Chapter III

Materials and Methods

1. Specimens

5-8 ml of EDTA-whole blood per donor obtained from 200 unrelated individuals were studied.

2. Materials

2.1 Polybutadiene Styrene conical centrifuge tubes , 15 ml. : (Nunc, USA)

2.2 Microcentrifuge tubes , 1.5 ml. : (Axygen, USA)

2.3 PCR tubes , 0.5 ml. : (Axygen, USA)

2.4 Polaroid film: (Type 667, Polaroid)

2.5 Automatic Processor Compatible Film (APC) : (Promega)

2.6 Glass pipettes , 10 ml.

2.7 Pasteur pipettes

2.8 Aerosol resistant pipette tips 10 µl, 20 µl, 200 µl, 1000 µl : (Molecular Bio-Products, USA)

2.9 Beakers, Flasks, Volumetric flasks, Cylinders, Reagent bottles

2.10 Disposable gloves

2.11 Microcentrifuge tube racks

2.12 Large metal binder clamps

2.13 Paper towel

2.14 Syringe 60 cc., 20 cc. and needles : (Terumo, Japan)

2.15 Plastic trays

2.16 Parafilm

2.17 Plastic wrap

3. Equipment

3.1 Heating block : (Multi-Block® Heater, LAB-LINE)

3.2 Low-speed centrifuge with swinging-bucket rotor : (Beckman)

3.3 Microcentrifuge, Biofuge 13 : (Heraeus, Sepatech)

3.4 Autoclave : (HICLAVE™, HIRAYAMA)

3.5 Incubator : (Memmert)

3.6 Freezer -70 °C : (Forma Scientific)

3.7 Refrigerator 4 °C, -20 °C : (SHARP, SJ439L)

3.8 Thermostat shaking-water bath : GFL)

3.9 Microwave : (Trx-2404, Turbora)

3.10 Balance : (Precisa 400 M, PAG OERLIKON AG)

3.11 pH meter : (Expandable ionAnalyzes EA 920, Orion Research)

3.12 Spectrophotometer : (UV-160A, SHIMADZU)

3.13 Quartz cuvettes

3.14 Vortex

3.15 Transilluminator : (LKB, Bromma)

3.16 UV absorbing face shield : (LKB, Bromma)

3.17 Dark room

3.18 Polaroid camera : (FotoDyne)

3.19 Fluorescent light box

3.20 X-ray film cassette : (Kodak)

3.21 DNA thermal cycler 480 : (Perkin Elmer, Cetus USA)

3.22 Thermal cycler : (Omnigene, Hybaid USA)

3.23 Laminar flow hood : (GelmanSciences)

3.24 Fume hood : (Model 252, NEWLAB®)

3.25 Horizontal^{TM}11•14 electrophoresis apparatus: (Gibco BRL, USA)

3.26 Power supply model EC105 : (E-C Apparatus corporation, USA)

3.27 Sequencing gel electrophoresis apparatus, Sequi-Gen® GT Nucleic Acid Electrophoresis Cell (30cm x 38cm x 0.4mm) : (BIO-RAD, USA)

3.28 Power supply model 2197 : (LKB, Bromma)

3.29 Water purification equipment : (LABCONCO)

3.30 Hot plate with magnetic field, stirring-magnetic bar : (IKAMAG® REC-G)

3.31 Pipet boy : (acuboy®, TECNOMARA)

3.32 Automatic adjustable micropipets ; P2 (0.1-2 µl), P10 (0.5-10 µl), P20 (5-20 µl), P200 (20-200 µl), P1000 (100-1000 µl) : (Gilson)

3.33 Timer

3.34 Ice-maker

3.35 Thermometer

4. Reagents

4.1 General reagents

Tris hydroxymethyl aminomethane : (Analytical grade, Fluka)

Hydrochloric acid

Sodium hydroxide

Ethylenediamine tetraacetic acid, disodiumsalt dihydrate ; EDTA : (Pure grade, Baker)

Sodium dodecyl sulfate : (Ultrapure grade, Amresco)

Sodium chloride : (Analytical grade, Mallinchrodt® Chemical works)

Absolute ethanol : (Merck)

Mineral oil : (Sigma)

Agarose : (Molecular grade, Promega)

Boric acid : (Analytical grade, Merck)

Ethidium bromide

Urea : (Ultrapure grade, USB™)

40% Acrylamide/Bis solution, 19:1 (5% C) : (BIO-RAD)

Glacial acetic acid : (Analytical grade, J.T.Baker, USA)

Sigmacote : (Sigma)

N,N,N',N'-Tetra-methylethylenediamine ; TEMED

Ammonium persulfate : (Analytical grade, Merck)

Bromophenol blue : (Merck)

Xylene cynanol FF : (Amresco)

Ficoll® (type400) : (Pharmacia)

4.2 Reagent Kits

- GenePrint™ STR Multiplex-CSF1PO,TPOX,TH01 : (Promega)

  STR 10X Buffer

  CTT Multiplex 10X primer pair mix

  CTT Allelic Ladders

  K562 DNA High Molecular Weight (10 ng/μl)

  STR 2X Loading solution

  pGEM® DNA Markers (20 ng/μl)

- GenePrint™ STR Systems-F13A01,F13B,FESFPS,LPL,vWA : (Promega)

  STR 10X Buffer

  Locus-specific 10X primer pair

  Locus-specific Allelic Ladder

K562 DNA High Molecular Weight (10 ηg/μl)

STR 2X Loading solution

pGEM® DNA Markers (20 ng/μl)

- DNA Silver staining System : (Promega)

Bind silane (Methacryloxypropyltrimethoxysilane)

Silver nitrate

Formaldehyde, 37%

Sodium thiosulfate

Sodium carbonate (A.C.S. grade)

4.3 Enzymes

Proteinase K : (Amresco)

Taq DNA Polymerase : (Promega)

5. Methods

- Sample collection

5-8 ml of whole blood were collected [in EDTA coated 15 ml tubes] from 200 unrelated individuals, who were blood donors at the National Blood Bank, Thai Redcross Society.

- DNA extraction (modified from Tanunyutthawongse C.[41])

1. Centrifuge each sample 10 minutes at 3000 rpm. Remove supernatant carefully, then freeze the samples at -70 °C at least 1 hour to lyse the red blood cells.

2. Quick thaw the frozen pellet at 37 °C until dissolved.

3. Add an equal volume (5-8 ml) of $T_{10}E_{10}$ (10 mM Tris pH 7.0 and 10 mM EDTA pH8.0). Vortex vigorously and centrifuge for 10 minutes at 3000 rpm. Discard the supernatant.

4. Resuspend the pellet in $T_{10}E_{10}$ (5-8 ml), mix, then spin down. Discard the supernatant. Repeat step 3 until the pellet appears white.

5. Add 2400 μl (300 μl /1 ml blood) of $T_{20}E_5$ (20 mM Tris and 5 mM EDTA). Add 160 μl (20 μl /1 ml blood) of 10% SDS and 48 μl (6 μl /1 ml blood) of 20 mg/ml proteinase K. Gently mix and incubate at 56°C in a shaking waterbath overnight for complete digestion.

6. Add 2000 μl (250 μl /1 ml blood) of saturated 6 M NaCl to deproteinate. Shake vigorously for 15 seconds. Allow tube to sit on the bench for 5 minutes, then centrifuge at 5000 rpm for 15 minutes.

7. Transfer DNA-containing supernatant to a new tube.

8. Add 2 volumes of 100% ethanol (room temperature) to each DNA tube.

9. Agitate the tubes to precipitate DNA (do not vortex) . You should be able to see the DNA. Remove ethanol by pipetting.

10. Rinse each DNA pellet with cold (stored in -20 °C) 70% ethanol two times to remove all residual salt.

11. Transfer the DNA sample to a labeled microcentrifuge tube. Allow the pellet to air dry briefly.

12. Resuspend the dry DNA pellet in approximately 100 μl distilled water, vary volume according to the pellet size. Incubate at 56 °C with periodic gentle mixing to dissolve.

13. Measure the optical density (OD) of samples at 260 nm and 280 nm.

14. Calculate the 260/280 ratio to show deproteination. The 260/280 ratio should be greater than 1.6.

- Calculation of DNA concentration

The OD reading at 260 nm is used for calculating the concentration of nucleic acid in the samples. An OD of 1 corresponds to approximately 50 μg/ml for double-stranded DNA. Therefore, DNA concentration can be calculated from the following formula

DNA concentration = OD X 50 X dilution ratio (μg /ml)

Dilute an aliquot of the original DNA to obtain the working DNA solution (working concentration approximately 10 ng/μl).

- PCR amplifications

The amplification of monoplex reactions of F13A01, FESFPS, F13B, LPL, vWA and multiplex reactions containing 3 loci of CSF1PO-TPOX-TH01 was performed according to the manufacturer's recommendations using the GenePrint™ STR systems (Promega Corporation, Madison, Wis.).[42] The PCR was carried out in 12-15 μl (modified from 25 μl without changing the concentration of components) reaction volumes containing 10 ng template DNA, 50 mM KCl, 10 mM Tris-HCl pH9.0 at 25 °C, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each of dNTP, locus-specific 1X primer pair or Multiplex 1X primer pair mix and 0.12 - 0.15 units of *Taq* DNA Polymerase (Promega) for monoplex reactions and 0.35 units for multiplex reactions.

The use of gloves and aerosol-resistant pipette tips is essential to prevent cross-contamination.

The setup of the PCR and the following electrophoresis analysis of the amplification reactions were carried out in two separate rooms. During amplification set-up, all of the reaction tubes were kept on ice.

To determine the number of reactions to be set up, this should include one positive and one negative control reaction tube. Add 1 or 2 reaction

volumes to this number to compensate for pipetting errors. While this approach does waste a small amount of each reagent, it ensures that you will have enough PCR Master Mix for all samples.

The positive amplification control contains K562 DNA as template. The negative amplification control contains sterile water (instead of template DNA).

Add 1 drop of mineral oil to each tube. Allow the mineral oil to flow down the side of the tube and form an overlay to limit sample loss and cross-contamination due to spattering.

The reactions were placed either into a Perkin Elmer 480 thermal cycler or a Hybaid Omnigene thermal cycler. The PCR was performed as follows (GenePrint™ STR Systems technical manual, Promega):[42]

GenePrint™ STR Systems	Recommended Protocols
Multiplex CTT	2
F13A01	2
F13B, LPL or vWA	7
FESFPS	1

Protocol 1	Protocol 2
96 °C for 2 minutes, then :	96 °C for 2 minutes, then :
94 °C for 1 minute	94 °C for 1 minute
60 °C for 1 minute	64 °C for 1 minute
70 °C for 1.5 minutes	70 °C for 1.5 minutes
For 10 cycles, then :	For 10 cycles, then :
90 °C for 1 minute	90 °C for 1 minute

60 °C for 1 minute

70 °C for 1.5 minutes

For 20 cycles.

64 °C for 1 minute

70 °C for 1.5 minutes

For 20 cycles.

Protocol 7

96 °C for 2 minutes, then :

94 °C for 1 minute

60 °C for 1 minute

70 °C for 1.5 minutes

For 10 cycles, then :

90 °C for 1 minute

60 °C for 1 minute

70 °C for 1.5 minutes

For 20 cycles, then :

60 °C for 30 minutes.

After the completion of the thermal cycling protocol, store all of the samples at -20 °C.

The locus-specific information and primer sequences of these eight STR loci are shown in Table. 1-3.

● Table 1. Locus-Specific Information[42]

STR locus	Chromosomal location	GenBank locus and locus definition	Repeat sequence 5' - 3'
CSF1PO	5q33.3-34	HUMCSF1PO Human c-fms proto-oncogene for CSF-1 receptor gene	AGAT
TPOX	2p23-2pter	HUMTPOX Human thyroid peroxidase gene	AAGT
TH01	11p15.5	HUMTH01 Human tyrosine hydroxylase gene	AAGT
TH01[a] Allele 9.3	11p15.5	HUMTH01 Human tyrosine hydroxylase gene	AAGT
F13A01	6p24-25	HUMF13A01 Human coagulation factor XIII a subunit gene	AAAG
FESFPS	15q25-qter	HUMFESFPS Human c-fes/fps proto-oncogene	AAAT
F13B	1q31-q32.1	HUMBFXIII Human factor XIII b subunit gene	AAAT
LPL	8p22	HUMLIPOL Human lipoprotein lipase gene	AAAT
VWA (formerly vWF)	12p12-pter	HUMVWFA31 Human von Willebrand factor gene	AGAT

a TH01 allele 9.3 is not an STR system. It is an amplified allele 9.3 from the TH01 locus and can be used as an additional sizing tool either separately or combined with the TH01 allelic ladders.

- Table 2. Additional Locus-Specific Information[42]

STR locus	Allelic ladder size range (bases)	STR ladder alleles (# of repeats)	K562 DNA allele sizes
CSF1PO	295-327	7,8,9,10,11,12,13,14,15	10,9
TPOX	224-252	6,7,8,9,10,11,12,13	9,8
TH01	179-203	5,6,7,8,9,9.3,10,11	9.3,9.3
F13A01	283-331	4,5,6,7,8,9, 11,12.13,14,15,16	4,5
FESFPS	222-250	7,8,9,10,11,12,13,14	12,10
F13B	169-185	6,7,8,9,10	10,10
LPL	105-133	7,9,10,11,12,13,14	12,10
vWA (formerly vWF)	139-167	13,14,15,16,17,18,19,20	16,16

- Table 3. Locus-Specific Primer Sequences

STR locus	Primer sequences
CSF1PO[35-36]	A: 5' AAC CTG AGT CTG CCA AGG ACT AGC 3' B: 5' TTC CAC ACA CCA CTG GCC ATC TTC 3'
TPOX[36]	A: 5' ACT GGC ACA GAA CAG GCA CTT AGG 3' B: 5' GGA GGA ACT GGG AAC CAC ACA GGT 3'
TH01[28,35-36]	A: 5' GTG GGC TGA AAA GCT CCC GAT TAT 3' B: 5' ATT CAA AGG GTA TCT GGG CTC TGG 3'
F13A01[35]	A: 5' GAG GTT GCA CTC GAG CCT TTG CAA 3' B: 5' TTC CTG AAT CAT CCC AGA GCC ACA 3'
FESFPS[35]	A: 5' GCT TGT TAA TTC ATG TAG GGA AGG C 3' B: 5' GTA GTC CCA GCT ACT TGG CTA CTC 3'
F13B[43]	A: 5' TGA GGT GGT GTA CTA CCA TA 3' B: 5' GAT CAT GCC ATT GCA CTC TA 3'
LPL[35]	A: 5' CTG ACC AAG GAT AGT GGG ATA TAG 3' B: 5' GGT AAC TGA GCG AGA CTG TGT CT 3'
VWA[44-45] (formerly vWF)	A: 5' CCC TAG TGG ATG ATA AGA ATA ATC 3' B: 5' GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG 3'

- Agarose gel electrophoresis[46]

PCR products were checked for successful amplification on 2% (w/v) agarose gels in 1X TBE buffer (50 ml for 3 mm gel thickness), containing 0.5 μg/ml ethidium bromide.

The agarose was melted in a microwave oven with occasional swirling until no granules of agarose remained visible, then poured at a temperature of 50 °C to 60°C. Be sure that the gel tray is leveled. Allow the gel to cool and solidify for 20 - 30 minutes.

Prepare the samples by mixing 5 μl of each sample with 2 μl of 6X loading solution (Capacity/Well 10 μl for Tooth Width 4.7 mm x Comb Thickness 1 mm x Gel Thickness 3 mm). Load each sample, allow the gel to run at 89 V for 1 hour in 1X TBE tank buffer. Submerge the gel at a depth of 1 to 2 mm. A molecular weight marker is always included in the first lane.

Using a UV transillumination (302 nm), photograph the gel with Polaroid film.

- Polyacrylamide gel electrophoresis[42]

♦ To prepare the 4% denaturing polyacrylamide gel with dimensions of 30cm x 38cm x 0.4mm (w x h x thickness), stepwise techniques should be followed:

Notes : Acrylamide is a neurotoxin and suspected carcinogen; avoid inhalation and contact with skin. Always wear gloves and safety glasses when working with acrylamide solutions. Methacryloxypropyltrimethoxysilane (bind silane) is toxic and should be used in a chemical fume hood.

1. Thoroughly clean the shorter and longer plates twice with paper towels saturated with 95% ethanol. Using the same paper towel, clean the spacers and combs.

2. Apply 250 μl of sigmacote onto the inner side of the **shorter** glass plate. With a dry paper towel, spread the sigmacote by circular motion over the entire surface. Always wear gloves while handling the glass plates during assembly to avoid fingerprints on the glass plates. Fingerprints will cause bubbles to form during gel casting.

3. In a chemical fume hood, prepare fresh binding solution by adding 1.5 μl of bind silane to 0.5 ml of 0.5% acetic acid in 95% ethanol in a 1.5 ml centrifuge tube. Then apply onto the inner side of the **longer** plate using a dry paper towel. Spread the binding solution by circular motion over the entire surface.

4. Wait 5 minutes for the binding solution to dry. Wipe the longer glass plate 2 times with 95% ethanol and a paper towel to remove excess binding solution

Note: Failure to wipe excess binding solution from the longer glass plate will cause the gel to stick to both plates, and the gel will be destroyed upon separation of the glass plates after electrophoresis.

5. Take special care not to allow the treated surfaces to touch each other. The two plates must kept apart at all times to prevent cross-contamination.

6. Assemble the glass plates by placing 0.4 mm side spacers between the plates and using clamps to hold them in place.

7. Prepare a 4% acrylamide solution (total of 60 ml) by adding together the ingredients listed below.

Urea	25.2	g	Final conc. 7 M
deionized H_2O	32	ml	Final conc. -
10X TBE	3	ml	Final conc. 0.5X
40% acrylamide:bis (19:1)	6	ml	Final conc. 4%

8. Add 40 μl of TEMED and 400 μl of 10% ammonium persulfate to the acrylamide solution and mix gently.

9. Pour the acrylamide solution into a syringe.

10. Carefully inject the acrylamide solution between the glass plates and maintain a constant flow of solution to prevent bubble formation.

11. Position the gel horizontally

12. Insert two 14 cm doublefine (49 point) sharkstooth combs, straight side into the gel, between the glass plates to the desired depth. Secure the combs with 4 large metal binder clamps.

13. Allow polymerization to proceed for at least 1 hour. Be sure that the gel is level. Check the polymerization control from the remaining acrylamide solution in the squeeze bottle to be sure that polymerization has occurred.

Note : The gel may be stored overnight if a paper towel saturated with deionized H_2O and plastic wrap are placed around the well end of the gel to prevent the gel from drying out (crystallization of the urea will destroy the gel).

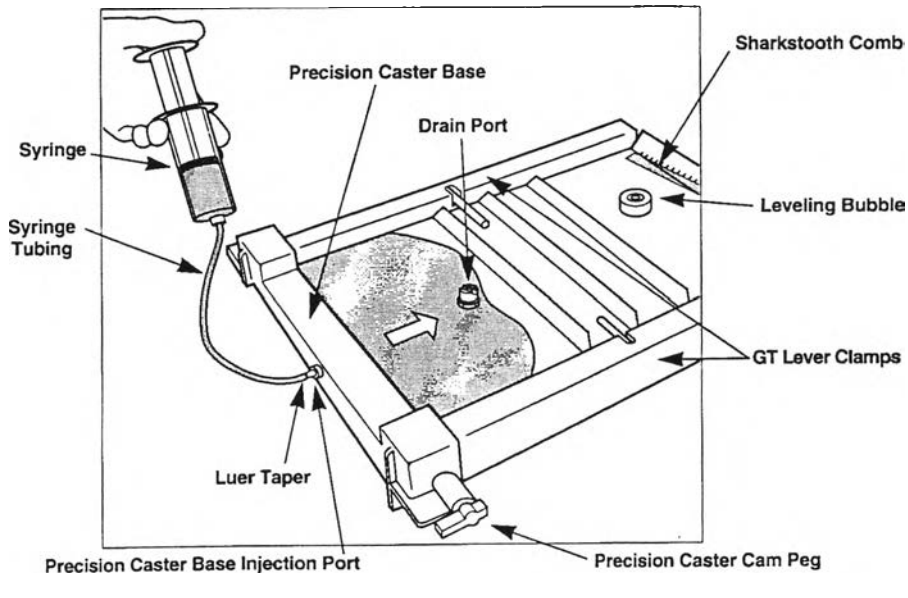

Fig. 5. Sequi-Gen GT gel casting parts.[47]

♦ Gel pre-run

1. Remove the clamps which secure the combs from the polymerized acrylamide gel.

2. Add 0.5X TBE to the bottom chamber of the electrophoresis apparatus.

3. Gently lower the gel (glass plates) into the buffer with the longer plate facing out and the well-side on top.

4. Secure the glass plates to the sequencing gel apparatus.

5. Add 0.5X TBE to the top chamber of the electrophoresis apparatus.

6. Remove the combs and shave any excess polyacrylamide away from the combs.

7. Using a 20 cc syringe filled with buffer, remove the air bubbles on the top of the gel. Be certain the well area is devoid of air bubbles and small pieces of polyacrylamide.

8. Pre-run the gel to achieve a gel surface temperature of approximately 50 ^{o}C which can be observed from a gel temperature indicator on the outer surface of the longer plate.

♦ Sample preparation

1. Prepare the PCR samples by mixing 10 μl of each sample with 5 μl of STR 2X Loading Solution.

2. Add 5 μl of pGEM® DNA markers to 2.5 μl of STR 2X loading solution for each marker lane. You should load pGEM® DNA markers into the first and last lanes of the gel.

3. Add 2.5 μl of the STR ladder to 2.5 μl of STR 2X loading solution for each ladder lane. The number of ladder lanes used depends on personal preference.

4. Briefly spin the samples in a microcentrifuge to bring the contents to the bottom of the tube.

◆ Sample loading

1. Denature the samples just prior to loading the gel by heating at 95 °C for 2 minutes and immediately chill on ice.

2. After the pre-run, use a syringe filled with buffer to flush the urea from the well area and carefully insert the sharkstooth comb teeth [approximately 1-2 mm] into the gel . Leave the comb inserted in the gel during both sample loading and electrophoresis.

3. Load 3-5 μl of each sample into the respective wells. The loading process should take no longer than 20 minutes to prevent the gel from cooling.

◆ Gel electrophoresis

1. After completion of loading, run the gel at 80 W for 40-60 minutes at approximately 50 °C. If the gel has to be loaded several times, the gel must be allowed to run 20-30 minutes before loading the next set of samples. This will prevent the samples from overlapping during electrophoresis.

Note : In a 4% gel, bromophenol blue migrates at approximately 40 bases and xylene cyanol migrates at approximately 170 bases.

2. Knowing the size range for each locus and migration characteristics of the dyes, electrophoresis can be stopped at any appropriate time. If more than one locus or a multiplex are run, be careful not to run the smallest locus off the bottom of the gel. The gel bands are detected by silver staining.

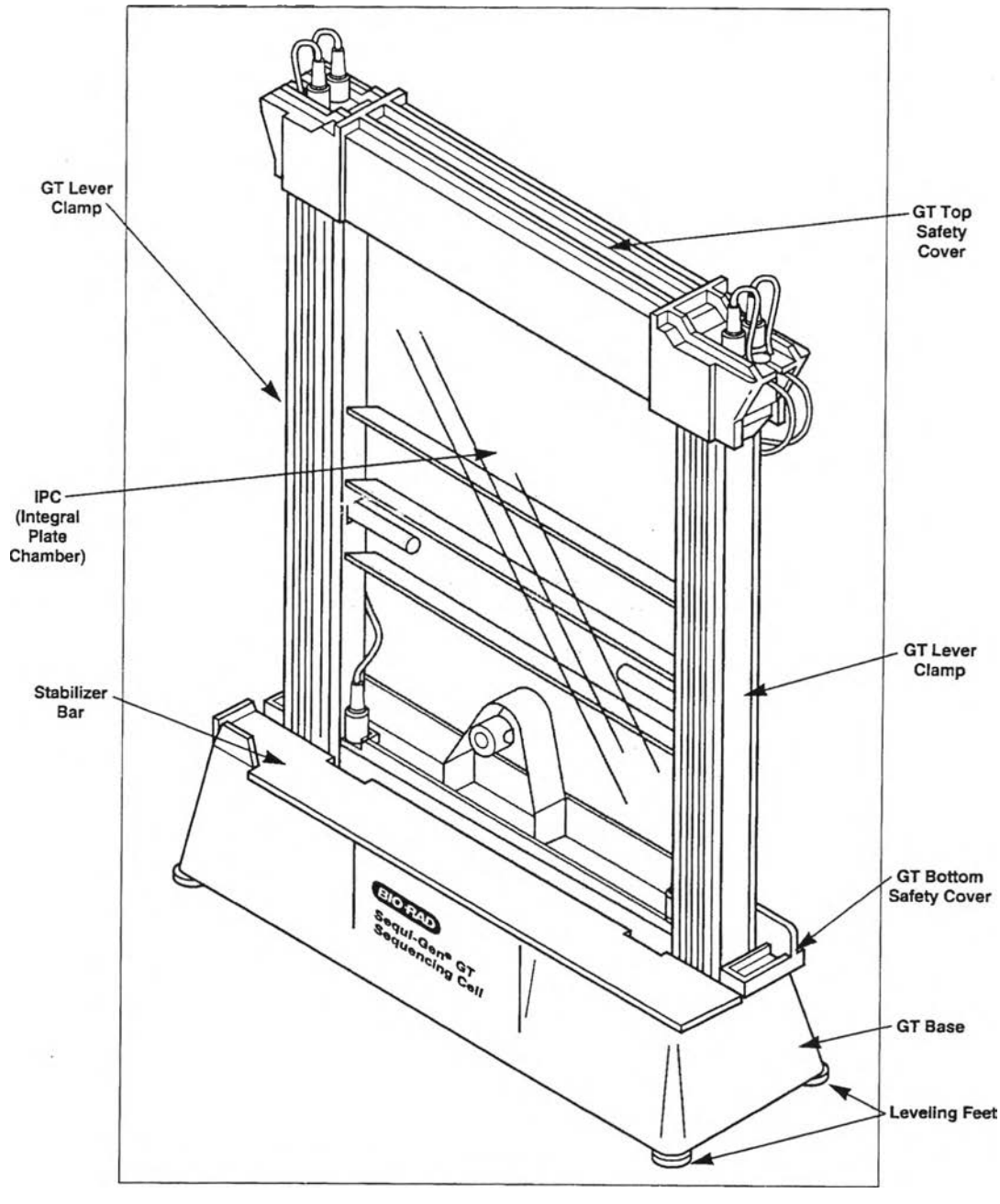

Fig. 6. Sequi-Gen GT nucleic acid electrophoresis cell.[47]

◆ Reuse of glass plates

1. Submerge the glass plates and affixed gel in deionized H_2O. Remove the gel from the longer glass plate by scraping the rehydrated gel with a plastic scraper. The plate should be cleaned thoroughly with deionized H_2O and a detergent.

2. All cleaning sponges for the longer glass plate should be kept separate from those for the shorter glass plate to prevent cross-contamination of the binding solution.

3. The longer glass plate preparation must be repeated for each gel. The shorter glass plate preparation must be repeated after every four gels.

- Silver staining[42]

Notes : -Steps involving solutions containing formaldehyde should be performed in a chemical fume hood.

- Chill the developer solution to 4-10 ^{o}C.

- Save the fix/stop solution from step 4a to use in step 4g, below.

- Gently agitate during each step.

- The duration of step 4e is important. The total time from immersion in deionized H_2O to immersion in developer solution should be less than 20 seconds. If the deionized H_2O rinse step does exceed 20 seconds, repeat step 4d again.

1. After electrophoresis, empty the buffer chambers and carefully loosen the gel clamps. Remove the glass plates from the apparatus.

2. Place the gel (glass plates) on a flat surface. Remove the combs and the side spacers. Carefully separate the two glass plates. The gel should be strongly affixed to the longer plate.

3. Place the gel (attached to the longer plate) in a plastic tray.

4. To perform the silver stain, follow steps a-h listed below :

Step	Solution	Time
a.	fix/stop solution	20 minutes
b.	deionized H_2O	2 minutes

c.	repeat step b, twice	2 X 2 minutes
d.	staining solution	30 minutes
e.	deionized H_2O	10 seconds
f.	developer solution (4 -10 ^{o}C)	up to 5 minutes (until allele ladders are visible)
g.	fix/stop solution	5 minutes
h.	deionized H_2O	2 minutes

5. Position the gel (longer plate) upright. To create film prints immediately, cover the gel with plastic wrap and expose the film.

- Exposure of film[42]

1. Place the dry, stained gel attached to the longer plate (gel side up) on a white fluorescent light box.

2. In the dark room, position the Automatic Processor Compatible (APC) film, emulsion side down, over the gel to be copied. The emulsion side of the film can be identified as the glossy white surface; the non-emulsion side has a gray tint.

3. Turn on the white light box and expose the film for 35 seconds, depending on the gel background level and intensity of the white light. (This step must be optimized for individual light boxes.)

4. Develop the film, APC film may be processed manually or with an automatic film processor. The processes like X-ray films processors.

Note : The image produced on APC film is the mirror image of the gel. Use of film allows the generation of multiple permanent images with more control over band and background intensity than does development of the gel alone.

5. If there is very little signal, decrease the exposure time. If the film appears brown or black, increase the exposure time.

- Genotype determinations[42]

A. pGEM® DNA markers

The pGEM® DNA markers are visual standards used to confirm allelic size ranges for the loci. The markers consist of fifteen DNA fragments with the following sizes (in base pairs):

2645	460	126
1605	396	75
1198	350	65
676	222	51
517	179	36

B. Controls

Observe the lanes containing the negative controls. They should be devoid of amplification products. The negative control confirms that there is no contamination.

Observe the lanes containing the positive K562 DNA controls. Compare the K562 DNA allelic repeat sizes with the locus-specific allelic ladder. The expected K562 DNA allele size(s) for each locus are listed in Table 2.

C. STR Ladders

Each locus or multiplex has a characteristic allelic ladder. The locus-specific allelic ladder information is listed in Table 2. The allelic ladders contain fragments of the same length and the same sequence as all known alleles for the locus which was run on the same electrophoretic gel.[13,38,48] Visual comparison between the allelic ladder and amplified samples of the locus allows precise assignment of alleles.

D. Genotype determinations

Alleles were classified by comparison of the sample fragments with those of the locus-specific allelic ladders. Allelic ladders are standard markers consisting of amplified fragments of the same length as either several or all known alleles for the locus.[38] These can be readily and accurately analyzed PCR amplification products, without the need for specialized equipment. Therefore, statistical analysis of populations based upon accurate, precise and confident allele determination is simplified.[38]

A sample in which two bands of different sizes can be observed is heterozygous. A sample in which one band of darker intensity can be observed is homozygous.

- Statistical analysis

1. The frequency of each allele for each STR was calculated by dividing the observed numbers of a specific allele by the total number of alleles.[49]

2. A standard χ^2 analysis of the observed and expected genotypes for each population was carried out in order to test for Hardy-Weinberg equilibrium. For a population to be in Hardy-Weinberg equilibrium (HWE), the alleles must be randomly inherited and allele frequencies remain constant from generation to generation.[49]

3. Heterozygotes are individuals with different alleles at the same locus. Homozygotes are individuals with two identical alleles at a given locus. Observed heterozygosity is also called frequency of heterozygotes and is represented by h in the following equation.[50]

$$h = n_h/n$$

where n_h is the number of individual observations with two alleles and n is the total number of individuals.

Since one is either a homozygote or a heterozygote, the frequency of heterozygote (h) plus the frequency of homozygote (H) is equal to one.[50]

$$H + h = 1$$

The unbiased estimate of the expected heterozygosity (h) was calculated from the formula[51-52]:

$$h = n(1-\sum_{i=1}^{n} x^2)/(n-1)$$

where x^2 = allele frequencies and n = total number of alleles observed.

4. The average power of exclusion is the probability that a falsely-accused putative father will be excluded as the biological father of a particular child. The higher the probability, the better the genetic system for parentage testing. This probability can be calculated for a trio when the genotypes of the alleged father, the mother and child are known, and can be calculated also for a parent/child duo. The general formulae for both situations are given below[53] :

$$\text{PE trio} = \sum_{i=1}^{n} Pi(1-Pi)^2 + \sum_{i=1, j=i+1}^{n} (PiPj)^2(3Pi+3Pj-4)$$

$$\text{PE duo} = \sum_{i=1}^{n} Pi^2(1-Pi)^2 + \sum_{i=1, j=i+1}^{n} 2PiPj(1-Pi-Pj)^2$$

where Pi = most common allele, Pj = next most common allele.

The combined power of exclusion for multiple loci can be calculated by the following equation :

$$\text{PE combined} = 1 - \prod_{i=1}^{n} (1-PE_i)$$

where n = the number of STR systems.

5. The probability of matching is the probability that two individuals selected at random from a given population will genotypically match at a particular locus or set of loci.[53] The smaller the value of Pm, the better the system for individualization purposes. This probability, Pm, is defined by the following formula :

$$Pm = \sum_{i=1}^{r} p_i^2$$

where p_i represents the frequencies of all possible genotypes.

The combined matching probability for more than one locus is the product of the individual matching probability at each locus, assuming they are not linked.

6. The discrimination power (DP) is the probability for two individuals chosen at random from a population to have different genotypes.[51,54]

$$\text{DP combined} = 1 - \sum(\text{expected genotype frequencies})^2$$

The combined power of discrimination for multiple loci can be calculated by the following equation[50]:

$$\text{DP combined} = 1 - \prod_{i=1}^{n} (1-DP_i)$$

where n = the number of STR systems.

7. The paternity index reflects how many more times likely it is that the person being tested is the biological father, rather than a randomly selected

individual. The typical paternity index is assigned to a locus rather than an individual case. Generally, a $PI_{typical}$ of less than one is indicative of non-relatedness. The $PI_{typical}$ is represented by the following equation [50]:

$$PI_{typical} = 1/2H$$

where H is the frequency of homozygotes (in this study the frequency of expected homozygotes).

The $PI_{typical}$ of several loci is the product of the individual $PI_{typical}$.

8. The polymorphism information content (PIC) measures how informative a marker is. The PIC of a marker is given by [11,55]:

$$PIC = 1 - \sum_{i=1}^{n} Pi^2 - \sum_{i=1}^{n} \sum_{j=i+1}^{n} 2Pi^2Pj^2$$

where Pi is the frequency of the *i* th allele.

A marker with a PIC of 0 is never informative, one with a PIC of 1 is always informative.