Chapter Two Materials And Methods

2.1 Blood samples

Blood samples of 210 healthy, unrelated individuals (70 Chinese, 70 Indians, and 70 Malays) were obtained from the blood bank of the University Hospital, Kuala Lumpur.

About 10 ml of each individual's blood was collected in a sterile universal bottle. A volume of 400 µl of 0.5 M disodium EDTA, pH 8.0, was added to prevent the blood from coagulation. The final concentration of EDTA was 20 mM. Each blood sample was then divided into two 5 ml portions and stored at either -20 or -70 °C.

2.2 Oligonucleotide primers

Table 1 lists the oligonucleotide primers used in this study. They were based on Monckton *et al.* (1993).

Table 1 : Primers.

Primer	Number of nucleotides	Sequence
32-O1RB (32-ORB)	25	5' CCTGTTCTGTAACCACCCTTCCCAG3'
32-S	20	5' GATGGAGCAATGGCCAGAGC3'
32-O2RH	24	5' CCTGAGTCACCTGTTCTGTAACCG3'
32-B	24	5' AAGCTCTCCATTTCCAGTTTCTGG3'
32-H1B	20	5' TTTGGTGCTGCAAAAGAAAG3'
32-NR (32-N)	20	5' AGTAGCCAATCGGAATTAGC3'
32-O	38	5' TCACCGGTGAATTCACCACCCTTCCCA CCAAACTACTC3'
32-H2AR	20	5' GTGCAGTCCAACCCTAGCCA3'
32-H2C	20	5' TGATGCGTCGTTCCCGTATC3'
32-H1C	20	5' TGGTGCTGCAAAAGAAATAC3'

Primer 32-O incorporates a 16 nucleotide 5' extension (underlined) which was designed for other experiments and is not essential for the present study.

All the primers were obtained from Genosys, U.S.A.; and New England Biolabs (NEB), U.S.A.

2.3 Enzymes

DNA polymerase (5 U/µl) from *Thermus* species by Advanced Biotechnologies Ltd., U.K. was used.

Table 2 shows the restriction endonucleases and buffers used.

Table 2 : Restriction endonucleases and buffers.

Restriction endonuclease	Buffer	Source
*Hin*fI	10x Buffer 2	NEB
*Bsp*1286I	10x *Bsp*1286I buffer, 100x Acetylated BSA	NEB
*Hae*III	10x Buffer 2	NEB
*Scr*FI	10x Buffer 4	NEB

2.4 Reagents and chemicals

All chemicals, reagents, and biochemicals used were of Analar grade or of the highest grade available commercially, obtained from Advanced Biotechnologies Ltd., U.K.; Amersham International Plc., England; Perkin Elmer, U.S.A.; BDH Chemicals Ltd., England; Boehringer Mannheim, Germany; Fluka, Switzerland; FMC, U.S.A.; GIBCO Bethesda Research Laboratories (BRL), U.S.A.; Hayman Limited, England; Kanto Chemicals Co., Japan; May and Baker Ltd., England; Merck, Germany; New England Biolabs (NEB), U.S.A.; Pharmacia, U.S.A.; Sigma Chemical Company, U.S.A.; AJAX Chemicals, Australia; and United States Biochemical Corp. (USB), U.S.A.

[γ-^{33}P]ATP with a specific activity of 1000-3000 Ci/mMol or 37-111 TBq/mMol and concentration of 10 mCi/ml or 370 Mbq/ml was from Amersham International Plc.

Mineral oil was obtained from Perkin Elmer.

Acrylamide, 2% dimethyldichlorosilane, isopropanol, sodium acetate (NaOAc), paraffin oil, sodium dodecyl sulphate (SDS), and xylene cyanol FF were from BDH.

Tris-HCl and Tris-base were obtained from Boehringer Mannheim.

Amberlite IR-120 (H), Amberlite IRA-400 (OH), ammonium persulphate (APS), ammonium acetate (NH_4OAc), disodium EDTA, 8-hydroxyquinoline, $MgCl_2$, NaCl, NN'-methylenebisacrylamide, TEMED, trisodium citrate dihydrate, and urea were from Fluka.

Agarose (LE) and NuSieve (GTG) were obtained from FMC.

Glycerol and 100 bp DNA ladder (1 μg/μl) were from GIBCO BRL.

Absolute EtOH was obtained from Hayman Limited.

Ammonium sulphate was from Kanto Chemicals Co.

Bromophenol blue was obtained from May and Baker Ltd.

Chloroform was obtained from Merck.

Acetylated BSA (100x), dNTPs, ddNTPs, and T4 polynucleotide kinase (10000 U/ml) were from NEB.

Size marker 20 bp DNA ladder (200 ηg/ml) was obtained from Advanced Biotechnologies Ltd. Size marker ϕX174 replicative form (RF) DNA/*Hae*III (440 μg/ml) was from Pharmacia.

Deionized formamide, EtBr, Ficoll (type 400), and 2-mercaptoethanol were from Sigma Chemical Company.

EtOH (95%) was obtained from AJAX Chemicals.

Phenol was obtained from USB.

2.5 Stock solutions and buffers

Stock solutions and buffers [e.g., 20x Sodium chloride-sodium citrate (SSC), 10% (w/v) SDS, Buffered phenol-chloroform, 80% (v/v) EtOH, 2 M NaOAc (pH 5.6), 2 M Tris-HCl (pH 7.5 and 8.8), 1 M Ammonium sulphate, 1 M $MgCl_2$, 0.5 M Disodium EDTA (pH 7.0, 8.0, and 8.8), 1 M Spermidine trihydrochloride, 6x Bromophenol blue (BPB) loading dye, 10 mg EtBr/ml, 20x Tris-borate EDTA buffer (TBE), 10% (w/v) APS, and 7.5 M NH_4OAc] commonly used in DNA extraction and manipulations were prepared according to the methods described by Sambrook *et al.* (1989).

2.5.1 11.1x PCR mixture (mix)

Table 3 shows the reagents used in 11.1x PCR mix.

Table 3 : Reagents for 11.1x PCR mix.

Reagent	Stock solution	Volume (μl)	Concentration in final reaction (11.1x)	Concentration in final reaction (1x)
Tris-HCl (pH 8.8)	2 M	167.0	494.10 mM	45.0 mM
Ammonium sulphate	1 M	83.0	122.80 mM	11.1 mM
$MgCl_2$	1 M	33.5	49.60 mM	4.5 mM
2-mercaptoethanol	100 %	3.6	74.40 mM	6.7 mM

EDTA (pH 8.0)	10 mM	3.4	0.05 mM	4.5 µM
dATP	100 mM	75.0	11.10 mM	1.0 mM
dCTP	100 mM	75.0	11.10 mM	1.0 mM
dGTP	100 mM	75.0	11.10 mM	1.0 mM
dTTP	100 mM	75.0	11.10 mM	1.0 mM
100x Acetylated BSA	10 mg/ml	85.0	1257.40 µg/ml	113.3 µg/ml
Total		676.0		

The buffer was stored in 100 ml portions at -20 °C.

2.5.2 10x Kinase buffer

Table 4 lists the reagents used in 10x Kinase buffer.

Table 4 : Reagents for 10x Kinase buffer.

Reagent	**Stock solution (M)**	**Volume (µl)**	**Concentration in final reaction (10x)**	**Concentration in final reaction (1x)**
Tris (pH 7.5)	2.0	12.5	0.5 M	0.05 M
$MgCl_2$	1.0	5.0	0.1 M	0.01 M
Spermidine	0.1	0.5	1.0 mM	0.10 mM
EDTA (pH 8.8)	0.5	0.1	1.0 mM	0.10 mM
sdH_2O	--	31.9	--	--
Total		50.0		

2.5.3 20x Base mix

Table 5 shows the reagents used in 20x Base mix.

Table 5 : Reagents for 20x Base mix.

Reagent	**Stock solution**	**Volume (µl)**	**Concentration in final reaction (20x)**	**Concentration in final reaction (1x)**
Tris-HCl (pH 8.8)	2 M	45.0	853.1 mM	42.7 mM
Ammonium sulphate	1 M	22.5	213.3 mM	10.7 mM
$MgCl_2$	1 M	9.0	85.3 mM	4.3 mM
2-mercaptoethanol	100 %	1.0	134.0 mM	6.7 mM
EDTA (pH 8.0)	10 mM	1.0	94.8 µM	4.7 µM
dATP	16 mM	1.0	151.7 µM	7.6 µM
dCTP	16 mM	1.0	151.7 µM	7.6 µM
dGTP	16 mM	1.0	151.7 µM	7.6 µM
dTTP	16 mM	1.0	151.7 µM	7.6 µM
100x Acetylated BSA	10 mg/ml	23.0	2180.1 µg/ml	109.0 µg/ml
Total		105.5		

2.5.4 4x PCR termination mix

Table 6 lists the reagents used in 4x PCR termination mix.

Table 6 : Reagents for 4x PCR termination mix.

Nucleotide	20x Base mix (µl)	ddNTPs (5mM) (µl)	sdH_2O (µl)	Final concentration of ddNTP (4x) (µM)	Final concentration of ddNTP (1x) (µM)
G	10	2.4	37.6	240	60
A	10	10.0	30.0	1000	250
T	10	13.2	26.8	1320	330
C	10	6.4	33.6	640	160

2.5.5 Stop solution (Formamide-dye mix)

Table 7 shows the reagents used in stop solution.

Table 7 : Reagents for stop solution.

Reagent	Stock solution	Volume (ml)	Concentration in final reaction (%)
Xylene cyanol FF	1 %	3.000	0.30
Bromophenol blue	1 %	3.000	0.30
Disodium EDTA (pH 7.0)	18.612 % or 0.5 M	0.199	0.37
Deionized formamide	--	3.801	--
Total		10.000	

2.5.6 Amberlite MB1

Amberlite IR-120 (H).....6.50 g

Amberlite IRA-400 (OH).....9.75 g

Total.....16.25 g

2.5.7 5x Acrylamide/bisacrylamide (38:2) [30% acrylamide stock solution]

A 30% acrylamide stock solution was prepared by dissolving 114 g of acrylamide and 6 g of NN'-methylenebisacrylamide in 250 ml of dH_2O. The volume was made up to 400 ml. Amberlite MB1, 16 g, was then added and the mixture was stirred gently for 5 min. The solution was filtered through nitrocellulose membrane filter (0.45 µm, 47 mm) (MFS, U.S.A.) to remove resin. The stock was stored in a dark bottle at 4 °C.

2.6 Sterilization

Glass pipettes in canisters were sterilized in a dry heat oven (Gallenkamp BS Oven-model OV-160) at 180 °C for 2 hr.

Solutions, deionized water, universal bottles, micropipette tips, microfuge tubes, microtubes (tubes for the polymerase chain reaction), Swinnex-25 filter holders, and cellulose acetate membrane filters (0.2 μm, 25 mm) were sterilized at 121 °C (15 psi) for 20 min in an autoclave.

Heat-labile solutions and autoclaved sterile deionized water (sdH_2O) were sterilized by filtering through sterile cellulose acetate membrane filters (0.2 μm, 25 mm) positioned in sterile Swinnex-25 filter holders.

2.7 Rapid mini-preparation of genomic DNA from blood samples

Each frozen blood sample was thawed before 600 μl of blood was transferred with a 'sawn off' micropipette tip to a 1.5 ml microfuge tube. Then 600 μl of 1x SSC was added. The mixture was mixed by flicking and then spun for 2 min at 15000 rpm in a microcentrifuge (Model Sigma 112, B. Braun Biotech International, Germany). The supernatant was carefully removed and discarded into a container containing Chlorox bleach.

The precipitated cells were suspended in 900 μl of 1x SSC by dragging until no clumps of cells were observed (i.e., dragging the tube on the surface of a plastic tube rack). The suspension was spun for 2 min at 15000 rpm. The supernatant was carefully removed and discarded.

The precipitated cells were resuspended in 400 μl of 1x SSC by dragging. Then 40 μl of 10% (w/v) SDS was added and the mixture was mixed gently by inverting the tube a few times. A clear viscous solution resulted because of cell lysis by SDS.

Buffered phenol-chloroform, 400 μl, was added, and the mixture was mixed gently by inverting the tube a few times until an emulsion resulted. The emulsion was left for > 1 min, mixed again, and spun for 5 min at 15000 rpm. After centrifugation, a solution with three phases was observed. A micropipette tip was used to remove about 300 μl of the lower phenol-chloroform, without removing the upper aqueous phase. The tube was spun again for 3 min at 15000 rpm. Then, 400 μl of the clear upper viscous

aqueous phase was removed, without interfering the interface, with a 'sawn off' micropipette tip and transferred into a new tube.

To the aqueous phase, 1 ml of room temperature absolute EtOH was added. The mixture was mixed gently while DNA strands began to form. The solution was spun briefly for < 10 sec to precipitate the DNA strands but not the dirt. The supernatant was discarded. The pellet was dissolved in 400 µl of sdH_2O over 30 min or longer with occasional flicking. If the pellet did not dissolve completely, the solution was incubated at 65 °C for 20 min and then at 37 °C for 30 min to dissolve the pellet.

To the DNA solution, 40 µl of 2 M NaOAc, pH 5.6, was added, followed by 1 ml of absolute EtOH. The mixture was mixed gently and DNA strands began to form. The solution was spun briefly for < 10 sec and the supernatant was discarded. The pellet was kept overnight in 1 ml of 80% (v/v) EtOH at -70 °C. The tube was then spun for < 10 sec and the supernatant was discarded. The pellet was vacuum dried in a Savant SpeedVac for 5 to 8 min. The pellet comprising genomic DNA was dissolved in 25 µl of sdH_2O. The DNA solution was then stored at -20 °C.

2.8 Determination of DNA concentrations

A concentrated DNA solution was diluted with sdH_2O and its spectrophotometric reading at 260 ηm was taken with uv-visible spectrophotometer (DU 7500i spectrophotometer, Beckman, U.S.A.). As OD_{260} of one unit corresponds to approximately 50 µg of double-stranded DNA/ml, the DNA concentrations were calculated with this formula:

$$OD_{260} \times 50\ \mu g/ml \times \text{Dilution factor}$$

2.9 Polymorphic site and haplotype assays

Each genomic DNA from blood samples of 210 healthy, unrelated Malaysian individuals (70 Chinese, 70 Indians and 70 Malays) was used for five polymorphic site (O2, *Hin*fI, Hump1, O1, and Hump2) assays. Each genomic DNA was diluted 3 times with sdH_2O (10 µl of genomic DNA in 20 µl of sdH_2O). The 3x diluted genomic DNA was amplified by PCR through O2 assay amplification (Section 2.9.2).

Out of the total 7.5 µl of O2 assay amplified PCR product, 5 µl was used for *Hin*fI digestion in *Hin*fI assay (Section 2.9.3). To the remaining 2.5 µl of PCR product, 2.5 µl of sdH_2O was added to make 5 µl. Of the 5 µl diluted PCR product, 4 µl was digested with *Hae*III in O2 assay (Section 2.9.2). The 1 µl of the remaining diluted PCR product was diluted 100x (1 µl of PCR product in 49 µl of sdH_2O). The 100x diluted PCR product was used for Hump1 (Section 2.9.4) and O1 (Section 2.9.5) assays. Diluted genomic DNA was used for Hump2 assay (Section 2.9.6) instead of 100x diluted PCR product.

For haplotype assays, only the diluted genomic DNA of double heterozygous individuals was amplified. Hence, in *Hin*fI-Hump1 (Section 2.9.7), *Hin*fI-Hump2 (Section 2.9.8), and Hump1-Hump2 (Section 2.9.9) assays, DNA of the double heterozygous individuals with *Hin*fI (+/-) and Hump1 (C/G), *Hin*fI (+/-) and Hump2 (C/T), and Hump1 (C/G) and Hump2 (C/T), respectively, were used.

2.9.1 General protocols for polymorphic site and haplotype assays

The PCR master mixture for each assay was prepared inside a clean sphere CA100 (SAFETECH LIMITED) which was irradiated with ultra-violet light to provide an environment free of DNA. The PCR master mixture was prepared by adding the solutions in the following sequence: (i) sdH_2O, (ii) 11.1x PCR mix, (iii) primers, and (iv) DNA polymerase. Solutions like 11.1x PCR mix and primers were thawed, vortexed, and then spun together with DNA polymerase for < 10 sec at 15000 rpm and kept in ice before each was dispenced into the PCR master mixture.

The PCR master mixture was then vortexed evenly and spun for < 10 sec at 15000 rpm before the mixture was dispensed into PCR tubes. About 10 µl of the mineral oil or paraffin oil was pipetted to each tube if DNA Thermal Cycler 480 (Perkin Elmer Cetus, U.S.A.) was used. No oil was required for GeneAmp PCR System 9600 (Perkin Elmer, U.S.A.). The PCR tubes were spun again as above before any DNA was added. DNA of various samples was then pipetted into their respective PCR tubes before the tubes were inserted into the PCR machine. To negative control tube, sdH_2O was added instead of DNA.

The PCR machine was preheated at 94 °C before the experiment. Once the PCR tubes were inserted into the PCR machine, the experiment can be carried out according to the parameters that were programmed.

The PCR products were then digested with the appropriate restriction endonucleases. The restriction endonuclease master mixture was prepared in the following sequence: (i) sdH_2O, (ii) 0.1 M spermidine, (iii) restriction endonuclease buffer, and (iv) restriction endonuclease. The spermidine and restriction buffer were thawed, vortexed, and then spun together with restriction endonuclease for < 10 sec at 15000 rpm and kept in ice before each was dispensed into the restriction endonuclease master mixture. The restriction endonuclease master mixture was vortexed evenly and spun for < 10 sec at 15000 rpm before the mixture was aliquoted into the PCR products. The restriction endonuclease digestion was carried out at 37 °C from 3 hr to overnight.

Each digested PCR product, after spinning, was added with 6x bromophenol blue (BPB) and spun again. The digested PCR products were then resolved by gel electrophoresis.

2.9.2 O2 assay

Table 8 shows the reagents used for one reaction in O2 assay.

Table 8 : PCR reagents for O2 assay.

Reagent	Volume (μl)
11.1x PCR mix	0.675
4 μM 32-B	0.750
4 μM 32-O2RH	0.750
DNA polymerase (5 U/μl)	0.075
sdH_2O	4.750
DNA	0.500
Total volume	7.500

PCR parameters:

TC480 : 94 °C, 1 min; 60 °C, 1 min; 70 °C, 2 min; x35 cycles

60 °C, 1 min; 70 °C, 5 min; x1 cycle

4 °C, soak

TC9600 : 94 °C, 45 sec; 60 °C, 30 sec; 70 °C, 1 min; x35 cycles

60 °C, 30 sec; 70 °C, 3 min; x1 cycle

4 °C, soak

Table 9 shows the reagents used for one reaction in *Hae*III digestion for O2 assay.

Table 9 : *Hae*III digestion for O2 assay.

Reagent	Volume (µl)
10x Buffer 2	1.20
*Hae*III (10 U/µl)	0.20
0.1 M spermidine	0.12
sdH_2O	6.48
PCR product	4.00
Total volume	12.00

Incubation : 37 °C, ~3 hr

6x BPB : 2 µl

Gel : 3% (w/v) NuSieve : 1% (w/v) Agarose

Voltage : 120-150 volts

2.9.3 *Hin*fI assay

Table 10 shows the reagents used for one reaction in *Hin*fI digestion for *Hin*fI assay.

Table 10 : *Hin*fI digestion for *Hin*fI assay.

Reagent	Volume (µl)
10x Buffer 2	1.20
*Hin*fI (10 U/µl)	0.30
0.1 M spermidine	0.12
sdH_2O	5.38
PCR product	5.00
Total volume	12.00

Incubation : 37 °C, ~3 hr

6x BPB : 2 µl

Gel : 3% (w/v) NuSieve : 1% (w/v) Agarose

Voltage : 120-150 volts

2.9.4 Hump1 assay

Table 11 shows the reagents used for one reaction in Humpl assay.

Table 11 : PCR reagents for Humpl assay.

Reagent	Volume (µl)
11.1x PCR mix	0.675
4 µM 32-N (32-NR)	0.750
4 µM 32-H1B	0.750
DNA polymerase (5 U/µl)	0.075
sdH_2O	4.750
Diluted PCR product	0.500
Total volume	7.500

PCR parameters:

TC480 : 94 °C, 1 min; 55 °C, 1 min; 70 °C, 2 min; x28 cycles

55 °C, 1 min; 70 °C, 5 min; x1 cycle

4 °C, soak

TC9600 : 94 °C, 45 sec; 55 °C, 30 sec; 70 °C, 1 min; x28 cycles

55 °C, 30 sec; 70 °C, 3 min; x1 cycle

4 °C, soak

Table 12 shows the reagents used for one reaction in *Bsp*1286I digestion for Humpl assay.

Table 12 : *Bsp*1286I digestion for Humpl assay.

Reagent	Volume (µl)
10x *Bsp*1286I buffer	2.0
*Bsp*1286I (5 U/µl)	0.5
0.1 M spermidine	0.2
100x Acetylated BSA (10 mg/ml)	0.2
sdH_2O	9.6
PCR product	7.5
Total volume	20.0

Incubation : 37 °C, ~3 hr

6x BPB : 4 µl

Gel : 3% (w/v) NuSieve : 1% (w/v) Agarose

Voltage : 120-150 volts

2.9.5 O1 assay

Table 13 shows the reagents used for one reaction in O1 assay.

Table 13 : PCR reagents for O1 assay.

Reagent	Volume (µl)
11.1x PCR mix	0.675
4 µM 32-S	0.750
4 µM 32-O1RB (32-ORB)	0.750
DNA polymerase (5 U/µl)	0.075
sdH_2O	4.750
Diluted PCR product	0.500
Total volume	7.500

PCR parameters:

TC480 : 94 °C, 1 min; 55 °C, 1 min; 70 °C, 1 min; x25 cycles

55 °C, 1 min; 70 °C, 5 min; x1 cycle

4 °C, soak

TC9600 : 94 °C, 45 sec; 55 °C, 30 sec; 70 °C, 30 sec; x25 cycles

55 °C, 30 sec; 70 °C, 3 min; x1 cycle

4 °C, soak

Table 14 shows the reagents used for one reaction in *Scr*FI digestion for O1 assay.

Table 14 : *Scr*FI digestion for O1 assay.

Reagent	Volume (µl)
10x Buffer 4	1.20
*Scr*FI (10 U/µl)	0.25
0.1 M spermidine	0.12
sdH_2O	5.43
PCR product	5.00
Total volume	12.00

Incubation : 37 °C, ~3 hr

6x BPB : 2 µl

Gel : 3% (w/v) NuSieve : 1% (w/v) Agarose

Voltage : 120-150 volts

2.9.6 Hump2 assay

Table 15 shows the reagents used for one reaction in Hump2 assay.

Table 15 : PCR reagents for Hump2 assay.

Reagent	Volume (μl)
11.1x PCR mix	0.675
4 μM 32-H2AR	1.500
4 μM 32-H2C	0.375
4 μM 32-O	0.375
4 μM 32-B	0.750
DNA polymerase (5 U/μl)	0.075
sdH_2O	3.250
Diluted PCR product	0.500
Total volume	7.500

PCR parameters:

TC480 : 94 °C, 1 min; 67 °C, 1 min; 70 °C, 2 min; x30 cycles

67 °C, 1 min; 70 °C, 5 min; x1 cycle

4 °C, soak

TC9600 : 94 °C, 45 sec; 67 °C, 30 sec; 70 °C, 1 min; x30 cycles

67 °C, 30 sec; 70 °C, 3 min; x1 cycle

4 °C, soak

No restriction endonuclease digestion was needed for Hump2 assay.

6x BPB : 2 μl

Gel : 3% (w/v) NuSieve : 1% (w/v) Agarose

Voltage : 120-150 volts

2.9.7 *Hin*fI-Hump1 assay

Table 16 shows the reagents used for one reaction in *Hin*fI-Hump1 assay. The samples used for this assay must be from double heterozygous individuals with *Hin*fI (+/-) and Hump1(C/G).

Table 16 : PCR reagents for *Hin*fI-Hump1 assay.

Reagent	Volume (μl)
11.1x PCR mix	0.675
4 μM 32-H1C	0.750
4 μM 32-O	0.750
DNA polymerase (5 U/μl)	0.075
sdH_2O	4.750
DNA	0.500
Total volume	7.500

PCR parameters:

TC480 : 94 °C, 1 min; 64 °C, 1 min; 70 °C, 2 min; x30 cycles

64 °C, 1 min; 70 °C, 5 min; x1 cycle

4 °C, soak

TC9600 : 94 °C, 45 sec; 64 °C, 30 sec; 70 °C, 1 min; x30 cycles

64 °C, 30 sec; 70 °C, 3 min; x1 cycle

4 °C, soak

Table 17 shows the reagents used for one sample in *Hin*fI digestion for *Hin*fI-Hump1 assay.

Table 17 : *Hin*fI digestion for *Hin*fI-Hump1 assay.

Reagent	**Volume (µl)**
10x Buffer 2	1.20
*Hin*fI (10 U/µl)	0.30
0.1 M spermidine	0.12
sdH_2O	2.88
PCR product	7.50
Total volume	12.00

Incubation : 37 °C, ~3 hr

6x BPB : 2 µl

Gel : 3% (w/v) NuSieve : 1% (w/v) Agarose

Voltage : 120-150 volts

2.9.8 *Hin*fI-Hump2 assay

Table 18 shows the reagents used for one reaction in *Hin*fI-Hump2 assay. The samples used for this assay must be from double heterozygous individuals with *Hin*fI (+/-) and Hump2 (C/T).

Table 18 : PCR reagents for *Hin*fI-Hump2 assay.

Reagent	**Volume (µl)**
11.1x PCR mix	0.675
4 µM 32-H2AR	0.750
4 µM 32-B	0.750
DNA polymerase (5 U/µl)	0.075
sdH_2O	4.750
DNA	0.500
Total volume	7.500

PCR parameters:

TC480 : 94 °C, 1 min; 67 °C, 1 min; 70 °C, 2 min; x30 cycles

67 °C, 1 min; 70 °C, 5 min; x1 cycle

4 °C, soak

TC9600 : 94 °C, 45 sec; 67 °C, 30 sec; 70 °C, 1 min; x30 cycles

67 °C, 30 sec; 70 °C, 3 min; x1 cycle

4 °C, soak

Table 19 shows the reagents used for one reaction in *Hin*fI digestion for *Hin*fI-Hump2 assay.

Table 19 : *Hin*fI digestion for *Hin*fI-Hump2 assay.

Reagent	**Volume (µl)**
10x Buffer 2	1.20
*Hin*fI (10 U/µl)	0.30
0.1 M spermidine	0.12
sdH_2O	2.88
PCR product	7.50
Total volume	12.00

Incubation : 37 °C, ~3 hr

6x BPB : 2 µl

Gel : 3% (w/v) NuSieve : 1% (w/v) Agarose

Voltage : 120-150 volts

2.9.9 Hump1-Hump2 assay

Table 20 shows the reagents used for one reaction in Hump1-Hump2 assay. The samples used for this assay must be from double heterozygous individuals with Hump1 (C/G) and Hump2 (C/T).

Table 20 : PCR reagents for Hump1-Hump2 assay.

Reagent	**Volume (µl)**
11.1x PCR mix	0.675
4 µM 32-H1C	0.750
4 µM 32-H2AR	0.750
4 µM 32-O	0.750
DNA polymerase (5 U/µl)	0.075
sdH_2O	4.000
DNA	0.500

Total volume	7.500

PCR parameters:

TC480 : 94 °C, 1 min; 64 °C, 1 min; 70 °C, 2 min; x30 cycles

64 °C, 1 min; 70 °C, 5 min; x1 cycle

4 °C, soak

TC9600 : 94 °C, 45 sec; 64 °C, 30 sec; 70 °C, 1 min; x30 cycles

64 °C, 30 sec; 70 °C, 3 min; x1 cycle

4 °C, soak

No restriction endonuclease digestion was needed for Hump1-Hump2.

6x BPB : 2 µl

Gel : 3% (w/v) NuSieve : 1% (w/v) Agarose

Voltage : 120-150 volts

2.10 Electrophoresis of DNA

The PCR products which were either digested or not digested with restriction endonucleases were resolved by gel electrophoresis.

The gel with the compositions of 3% (w/v) NuSieve: 1% (w/v) Agarose was dissolved in 0.5x TBE inside a Schott bottle by heating in a Samsung microwave oven with 50% power for 1.5 to 2 min. Then, 3 µl of 10 mg/ml EtBr was incorporated into a 60 ml gel to form EtBr with concentration of 5 µg/ml. The gel was then heated again as above. After the gel boiled, the cap of the bottle was tightened immediately and cooled in a running water until 50 to 60 °C. The bubbles inside the gel were released before the gel was poured into a gel tray.

The gel tray and 1 mm thickness combs were used for casting the gel. The height of the combs from the gel tray was 0.5 to 1 mm. Gel was set for 30 min. Since the gel was of high concentration and brittle in nature, dH_2O was sprayed to the wells that the combs stuck inside before the combs were pulled out. This can prevent the gel from cracking.

The horizontal gel tank (BRL, U.S.A.) was balanced with a spirit-level before 0.5x TBE was poured and covered over the gel. The PCR products together with

6x BPB were loaded into the gel and electrophoresis was carried out at room temperature from cathode (-) to anode (+) at 120 to 150 volts for 30 to 45 min. The voltage across a known distance can be measured with a multimeter (Sanwa CD-720C, Sanwa Electric Instrument Co., Ltd., Japan). The power for the electrophoresis was supplied by a power pack EC-105 from E-C Apparatus Corp., U.S.A.

After the electrophoresis, the gel was viewed on a 302 ηm ultra-violet light transilluminator (Model TM-36, UV Products., U.S.A.). The gel was photographed by using a Polaroid MP-4 Land Camera (Polaroid Corporation, U.S.A.) fitted with a Kodak red filter and Polaroid 665 instant black and white films. The photo was taken at shuttle speed B, aperture 4.5 and exposure time of 30 to 60 sec. The positive of the photo was coated with print coater and the negative of the photo was cleaned with the tap water.

2.11 Protocol for direct DNA sequencing

The method used is modified from Katrina's sequencing protocol was provided by Professor Koh Chong Lek. Katrina's method was adapted from Gyllensten and Erlich (1988), Bachmann *et al.* (1990), and the standard 'sequenase' protocol. This method allows direct sequencing of PCR amplified DNA, incorporating a step for generation of single-stranded DNA by PCR (1/2 PCR). One of the pair of primers used in the original amplification is used in the 1/2 PCR; the other one is used in the sequencing reactions.

2.11.1 Preparation of double-stranded DNA (dsDNA)

The dsDNA fragment was amplified from 3x diluted genomic DNA by using standard PCR protocol with primers 32-H1C and 32-O. The reaction volume of 5x was used for each sample except the negative control.

Table 21 shows the PCR reagents used in one reaction for dsDNA amplification.

Table 21 : PCR reagents for dsDNA amplification.

PCR	x1 (μl)
11.1x PCR mix	0.675

4 μM 32-H1C	0.750
4 μM 32-O	0.750
DNA polymerase (5 U/μl)	0.075
sdH_2O	4.750
DNA	0.500
Total volume	7.500

PCR parameters:

TC9600 : 94 °C, 45 sec; 64°C, 30 sec; 70 °C, 1 min; x30 cycles

64°C, 30 sec; 70 °C, 3 min; x1 cycle

4 °C, soak

The dsDNA was removed from the overlaying paraffin oil/mineral oil if it were applied. The dsDNA was precipitated by addition of 9.975 μl of 7.5 M ammonium acetate (NH_4OAc) and 47.25 of μl isopropanol. Incubation was carried at -70 °C for 20 min and the dsDNA was centrifuged at 15000 rpm for 5 min. The pellet was washed with 500 μl of 80% (v/v) EtOH and vacuum dried for 5 to 8 min. DNA was suspended in 10 μl of sdH_2O and 1 μl of DNA was electrophoresed to check the efficiency of amplification and precipitation.

2.11.2 Preparation of single-stranded DNA (ssDNA) or 1/2 PCR

A 1/2 PCR was carried out with primer 32-O for the first set of experiment and primer 32-H1C for the second set of experiment (reverse strand). dsDNA was used as templatè.

Table 22 shows the reagents used in one reaction for 1/2 PCR.

Table 22 : Reagents for 1/2 PCR.

1/2 PCR	x1 (μl)	Final concentration
11.1x PCR mix	4.5	1 x
4 μM 32-O or 32-H1C	12.5	1 μM
DNA polymerase (5 U/μl)	0.4	2 units
sdH_2O	29.6	--
dsDNA	3.0	200-500 ηg
Total volume	50.0	--

The PCR parameters were similar to section 2.11.1 except the DNA was amplified for 16 cycles. The ssDNA was precipitated as in section 2.11.1 except the

ssDNA was centrifuged for 20 min. The ssDNA was now ready for use in cycle sequencing.

2.11.3 Cycle sequencing

2.11.3.1 End labelling of primer

10 μM primer 32-O or 32-H1C.....1.0 μl

10x Kinase buffer.....2.0 μl

T4 Polynucleotide kinase (10000 U/ml).....0.5 μl

[γ-^{33}P]ATP.....1.0 μl

sdH_2O.....15.0 μl

Total.....20.0 μl

The primer for end labelling was incubated for 1 hr in a 37 °C Grant water bath.

2.11.3.2 Sequencing reaction

Table 23 shows the reagents used for one reaction in sequencing reaction.

Table 23 : Reagents for sequencing reaction.

Reagent	Base (μl)				Master mix of 1 sample (μl)
	G	A	T	C	
4x PCR termination mix	1.875	1.875	1.875	1.875	
ssDNA	1.000	1.000	1.000	1.000	
sdH_2O	3.525	3.525	3.525	3.525	14.1
DNA polymerase (5 U/μl)	0.100	0.100	0.100	0.100	0.4
Hot primer 32-O or 32-H1C	1.000	1.000	1.000	1.000	4.0
Total	7.500	7.500	7.500	7.500	18.5

Out of 18.5 μl of the master mix, 4.625 μl was aliquoted into each tube which was ready with 1.875 μl of 4x PCR termination mix and 1 μl of ssDNA. PCR was performed with 16 cycles by using the normal reaction time and temperature as in section 2.11.1.

Each 7.5 μl of PCR product was later added with 8 μl of stop solution before the mixture was denatured at 95 to 100 °C for 3 min. Subsequently, 4 μl of the reaction solution was loaded on the sequencing gel.

2.11.4 Sequencing gel

The model S2 sequencing gel electrophoresis system with two large glass plates (38.5 x 31 cm) from BRL, U.S.A., was used to perform the polyacrylamide gel electrophoresis (PAGE).

2.11.4.1 Assembly of glass plates

As the glass plates must be spotlessly clean before use, they were carefully cleaned with dilute non-abrasive detergent (Teepol), water and then diluted ethanol. Scratching of the surface of the plates must be avoided. The glass surface was rinsed with dH_2O and wiped dry with lint-free cloth (Sontara, MicroPure, Dupont) or tissue in a fumehood. No fingers were touched on the plates to ensure very clean plates were obtained.

One side of the short glass plate was wiped with 2% dimethyldichlorosilane (BDH Chemical Ltd., U.K.) in a fumehood. The plates was dried in the hood for 10 min before the plate was rinsed with dH_2O and wiped dry.

The glass plates were assembled with the clean surface facing inside. Two long side spacers (0.4 mm) were placed between the plates. The sides and bottom of the plates were taped firmly to prevent leakage of acrylamide. The sides were clamped evenly during and after the assembly, and the clamping was done only over the spacers. A horizontal line of 5 mm below the top of the short plate was drawn on its surface.

2.11.4.2 Preparation of a 6% polyacrylamide sequencing gel

Table 24 shows the reagents used for 6% polyacrylamide sequencing gel.

Table 24 : Reagents for 6% polyacrylamide sequencing gel.

Reagent	Amount
Urea	37.5 g
dH_2O	22.5 ml
10x TBE	7.5 ml
5x Acrylamide/bisacrylamide (38:2)	15.0 ml
TEMED	37.5 µl
10% Ammonium persulphate	450.0 µl
Total	75.0 ml

Firstly, the urea was added with dH_2O and 10x TBE. The mixture was heated briefly to about 37 °C in a microwave oven to dissolve urea. It should not be overheated.

5x acrylamide/bisacrylamide (38:2) was then added into it. The mixture was filtered through a 0.45 μm, 47 mm diameter, nitrocellulose membrane filter (Advantec, MFS, U.S.A.) positioned in a filter holder (Sartorius, Germany). The volume of the mixture was adjusted to 75 ml with dH_2O in a 100 ml graduated measuring cylinder. This was followed by addition of NNN'N'-tetramethylethylenediamine (TEMED) and freshly prepared 10% ammonium persulphate solution. The mouth of the cylinder was sealed with a piece of Whatman's parafilm and mixed thoroughly.

After the clamps were removed from the assembled glass plates, the gel was poured immediately down along one of the side spacers of the assembled glass plates. The even flow was maintained so that the solution was slowly fill from the bottom without trapping air bubbles. The gel was poured on a bench lined with an absorbent paper coated one side with polyethylene (e.g., Whatman's Benchkote). When air bubbles were trapped, the plates were placed vertically and the bubbles were worked to the top by gently tapping on the glass plates. After the gel solution had reached the top of the plates, the plates were laid down flat on a table. Immediately, the flat edge of the two clean sharktooth combs was inserted between the plates to a depth of 5 mm below the short plate. The plates were left horizontally for the gel to polymerise for at least 1 hr.

After the gel has polymerised, the clips and the tape at the bottom of the plates were removed. The top of the gel around the combs was flooded with dH_2O. The excess polyacrylamide was removed from around the combs with a blade. The combs were slided out carefully from between the plates without pressing one end of the comb into the gel during the process. The top surface of the gel was flushed with dH_2O to remove urea and any unpolymerised acrylamide. The combs were rinsed with dH_2O to clean off any acrylamide before the combs were inserted between the plates until the teeth just touched the surface of the gel. The teeth were not allowed to pierce the gel surface. A slight indentation of the gel surface were observed if the combs had been properly inserted.

2.11.4.3 Polyacrylamide gel electrophoresis (PAGE) and autoradiography

The gel sandwich was secured to the electrophoresis apparatus with the shorter plate facing the apparatus. The surface of the short plate was cleaned before fixing. The upper buffer chamber drain valve was closed before 1x TBE buffer was filled inside the upper and lower chambers. No leakage from the upper chamber should occur and no air bubbles were allowed to trap below the glass plates. The sample wells were flushed with the buffer by using a syringe with needle (Becton Dickinson, Singapore). The gel was pre-run for 30 min at 55 watts constant power to heat up the gel.

The outer glass plate was then labelled with G, A, T, and C, for each template-primer reaction. The sequencing reaction mixes were heated at 94 °C by using a PCR machine or a heat block (Thermolyne, Sybron) for 3 min in order to denature the DNA. Immediately prior to the loading 4 µl of each sample onto the sequencing gel, the sample wells were flushed with 1x TBE to remove urea that has leached into the wells. All samples were loaded into the appropriate wells quickly and the order of all samples loaded was recorded.

The gel was run in 1x TBE at 60 watts constant power (30 to 45 mA, and 1500 to 1900 volts). For two loadings, in order to read longer nucleic acid sequence, first run was carried out until xylene cyanol migrated 2/3 length of the gel. PAGE was stopped, the samples were reheated as above and the new sample wells alongside the previous wells were flushed with 1x TBE. After the samples were loaded, the PAGE was resumed at 60 watts until bromophenol blue almost reached the bottom of the gel. The samples were loaded into the appropriate wells in an asymmetrical way so that the orientation of the samples was tracked in the autoradiograph.

After PAGE, the power supply was turned off, the sequencing apparatus was disconnected from power supply and all the power cords were removed. The upper buffer chamber drain valve was opened to release the content of the upper buffer chamber into the rear portion of the lower buffer chamber. The gel sandwich was removed from the electrophoresis apparatus and placed on Benchkote on the laboratory bench in flat manner with the short plate facing up. The lower buffer chamber tray was removed from the electrophoresis apparatus and the radioactive liquid in the lower

chamber and non-radioactive liquid in the upper chamber were disposed in a careful, appropriate manner. The tray was rinsed and replaced in the unit.

Then, one side spacer was removed and the glass plates were separated carefully by prying them apart with a spatula. The short (siliconised) plate was removed in fast manner, so that the gel will stick to only one plate. The second side spacer was then removed. A sheet of Whatman 3 MM chromatography paper, precut to the size of the gel, was laid slowly on top of the gel, starting at the centre of the gel towards the two ends. A pipette was gently rolled over the top of the filter paper to squeeze out any air bubbles. The 3 MM paper was peeled off the glass plate with the gel stuck to the paper. The gel on the paper was covered with Glad's cling wrap. Air bubbles and wrinkles were smoothed away gently. The gel on the paper was then placed in a Savant's slab gel dryer with the gel and cling wrap facing up. The dryer was pre-heated for 30 min before the gel was dried thoroughly on the dryer under vacuum at 80 ^{o}C for 3 hr. After the gel was dried and cooled, the cling wrap was removed and the gel was placed in a film cassette (Kodak X-Omatic regular cassette).

In the dark room with a safelight (Paterson, England), a sheet of X-ray film (Hyperfilm-MP, Amersham) was placed directly in contact with the gel. The cassette was stored in a dark location at room temperature for 4 to 7 days (depends on the specific activity of the radioisotope). The film was then removed in the dark room with the safelight on. The film was immersed completely and developed in Kodak GBX Developer and Replenisher for 2 min. The film was then rinsed in tap water for 30 sec. Next, the film was immersed completely and fixed in Kodak GBX Fixer and Replenisher for 2 min. During the developing and fixing, non stop agitation of the film in the solution was carried to ensure better result. The film was washed with tap water for 30 sec before it was air dried in a dust-free room.

The sequence was read from the autoradiograph, starting from the first band at the bottom of the gel. This gives the sequence in a 5' to 3' direction from the primer.

2.12 Statistical analyses

The genotype distributions were checked against simple Hardy-Weinberg equilibrium expectations by using a χ^2 test (Falconer, 1981; Freund, 1984).

The expected heterozygosity or allelic diversity value (h) was calculated as $[1-\Sigma (Xi)^2]/[n/n-1]$, where Xi = allele frequencies and n = sample size (number of alleles), and the power of discrimination (Pd) was calculated as $1-\Sigma (Pi)^2$, where Pi = the frequency of each genotype (Falconer, 1981; Freund, 1984).

Pairwise comparisons between samples were estimated by using the heterogeneity G-test described in Sokal and Rohlf (1981).