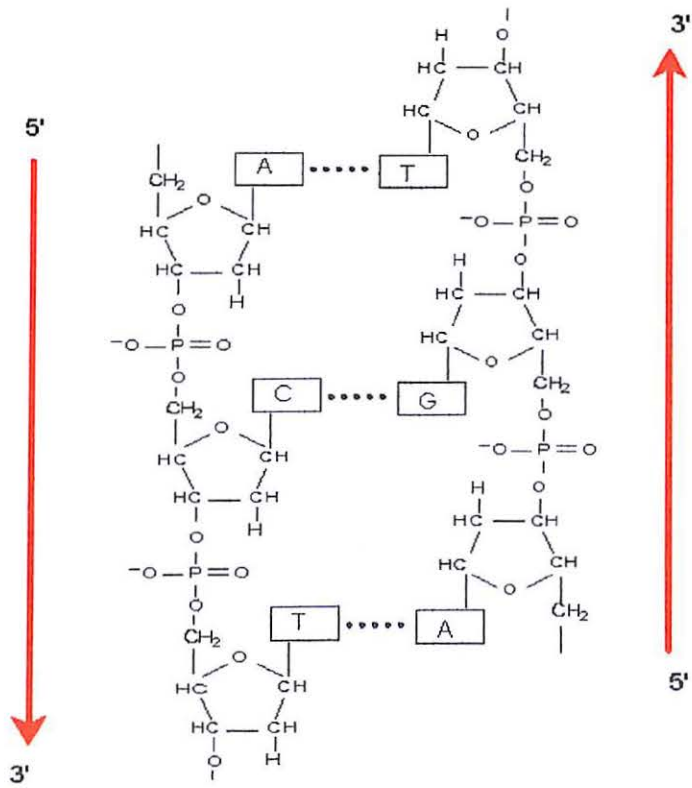
Figure .1b. Schematic drawing showing base pairing between two strands of DNA.

OBJECTIVE OF THE STUDY

- To develop a database for three STRs – F13A01, FESFPS, vWA for Chinese population of Malaysia that can be referred throughout world.

- To compare the allelic distribution pattern of STR of Chinese population with other populations.

MATERIALS AND METHODS

Saliva samples (buccal swabs) from 100 random Chinese individuals

DNA extraction using salting out procedure

PCR amplification of DNA samples using FFv multiplex primer kit.

Electrophoresis of amplified products

Developing the allele band with silver staining technique

Documentation and Statistical analysis

Figure .2. Schematic representation of analytical protocol.

1.0 MATERIALS

Materials and reagents were used in this study were made sterile by autoclaving. The STR typing was done according to the guidelines given by the manufacturer of STR kits (GenePrint STR System Technical Manual D004, Promega, USA).

1.1 Sample source

Buccal swabs were taken from 100 unrelated random Chinese individuals within Malaysia. Sterile cotton buds were used. Each individual was asked to streak the cotton buds from inside their mouth towards cheek at least 10 seconds. The cotton buds were air dried at room temperature away from direct sunlight and kept in an envelope (9cm x 15cm) labeled with particulars such as subject's name, father's name, age, sex and the place of domicile. Two samples (buccal swab) were taken from each individual.

1.2 Reagents

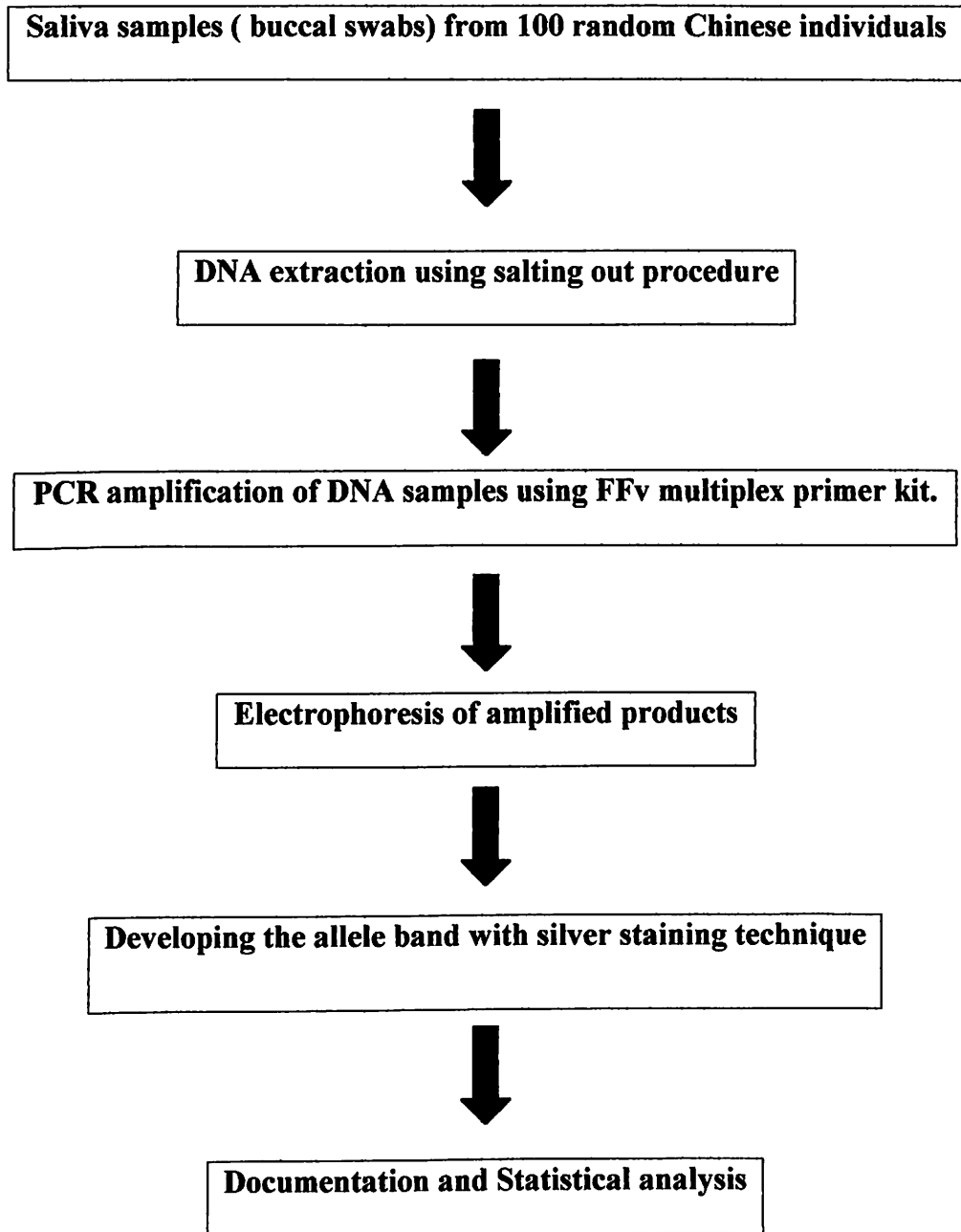Proteinase K (20µg/µl) (Promega, USA), Digestion buffer (1M Tris HCl pH 7.5, 0.5M EDTA, 20% SDS, 5M NaCl), 3M sodium acetate, 2M sodium acetate, 70% ethanol, TE buffer, 10% NaOH, 0.5% acetic acid in ethanol, chloroform - isoamyl alcohol (24:1), bind saline (Promega, USA), 10% ammonium persulphate, 40% acrylamide, 10X TBE buffer, 0.5X TBE buffer, FFv Multiplex kit consisting of STR 10X buffer (500mM KCl , 100mM Tris-HCl (pH 9), 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), 15mM

MgCl$_2$, FFv Allelic Ladder Mix (Promega , USA), silver nitrate staining solution, fix/stop solution, acrylamide solution, gel developer , rain X (Blue Coral-Slick 50 , USA) .

1.3 Chemicals

NaOH (Merck, Germany), NaCl (Merck, Germany), chloroform (Merck, Germany), isoamyl alcohol (Merck, Germany), sodium acetate (Merck, Germany), concentrated HCl, Na$_2$EDTA (Promega, USA), glacial acetic acid (Merck, Germany), absolute ethanol (Merck, Germany), EDTA (Promega, USA), sodium dedocyl sulphate (SDS) (Bio-Rad Lab.), ammonium persulphate (Promega, USA), acrylamide (Promega, USA), bisacrylamide (Promega, USA), boric acid (Promega, USA), urea (Promega, USA), silver nitrate (Promega, USA), tris base (Promega, USA), 37% formaldehyde (Promega, USA), sodium thiosulphate (Promega, USA), sodium carbonate (Promega, USA) phenol (PIERCE, USA) and TEMED (N,N N'N' tetramethylethylene diamine)(Promega, USA).

1.4 Apparatus

Vortex mixer EVM-6000 (ERLA), Biometra-Gene Ray UV-photometer , cuvette, Gilson varying volume pipette (1000μl, 200μl, 100μl, 20μl and 10μl), parafilm (American National Can), microfuge 16M (National Labnet Co.), MJ Research PTC-200 Peltier Thermal Cycler (Gradient cycler) used for 0.2μl tubes, Desiccators (Nalgene/Syborn Corp), 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, High voltage powepask (EC 3000-90) , Hitachi refrigerator, Mammart

water bath, fumehood (Model : RICO), plastic trays for staining , stopwatch SA 32 Electrophoresis apparatus (GIBCO BRL Sequencing System) .

2.0 REAGENT PREPARATION AND METHODOLOGY

2.1 DNA Extraction Reagents

2.1.1 Reagent preparations

<u>1M Tris HCl pH 7.5</u>

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

<u>0.5M EDTA</u>

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

<u>5M NaCl</u>

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

<u>20% SDS</u>

100g sodium dodecyl sulphate (SDS) was dissolved in 400ml sterile deionized water. The solution was made up to 500ml.

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.

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 is 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 added to 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 were cut into small pieces and placed into a 1.5ml microfuge tube. 500μl digestion buffer and 12μl Protease K (20mg/ml) was added into the sample and incubated overnight at 56°C. Next morning, 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 was transferred to another 1.5 ml tube. To this, 1 volume (250μl) of buffered phenol and chloroform- isoamyl was added. The contents were mixed vigorously and centrifuged at 10,000 rpm for 3 minutes. Using cut tips, the supernatant was transferred to a new tube. 1 volume of Chloroform – isoamyl alcohol (same volume as supernatant) was added to the sample and mixed vigorously. Then the contents in the tube centrifuged at 10 000 rpm for 5 minutes. Supernatant was transferred to a new microfuge tube using cut tips. 500μl chilled ethanol and 50μl 2M sodium acetate were added to the sample. The sample was mixed by inverting the tube slowly. The sample was then centrifuged for 10 minutes at 10 000 rpm. The supernatant was then discarded and 0.5 ml 70% ethanol is added. The precipitated DNA pellet was dislodged and centrifuged at 10 000 rpm for 3 minutes. Supernatant was discarded. The tubes were sealed with parafilm and the pellets are dried using vacuum pump. Upon drying, 50μl TE Buffer was added and kept overnight at 37°C. The samples were stored at in -20°C for further use.

2.2 Quantification of DNA

2.2.1 Reagents preparations

1g of ethidium bromide working solution were dissolved to 100ml of deionized water.

2.2.2 Preparatory agarose gel electrophoresis and spectrophotometer

DNA for its presence was detected using preparatory agarose gel electrophoresis. 5μl of extracted DNA sample was used. The DNA samples were quantified spectrophotometrically at 260nm. DNA sample appropriately diluted, so that 1μl of DNA sample contained 10ng of DNA.

2.3 PCR Amplification

The amplification of HUMF13A01, HUMFESFPS and HUMvWA was performed according to the manufacturer's recommendations (Promega Corporation, Madison, USA). The STR 10X Buffer and STR 10X FFv Primer pairs are thawed and kept on ice. The number of reactions to be set up was determined. 1 or 2 reactions are added to this number to compensate for pipetting error. The required amount for each component of the PCR Master mix was calculated (Table 1). The volume per sample was multiplied by the total number of reactions to obtain the final volume.

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 on ice. 2.5μl of sample is pipetted into the respective tubes containing 22.5 μl of the PCR master mix. The samples are spinned briefly to bring the contents to the bottom of the tube. The recommended PCR protocol was used for amplification (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 60°C, 1 minute 70°C, 1.5 minute	90°C, 1 minute 60°C, 1 minute 70°C, 1.5 minute	60°C,30 minute	4°C

The tubes are then placed in the thermal cycler for amplification. The thermal cycler is shown in Figure 3.

Figure.3. Thermal cycler

Figure.4. SA 32 sequencing electrophoresis (GIBCO BRL   Sequencing system)

2.4 Electrophoresis

2.4.1 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 added to 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 strored in an amber bottle.

10X TBE buffer

107.8g of Tris base and 7.44g of Na$_2$EDTA 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.

2.4.2 Electrophoresis methodology

A long and a short glass plates are used. At the right corner on non-treated side of each glass plate is marked with permanent marker to distinguish the treated sides of the glass plates. Long and short plate was kept apart during preparation process to prevent cross-contamination and to avoid the plate sticking to each other when the short plate is applied with bind saline.

Long plate: First, the long plate was cleaned with two times with 95% ethanol using tissue paper. On drying the plate was treated with Rain – X (water repellent) using circular motion over the entire glass surface. The plate was allowed to stand for 5 minutes

for the solution to dry. After that the long plate was wiped with paper towel saturated with deionised water.

Short plate: The short plate was wiped with 95 % ethanol two times using tissue paper. The short plate is wiped using tissue paper with bind silane solution. After 5 minutes, the plate was cleaned with 95 % of ethanol for three or four times.

0.4 mm side spacers were placed vertically between the long plate and short plate. The plates were properly sealed with sealing tapes to prevent any leakage while pouring the gel between both plates.

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**

40µl TEMED (N, N N'N' tetramethylethylene diamine) and 400µl of 10% ammonium persulphate are added to the 6% gel solution. Using a 50cc disposable syringe with 21G needle, the acrylamide solution is poured between the glass plates. The gel is positioned in a slant and the straight side of the sharktooth comb is inserted to make the well. The gel is left for polymerization for two hours. Polymerization is dependent on the presence