Chapter Two

MATERIALS AND METHODS
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2.1 Blood samples

Human blood was used as the source of DNA. Blood samples were collected from 310 healthy unrelated individuals, comprising 103 Malays, 106 Chinese, and 101 Indians, from the Malaysian population. Each blood sample, 10 ml, was mixed with 0.4 ml of 0.5 M EDTA (final 20 mM EDTA in 10 ml of blood) to prevent clotting and stored at -20°C until DNA extraction was performed.

2.2 Materials

All chemicals and solvents used were of Analar Grade or of the highest grade available commercially, purchased from Aldrich Chemical Co., U.S.A.; BDH Chemicals Ltd., England; Clontech Laboratories, Inc., U.S.A.; Fluka BioChemika, Switzerland; GIBCO BRL, U.S.A.; Hopkin and William Ltd., England; Riedal-de Haen AG, Germany; and United States Biochemical Corp. (USB), U.S.A.

Cellulose acetate membrane filters with pore size 0.2 μm and diameter 25 mm were purchased from MFS (Micro Filtration Systems), U.S.A.

NuSieve GTG agarose and SeaKem LE agarose were purchased from FMC BioProducts, U.S.A., and acrylamide was from GIBCO BRL.

Restriction endonucleases used are listed in Section 2.3.4.1. Size standards of 1 kb and 100 bp DNA ladders were purchased from GIBCO BRL, and the 20 bp DNA ladder was from Advanced Technologies, U.K.

2.3 Stock solutions and buffers

2.3.1 Stock solutions and buffers for DNA extraction

2.3.1.1 20x SSC (3 M NaCl, 0.3 M sodium citrate)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>175.32 g</td>
</tr>
<tr>
<td>Trisodium citrate.2H₂O</td>
<td>88.32 g</td>
</tr>
</tbody>
</table>
Distilled $\text{H}_2\text{O}$ to 1000 ml

The solution was dispensed into aliquots and autoclaved.

2.3.1.2 2x SSC
Sterile 20x SSC 10 ml
Distilled $\text{H}_2\text{O}$ 90 ml

2.3.1.3 10% (w/v) SDS

SDS (sodium dodecyl sulphate) or SLS (sodium lauryl sulphate), 10 g, was dissolved in distilled $\text{H}_2\text{O}$ to a final volume of 100 ml. The solution was heated to 68°C to assist dissolution. The solution was then kept at room temperature. (Caution: Mask should be worn when handling SDS powder.)

2.3.1.4 80% (v/v) ethanol
Absolute ethanol 80 ml
Distilled $\text{H}_2\text{O}$ 20 ml

2.3.1.5 Buffered phenol-chloroform
Phenol (colourless) 100 ml
Chloroform 100 ml
8-hydroxyquinoline 100 mg
Distilled $\text{H}_2\text{O}$ 20 ml

1 M Tris-Cl (pH 8.8), 0.5 volume, was added to the phenol-chloroform mixture, and the resultant mixture was stirred for 30 min. The phases were allowed to separate and the upper aqueous phase was removed by aspiration. An equal volume of 0.1 M Tris-Cl, pH 8.0, was added to the phenol-chloroform mixture and the resultant mixture was stirred for 30 min. The phases were allowed to separate and the pH of lower organic phase was checked with pH paper. Extraction was repeated until the organic phase reached a pH > 7.8. The solution was stored at 4°C in a brown bottle.
2.3.1.6 2 M NaOAc, pH 7 or 5.6

Sodium acetate (anhydrous) 16.41g
Distilled H₂O to 80 ml

Sodium acetate was dissolved and the solution was adjusted to pH 7 or 5.6 with glacial acetic acid. The solution was adjusted to a final volume of 100 ml with distilled H₂O and the resultant solution was dispensed into aliquots before autoclaving.

2.3.2 Stock solutions and buffers for 11.1x PCR mix

2.3.2.1 2 M Tris-base

Tris crystals 193.6 g
Distilled H₂O to 1000 ml

2.3.2.2 2 M Tris-HCl, pH 7.5

Tris base crystals, 242.28 g, were dissolved in 800 ml of distilled H₂O. The pH was adjusted to 7.5 with concentrated HCl and the total volume adjusted to 1 litre with distilled H₂O. The solution was dispensed into aliquots and autoclaved.

2.3.2.3 2 M Tris-HCl, pH 8.8, 25°C

2 M Tris-Base 817 ml
2 M Tris-HCl, pH 7.5 183 ml

2.3.2.4 1 M MgCl₂

MgCl₂·6H₂O 20.33 g
Distilled H₂O to 100 ml

2.3.2.5 0.5 M EDTA (pH 8.0)

EDTA (ethylenediaminetetraacetate) 16.81 g
Distilled H₂O to 100 ml
2.3.2.6 10 mM EDTA, pH 8.0

0.5 M EDTA 10 ml
Distilled H₂O to 450 ml

2.7.1 Stock solutions and buffers for PCR

2.3.3.1 11.1x PCR mixture (mix)

Table 2 shows the reagents used in 11.1x PCR mix.

Table 2: 11.1x PCR mix.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
<th>Conc. in final reaction (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 M Tris-HCl, pH 8.8</td>
<td>167.0</td>
<td>45 mM</td>
</tr>
<tr>
<td>1 M (NH₄)₂SO₄</td>
<td>83.0</td>
<td>11.1 mM</td>
</tr>
<tr>
<td>1 M MgCl₂</td>
<td>33.5</td>
<td>4.5 mM</td>
</tr>
<tr>
<td>2-mercaptoethanol (100%)</td>
<td>3.6</td>
<td>6.7 mM</td>
</tr>
<tr>
<td>10 mM EDTA, pH 8.0</td>
<td>3.4</td>
<td>4.5 µM</td>
</tr>
<tr>
<td>100 mM dATP</td>
<td>75.0</td>
<td>1 mM</td>
</tr>
<tr>
<td>100 mM dCTP</td>
<td>75.0</td>
<td>1 mM</td>
</tr>
<tr>
<td>100 mM dGTP</td>
<td>75.0</td>
<td>1 mM</td>
</tr>
<tr>
<td>100 mM dTTP</td>
<td>75.0</td>
<td>1 mM</td>
</tr>
<tr>
<td>10 mg/ml BSA</td>
<td>85.0</td>
<td>113 µg/ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>676.5</td>
<td></td>
</tr>
</tbody>
</table>

The buffer was stored at -20°C.

2.3.3.2 Primers

Table 3 lists the oligonucleotide primers used in this study. They are based on Neil and Jeffreys (1993).
Table 3: Primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Number of nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>31A</td>
<td>5’CCCTTTTGACCGCTGGACGTTGGCG3’</td>
<td>24</td>
</tr>
<tr>
<td>31-TAG-A</td>
<td>5’AGGTGGAGGTGTCTGTGAaggctgt3’</td>
<td>27</td>
</tr>
<tr>
<td>31Rsal</td>
<td>5’GGCAGGAAACCCACAGCGAAGCCGTA3’</td>
<td>24</td>
</tr>
<tr>
<td>31F</td>
<td>5’CCACTCGGAACCACCTGCAG3’</td>
<td>20</td>
</tr>
<tr>
<td>31E</td>
<td>5’TGGACAGCCCAAGGCAGGTCCC3’</td>
<td>22</td>
</tr>
<tr>
<td>31Hgal+t</td>
<td>5’aatagactcactatagttacctccccactcagcg3’</td>
<td>36</td>
</tr>
</tbody>
</table>

2.3.3.3 Taq DNA polymerase

Taq DNA polymerase (5 U/μl) from Thermus aquaticus was from Perkin Elmer and Advanced Technologies.

2.3.4 Restriction endonucleases and buffers for digestion of PCR products

2.3.4.1 Restriction enzymes and assay buffers

Table 4 shows the restriction endonucleases and buffers used.

Table 4: Restriction endonucleases and buffers.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Cleavage position</th>
<th>Units/μl</th>
<th>Assay buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu</td>
<td>New England BioLab.</td>
<td>AG^CT</td>
<td>8</td>
<td>NEBuffer 1</td>
</tr>
<tr>
<td>Rsal</td>
<td>New England BioLab.</td>
<td>GT^AC</td>
<td>10</td>
<td>NEBuffer 1</td>
</tr>
<tr>
<td>Psp1406I</td>
<td>Fermentas (MBI)</td>
<td>AA^CGTT</td>
<td>16</td>
<td>NEBuffer Y</td>
</tr>
</tbody>
</table>

Note: NEBuffer Y was used together with BSA.

NEBuffer 1 (1x concentration) contained:

Bis Tris Propane-HCl 10 mM

34
MgCl₂ 10 mM
DTT (dithiothreitol) 1 mM

NEBuffer Y (1x concentration) + BSA contained:

Tris-HCl, pH 7.4 10 mM
KCl 100 mM
DTT 1 mM
EDTA 1 mM
BSA 0.2 mg/ml
Glycerol 50%

2.3.4.2 10% (w/v) Spermidine [N (3-Aminopropyl)-1, 4-butanediamine]

Spermidine 10 g
Distilled H₂O to 100 ml

2.3.5 Stock solutions, buffers and gel for electrophoresis

2.3.5.1 5x TBE buffer

Tris base 54 g
Boric acid 27.5 g
Na₂EDTA.2H₂O 4.65 g
Distilled H₂O to 1000 ml

The solution was stored at room temperature. The solution was discarded as soon as precipitation was seen (formed after an extensive storage period). For agarose gel electrophoresis, 0.5x TBE containing 0.045 M Tris-borate, 1 mM EDTA, pH 8, was used.
2.3.5.2 EtBr (Ethidium bromide solution - 10 mg/ml)

EtBr powder, 100 mg, was added to a graduated Sterilin tube and dissolved with 0.1 ml of 95% (v/v) ethanol. The volume was adjusted to 10 ml with distilled H₂O to give a concentration of 10 mg/ml. The solution was filtered through a cellulose acetate membrane filter, size 0.2 µm. As EtBr is light-sensitive, the tube was wrapped in aluminium foil and stored refrigerated in the dark.

2.3.5.3 Agarose gel

For flanking gene assays, 4% (w/v) agarose gel (NuSieve GTG: SeaKem LE, 3:1) was used except for Hgal assay in which 5% (w/v) agarose gel (NuSieve GTG: SeaKem LE, 3:2) was used. To prepare 60 ml of gel for 3:1 (NuSieve GTG: SeaKem LE):

 NuSieve GTG agarose              1.8 g
 SeaKem LE agarose                0.6 g
 0.5x TBE buffer to                60 ml.

3.0 µl of EtBr (10 mg/ml) was added into the gel.

To prepare 60 ml of gel for 3:2 (NuSieve GTG: SeaKem LE):

 NuSieve GTG agarose              1.8 g
 SeaKem LE agarose                1.2 g
 0.5x TBE buffer to                60 ml.

3.0 µl of EtBr (10 mg/ml) was added into the gel.
2.3.5.4 6x Gel loading dye

Ficoll (type 400) 0.45 g
1% (w/v) Bromophenol blue 0.90 ml
100% Glycerol 2 ml
Distilled H₂O to 5 ml

2.3.5.5 30% (w/v) Acrylamide

Acrylamide 29 g
N,N-methylene-bis-acrylamide 1 g
Distilled H₂O to 100 ml

The solution was heated to 37°C to dissolve.

2.3.5.6 10% (w/v) Ammonium persulphate

Ammonium persulphate 1 g
Distilled H₂O to 10 ml

The solution was kept at 4°C.

2.3.5.7 5% (w/v) Polyacrylamide gel

30% Acrylamide 16.6 ml
H₂O 62.7 ml
5x TBE 20.0 ml
10% (w/v) Ammonium persulphate 0.7 ml
2.3.6 Stock solutions and buffers for sequencing reaction

2.3.6.1 5 M Ammonium acetate

Ammonium acetate 38.54 g
Distilled \( \text{H}_2\text{O} \) to 100 ml

2.3.6.2 5x Sequenase buffer

Tris-HCl, pH 7.5 200 mM
\( \text{MgCl}_2 \) 100 mM
NaCl 250 mM

2.3.6.3 Termination mixes

dNTP 80 \( \mu \text{M} \)
NaCl 50 \( \mu \text{M} \)
ddNTP 8 \( \mu \text{M} \)

2.3.6.4 Chase mixes

dNTP 0.25 mM
NaCl 50 mM
NP-40 1% (v/v)

2.3.6.5 Labelling mixes

dCTP 7.5 \( \mu \text{M} \)
dGTP 7.5 \( \mu \text{M} \)
dTTP 7.5 \( \mu \text{M} \)
2.3.7 Sterilisation

All glassware was sterilized in a dry heat oven at 180°C for 2.5 hr. Solutions were steam-sterilized at 121°C (15 psi) for 20 min in an autoclave. Distilled or deionized water, polypropylene centrifuge tubes, micropipette tips, microfuge tubes and PCR tubes were autoclaved for 30 min. Swinnex-25 filter holders and 0.2 μm (pore size) membrane filters were autoclaved for 15 min.

All stock solutions for DNA extraction and PCR were sterilized by filtration through sterile 0.2 μm (pore size) membrane filters positioned in sterile Swinnex-25 filter holders.

The master mix for PCR was prepared inside a Cleansphere CA100 chamber (Safetech Limited) to prevent contamination.

2.4 Extraction of DNA from blood cells

Whole blood sample, 0.6 ml, was thawed and transferred to a 1.5 ml microfuge tube containing 0.6 ml of 1x SSC. The tube was flicked and then centrifuged for 1 min at 11,000 revolutions per min (rpm). The supernatant was removed and discarded. Again, 1x SSC, 0.9 ml, was added and mixed by dragging. The cell suspension was centrifuged for 1 min (11,000 rpm). The supernatant was removed carefully.

The washing step was repeated with 0.4 ml of 1x SSC. Once the pellet was completely dissolved, 0.04 ml of 10% (w/v) SDS was added and mixed. A clear viscous solution was obtained.

An equal volume of phenol-chloroform was added and mixed gently by inverting the capped tubes several times to emulsify the solution. The tube was left for more than 1 min, and then centrifuged (11,000 rpm) for 5 min. The lower phenol-
chloroform layer was removed and the solution was centrifuged again for another 3 min. The upper viscous aqueous phase (about 0.4 ml) was transferred to a fresh tube. Absolute EtOH (room temperature), 1 ml, was added and mixed until a pellet was seen. The pellet was centrifuged briefly (less than 10 sec).

The supernatant was discarded and the pellet was dissolved in 0.4 ml of distilled H₂O over 30 min or longer with occasional flicking. Next, 0.04 ml of 2 M NaOAc, pH 7 or 5.6, was added and mixed, then followed by 1 ml of absolute EtOH. The content of the tube was again mixed to precipitate the DNA. After centrifugation (spinning) for less than 10 sec, the supernatant was discarded. The pellet was then left overnight in 1 ml of 80% (v/v) EtOH at room temperature.

The supernatant was carefully removed and the pellet was dried in a DNA vacuum concentrator for 5 to 8 min. The pellet was then resuspended in 25 μl of distilled H₂O and left overnight at 4°C to dissolve.

2.5 O.D. 260/280 assay

This assay determines the concentration of a given DNA sample in solution. The assay is based on the fact that double stranded DNA at a concentration of 50 μg/ml has an optical density reading (O.D.) of 1.0 when measured at 260 nm in Quartz cuvettes with a 1 cm light path.

A 1 ml portion of an appropriately diluted DNA sample was measured at 260 and 280 nm in an ultraviolet spectrophotometer (model DU 7500i, Beckman, U.S.A.). The 260 nm reading is indicative of DNA concentration and the 280 nm reading indicates protein contamination. The 260/280 ratio of pure DNA should read approximately 1.8 but any sample with a reading in the range 1.6-2.0 was considered to be of sufficient purity. A sample reading below 1.5 was assumed to be contaminated with protein and was treated with phenol to further remove the protein.
The readings were taken against a blank of 1 ml of sdH₂O at 260 nm. The 260 nm reading for sample was multiplied by the dilution factor and the concentration calculated by proportion.

\[
\text{E.g., } \quad \text{O.D. 260} = 0.25 \text{ (using } 1/200\text{ dilution)} \\
1.0 = 50 \mu g/ml \\
0.25 = 50 \times 0.25 \times 200 \\
= 2500 \mu g/ml \\
\text{Concentration} = 2.5 \mu g/\mu l
\]

2.6 Flanking DNA polymorphism assays (Neil and Jeffreys, 1993)

Extracted genomic DNA was diluted 3x with distilled H₂O. A few DNA samples were picked randomly and their concentrations were determined. The samples contained approximately 100 ng of DNA per 0.5 µl.

2.6.1 PCR

PCR amplifications were performed according to the steps carried out by Neil and Jeffreys (1993) with minor modifications (Koh, C.L., personal communication). PCR amplification was carried out in a tube with 7 µl of reaction mixture, containing 3x dilution of genomic DNA (about 100 ng), 1x PCR mix, 0.4 µM of each of the specified primers and 0.25 unit of Taq DNA polymerase, placed in a Thermalcycler 480 or Geneamp 9600 (Perkin Elmer Cetus). Samples were overlaid with mineral oil when necessary. The primer sequences are given in Table 3 and in the legend of Figure 5. Three polymorphic sites in the 5' flanking region of MS31A were studied, that is \textit{Alul}, \textit{Hgal} and \textit{Psp}1406I. The volume of reaction and parameters for each polymorphic site are as follows:
2.6.1.1 *Alu I +/- assay*

<table>
<thead>
<tr>
<th>PCR</th>
<th>x1 (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1x PCR mix</td>
<td>0.63</td>
</tr>
<tr>
<td>4 μM 31-TAG-A</td>
<td>0.7</td>
</tr>
<tr>
<td>4 μM 31A</td>
<td>0.7</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μl)</td>
<td>0.05</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>4.42</td>
</tr>
<tr>
<td>DNA</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>7.0</td>
</tr>
</tbody>
</table>

PCR parameters:

**Thermalcycler 480**  : 94°C, 1 min; 68°C, 45 sec; 70°C, 45 sec; x 35 cycles
  68°C, 1 min; 70°C, 5 min; x 1 cycle
  4°C.

**Geneamp 9600**   : 94°C, 45 sec; 68°C, 30 sec; 70°C, 30 sec; x 35 cycles
  68°C, 30 sec; 70°C, 3 min; x 1 cycle
  4°C.

2.6.1.2 *Hga I +/- assay*

<table>
<thead>
<tr>
<th>PCR</th>
<th>x1 (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1x PCR mix</td>
<td>0.63</td>
</tr>
<tr>
<td>4 μM 31F</td>
<td>0.7</td>
</tr>
<tr>
<td>4 μM 31Rsal</td>
<td>0.7</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μl)</td>
<td>0.05</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>4.42</td>
</tr>
<tr>
<td>DNA</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>7.0</td>
</tr>
</tbody>
</table>

PCR parameters:

**Thermalcycler 480**  : 94°C, 1 min; 68°C, 30 sec; 70°C, 30 sec; x 40 cycles
  68°C, 1 min; 70°C, 5 min; x 1 cycle
  4°C.

**Geneamp 9600**   : 94°C, 45 sec; 68°C, 20 sec; 70°C, 20 sec; x 40 cycles
  68°C, 30 sec; 70°C, 3 min; x 1 cycle
  4°C.
2.6.1.3 *Psp* 1406I +/- assay

<table>
<thead>
<tr>
<th>PCR</th>
<th>x1 (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1x PCR mix</td>
<td>0.63</td>
</tr>
<tr>
<td>4 µM 31E</td>
<td>0.7</td>
</tr>
<tr>
<td>4 µM 31F</td>
<td>0.7</td>
</tr>
<tr>
<td><em>Taq</em> DNA polymerase (5 U/µl)</td>
<td>0.05</td>
</tr>
<tr>
<td>Distilled H2O</td>
<td>4.42</td>
</tr>
<tr>
<td>DNA</td>
<td>0.5</td>
</tr>
<tr>
<td>Total volume</td>
<td>7.0</td>
</tr>
</tbody>
</table>

PCR parameters:

- Thermalcycler 480: 94°C, 1 min; 66°C, 45 sec; 70°C, 1 min; x 30 cycles
  - 66°C, 1 min; 70°C, 5 min; x 1 cycle
  - 4°C.
- Geneamp 9600: 94°C, 30 sec; 66°C, 30 sec; 70°C, 45 sec; x 30 cycles
  - 66°C, 30 sec; 70°C, 3 min; x 1 cycle
  - 4°C.

2.6.2 Digestion of amplified products

PCR products were digested by adding 3 units of *Alu*I, *Rsa*I, or *Psp*1406I, 1 µl of their respective recommended buffer (refer Section 2.3.4.1) and 1 µl of 10% (w/v) spermidine, directly into the 7 µl of PCR products, followed by incubation at 37°C for at least 3 hr. The specific volume of reactions for each assay per tube is as follows:

*Alu*I digestion

<table>
<thead>
<tr>
<th></th>
<th>(µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x NEBuffer 1</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Alu</em>I (8 U/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>10% (w/v) spermidine</td>
<td>0.1</td>
</tr>
<tr>
<td>Distilled H2O</td>
<td>1.5</td>
</tr>
<tr>
<td>PCR product</td>
<td>7.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>10.0</td>
</tr>
</tbody>
</table>
**Rsal digestion**

<table>
<thead>
<tr>
<th></th>
<th>(µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x NEBuffer 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Rsal (10 U/µl)</td>
<td>0.3</td>
</tr>
<tr>
<td>10% (w/v) spermidine</td>
<td>0.1</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>1.6</td>
</tr>
<tr>
<td>PCR product</td>
<td>7.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>10.0</td>
</tr>
</tbody>
</table>

**Psp1406I digestion**

<table>
<thead>
<tr>
<th></th>
<th>(µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x NEBuffer Y</td>
<td>1.0</td>
</tr>
<tr>
<td>Psp1406I (16 U/µl)</td>
<td>0.25</td>
</tr>
<tr>
<td>10% (w/v) spermidine</td>
<td>0.1</td>
</tr>
<tr>
<td>BSA (10 mg/ml)</td>
<td>0.1</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>1.55</td>
</tr>
<tr>
<td>PCR product</td>
<td>7.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>10.0</td>
</tr>
</tbody>
</table>

2.6.3 Electrophoresis

After incubation, 2 µl of 6x bromophenol blue (BPB) was added to the product and the resulting fragments were then electrophoresed through a 4% (w/v) (NuSieve GTG: SeaKem LE, 3:1) agarose gel for Alul and Psp1406I assays. For Hgal assay, a 5% (w/v) (NuSieve GTG: SeaKem LE, 3:2) agarose gel was used. Agarose gel electrophoresis was carried out with 0.5x TBE buffer containing EtBr (0.5 µg/ml) for 1.5 hr at 130 volts.

2.6.4 Statistical analysis

Mendel's laws of segregation and independent assortment enable one to predict the equilibrium distribution of genotypes in a population from allelic frequencies. The equilibrium distribution, \( p^2 + 2pq + q^2 = 1 \), is called the Hardy-Weinberg equilibrium. The frequency of each allele in the population was calculated
from the number of each genotype that was collected in the sample set. From the allele frequency data, the expected number of genotype frequency was calculated under the assumption of Hardy-Weinberg (H-W) expectations. Possible divergence of genotype frequencies from the H-W expectation was determined by calculating the unbiased estimate of the expected heterozygous frequency which is equivalent to the allele diversity value \( h \) or heterozygosity:

The power of discrimination (Pd) value was calculated. Pd is the probability that two unrelated individuals drawn at random from the same breeding population have different genotypes at the site under investigation.

The calculations of the data of MS31A AluI assay in the Malaysian Malay population as an example are illustrated as below:

a) $\chi^2$ test

i) Null hypothesis ($H_0$): The number observed for the genotypes in the MS31A AluI site are according to the Hardy-Weinberg equilibrium.

ii) Alternative hypothesis: The numbers observed for the genotypes in the MS31A AluI site are not according to the Hardy-Weinberg equilibrium.

iii) Level of significance: $\alpha = 0.05$

iv) Criterion: Reject the null hypothesis if $\chi^2 > 3.841$, the value of $\chi^2_{0.05}$ for 1 degree of freedom, and

$$\chi^2 = \sum_{i=1}^{n} \frac{(O - E)^2}{E}$$

or otherwise accept it.

v) Expected numbers:

For $+/+$ (A/A), $E = p^2 \times 103$

$$= (0.257)^2 \times 103$$

$$= 6.80$$
For +/- (A/G), \( E = 2pq \times 103 \)
\[ = 2 (0.257)(0.743) \times 103 \]
\[ = 39.34 \]

For -/- (G/G), \( E = q^2 \times 103 \)
\[ = (0.743)^2 \times 103 \]
\[ = 56.86 \]

vi) Calculation:

\[
\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E)^2}{E} \\
= 0.712 + 0.479 + 0.668 \\
= 1.859
\]

vii) Decision: Since \( \chi^2 = 1.859 \) does not exceed 3.841, the null hypothesis is accepted.

b) Power of discrimination (Pd):

\[
Pd = 1 - \sum_{i=1}^{n} (P_i)^2 \\
= 1 - 0.449 \\
= 0.551
\]

c) Heterozygosity:

\[
h = n \left[ 1 - \sum_{i=1}^{n} \frac{(n_i/n)^2}{n-1} \right]
\]
\[ = 0.345 \]

Where \( n_1, n_2 \ldots n_i \) are the allele counts of the D7S21 alleles in a sample of \( n \) chromosomes, \( P_i \) = allele frequencies. In a population following H-W expectations, \( h \) should be mathematically equivalent to the frequency of observed heterozygotes.
2.8 Haplotype assays of flanking DNA polymorphism (Neil and Jeffreys, 1993)

2.7.1 PCR

The standard amplification program was used for haplotype assay which combined the annealing and extension time based on the method of Neil and Jeffreys (1993). Two haplotype assays were studied, Hgal-Alul and Hgal-Psp1406I. The PCR parameters and volume of the reaction are as follows:

**PCR parameters:**
- Thermalcycler 480: 94°C, 1 min; 72°C, 1 min 30 sec; x 35 cycles
  - 68°C, 1 min; 70°C, 5 min; x 1 cycle
  - 6°C.
- Geneamp 9600: 94°C, 30 sec; 70°C, 1 min; x 35 cycles
  - 6°C.

<table>
<thead>
<tr>
<th>PCR</th>
<th>x 1 (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1x PCR mix</td>
<td>0.63</td>
</tr>
<tr>
<td>4 μM 31-TAG-A</td>
<td>0.7</td>
</tr>
<tr>
<td>4 μM 31Hgal+t</td>
<td>0.7</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μl)</td>
<td>0.05</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>4.42</td>
</tr>
<tr>
<td>DNA</td>
<td>0.5</td>
</tr>
<tr>
<td>Total volume</td>
<td>7.0</td>
</tr>
</tbody>
</table>

2.7.2 Digestion of amplified products

PCR products were digested by adding 3 units of enzyme. For Hgal-Alul assay, Alul was used, whereas for Hgal-Psp1406I assay, Psp1406I was used. The volumes and conditions of reactions are as stated in Section 2.6.2.

2.7.3 Electrophoresis

The resulting fragments were then electrophoresed through a 5% (w/v) (NuSieve GTG: SeaKem LE, 3:2) agarose gel for Hgal-Alul assay and a 4% (w/v) (NuSieve GTG:
SeaKem LE, 3:1) agarose gel for \textit{Hgal-Psp14061} assay. Agarose gel electrophoresis was carried out with 0.5x TBE buffer containing 0.5 \( \mu g/ml \) EtBr for 1.5 hr at 130 volts.

2.7.4 Statistical analysis for haplotyping

Chi-square analysis (\( \chi^2 \)) with two-way classification, called 2x2 contingency tables, was used to investigate the association between two restriction sites. In statistical or probabilistic independence, if two events are independent, the probability of their occurring together can be computed as the product of their separate probabilities. If two properties are not independent of each other, they are associated.

Two hypotheses are involved. The null hypothesis shows the independence between the two variables and vice-versa for the other hypothesis (\( H_A \)).

\[
H_0 = \text{Variables are independent} \\
H_A = \text{Variables are not independent}
\]

The method used was according to Sokal and Rohlf (1981). The calculations of the Malaysian haplotype are illustrated as below:

\( \chi^2 \) test

i) Null hypothesis (\( H_0 \)): All the polymorphic sites (\textit{AluI}, \textit{Hgal}, and \textit{Psp1406I}) are not associated together.

ii) Alternative hypothesis: All the polymorphic sites (\textit{AluI}, \textit{Hgal}, and \textit{Psp1406I}) are associated together.

iii) Level of significance: \( \alpha = 0.05 \)

iv) Criterion: Reject the null hypothesis if \( \chi^2 > 3.841 \), the value of \( \chi^2_{0.05} \) for 1 degree of freedom, and

\[
\chi^2 = \sum_{i=1}^{n} \frac{(O - E)^2}{E}
\]

or otherwise accept it.

v) Calculation: See Section 3.2.1.
2.8 Agarose gel electrophoresis of DNA (Sambrook et al., 1989)

Agarose gel electrophoresis was carried out for 1.5 hr in 0.5x TBE with EtBr (0.5 μg/ml). The agarose gels were then viewed on a uv transilluminator (Ultra-violet Product, Inc., model TM-36) and photographed by using Polaroid 665 instant films and a red filter (Kodak), with an exposure time of 30 sec.

2.9 Direct sequencing

A total of 11 samples were selected for sequencing. All 11 samples were from Hgal flanking polymorphism assay, which included 7 samples that showed irregular bands (refer Section 3.3). The other 4 samples were randomly chosen to represent normal controls.

Sequencing was carried out with an ALFexpress DNA sequencer (automated sequencer) and the procedures were essentially those described in the Pharmacia Biotech manual (1995). The process involved:

(i) PCR

PCR amplification of the fragment to be sequenced was performed in the presence of two primers, 31F and 31Rsal (Table 2). The amplification was carried out in 30 μl of reaction mixture containing about 200 ng of genomic DNA, 1x PCR mix, 0.4 μM of each primer, and 1 unit of Taq DNA polymerase. The specific amplification program for Hgal assay was used to amplify these fragments.

<table>
<thead>
<tr>
<th>PCR</th>
<th>x1 (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1x PCR mix</td>
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<tr>
<td>4 μM 31F</td>
<td>3.0</td>
</tr>
<tr>
<td>4 μM 31Rsal</td>
<td>3.0</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μl)</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled H2O</td>
<td>20.1</td>
</tr>
<tr>
<td>DNA</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>30.00</strong></td>
</tr>
</tbody>
</table>

(ii) Primer annealing, sequencing reactions, labelling mix and termination reaction were
carried out according to the standard procedures provided in Pharmacia Biotech manual (1995).

(iii) Loading the gel

The reaction tubes were heated at 80-85°C for 2-3 min and immediately chilled on ice. Approximately 6-8 μl of each sample was loaded into the appropriate well of a 5% polyacrylamide gel. Samples were electrophoresed in an ALFexpress DNA sequencer (automated sequencer). The electrophoresis was carried out at 1500 volts, 38 mA, 34 watts and 40°C. The nucleotide sequences were analysed by using the ALFwin sequence analyser, Ver. 2.10.

2.10 Flowcharts

The summary of experiments that were carried out in this project is shown in Figures 7 and 8.
Figure 7: Flowchart of work performed for flanking region assay and haplotyping of the MS31A minisatellite.
Selection of samples with irregular bands from \textit{Hgal} assay

- PCR
- Primer annealing
- Sequencing reactions
- Labelling mix
- Termination reaction
- Loading the gel
- Sequence analysis

Figure 8: Steps involved in preparation of sample for sequencing.