© Kamla-Raj 2018 J Hum Ecol, 63(1-3): 9-33 (2018)
PRINT: ISSN 0970-9274 ONLINE: ISSN 2456-6608 DOI: 10.31901/24566608.2018/63.1-3.3056

DNA Fingerprinting and Profiling

Satish Kumar[1] and M. K. Bhasin[2*]

[1]Department of Human Genetics & South Texas Diabetes and Obesity Institute, UTRGV School of Medicine, Edinburg, TX – 78541, USA
E-mail: satish.kumar@utrgv.edu
[2]Department of Anthropology, University of Delhi, Delhi 110 007, India
E-mail: mkbhasin17@gmail.com

KEYWORDS Genomes. Human Phenotypic Variation. Genetic Fingerprint. Tandem Repeat. Biological Sample

ABSTRACT Scientific study of human blood started around the beginning of the 20th century. Since then, there have been many discoveries of the typology of blood, and the multiple factors present in it. Laboratory (as well as field) techniques of collecting and anlysing blood have also undergone considerable refinements. A plethora of literature on the distribution of blood types in different ethnic groups world-wide was published during the 20th century. Forensic scientists used the genetic markers present in human blood to illumine cases of rape, murder and other crimes including paternity disputes. This article reviews the DNA fingerprinting and profiling methodologies used in forensic and phylogenetic investigations.

1. INTRODUCTION

The discovery of deoxyribonucleic acid (DNA) double helical structure by James Watson and Francis Crick in 1953 (Watson and Crick 1953), gave rise to modern molecular biology. Following this discovery, knowledge of the genetic material's composition, organization and function has accumulated at a very rapid pace. Genetic variation studies in the early 1980s showed that majority of the human DNA does not vary between individuals, however, the small percentage that does vary presents enormous potential to understand human phenotypic variation and disease. More recently, in the 1000 genome project the difference between two unrelated individuals was estimated at about 20 million base pairs, in other words ~ 0.6% of the 3.2 billion base pairs of the human genome is unique to an individual (Genomes Project et al. 2015). The techniques of DNA finger printing utilize this DNA variation for individual, phylogenetic and taxonomic identification (Chambers et al. 2014; Jamieson 2000; Roewer 2013).

While studying the myoglobin gene, Alec Jeffreys and his co-workers at the University of Leicester, UK, discovered that a short sequence of DNA located in one of the myoglobin gene introns, was tandemly repeated. The number of these tandem repeats varied between individuals. They isolated the piece of DNA containing this tandem repeat region and used it as a probe in DNA analyses, the probe bound to a large number of DNA regions/loci and produced a banding pattern unique to an individual and thus 'the genetic fingerprint' was discovered (Jeffreys et al. 1985a,b) . Later, a number of multi allelic "variable number of tandem repeats" (VNTR) loci that produced an allele specific banding pattern were identified. A multi-locus VNTR analysis is the principle behind today's DNA profiling technology.

2. DNA EXTRACTION

Before any type of DNA analysis, DNA must be isolated from the biological material. The source of DNA can be any biological sample (for example, whole blood, bloodstains, sperms, hairs, and other tissues) containing nucleated cell(s). The mitochondrial- and other organellar-DNA can be isolated from enucleated cells. The success and choice of the subsequent DNA profiling procedure(s) depends on the availability of sufficient amounts of DNA and its quality. For example, a "restriction fragment length polymorphism" (RFLP) analysis would require 10-50 ng of high molecular weight (MW > 20-23 kb) DNA, whereas, a polymerase chain reaction (PCR) based analysis can be successfully performed using a very small amount (0.2 - 0.5 ng) of purified DNA, and the average fragment size

*Retired Professor and Ex-Head

can be significantly smaller (≤ 2 kb). Many different methods and technologies were developed to isolate and purify genomic and mitochondrial DNA from biological samples. Typically, every method involves disruption and lysis of the cells contained in the biological sample, followed by removal of proteins and other contaminants and finally isolation of purified DNA. We have discussed several of these commonly used DNA isolation methods in this chapter, so that readers can make an informed choice based on their available biological sample and downstream analysis. The time and expense of DNA isolation may also be important factors in some analysis.

2.1 Salting out DNA Extraction (after Sunnucks and Hales 1996; and Aljanabi and Martinez 1997)

The Salting out DNA extraction procedure uses an alkaline cell lysis buffer to lyse nucleated cells contained in the biological sample, followed by a protein digestion step, performed at 56 °C in the presence of proteinase K and sodium dodecyl sulfate (SDS). The protein digestion step partially digests cellular proteins and loosen the association between DNA and the proteins. The partially digested proteins are precipitated by raising the salt concentration and the sample is centrifuged to remove the precipitated proteins. The supernatant containing the DNA is then separated and treated with isopropanol or ethanol to precipitate the DNA. The precipitated DNA is removed and washed with 70% ethanol to remove any salt and protein contaminants. The isolated DNA is then dried and dissolved in water or TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA extracted by this method is generally high molecular weight (average fragment size >20 kb) and double stranded and therefore is suitable for either RFLP analysis or PCR amplification. However, may still contain more than desired salt and/or protein contaminants for certain downstream applications.

2.2 Phenol-Chloroform DNA Extraction (after Sambrook et al. 1989)

Similar to the salting out DNA extraction procedure, cells are lysed in an alkaline lysis buffer and then incubated at 56 °C in the presence of proteinase K and SDS to partially digest cellular proteins and loosen the association between DNA and the proteins. Additionally, depending upon the sample source RNase A may also be added to the protein digestion reaction to degrade cellular RNA. After the protein digestion step the cell lysate is then extracted using buffer-saturated phenol-chloroform solution. During the phenol-chloroform extraction step the DNA remains in the aqueous phase while the cellular proteins are extracted into the organic phase. The phenol-chloroform extraction of the aqueous phase is often repeated a second time to ensure complete removal of the proteins. The DNA present in the final aqueous phase, is then precipitated using equal volume of isopropanol or ethanol. The precipitated DNA is then spooled out or removed by centrifugation and washed with 70 % ethanol to remove any remaining salt and/or protein contaminants. The isolated DNA is then dried and dissolved in water or TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA isolated using the phenol-chloroform extraction method is generally of high molecular weight (average fragment size >20 kb), double stranded and is devoid of salt and protein impurity, and therefore is suitable for most downstream applications. If the phenol-chloroform extraction of the aqueous phase is not performed properly, phenolic contaminants may interfere with some downstream applications.

2.3 Chelex Extraction (after Walsh et al. 1991)

In the Chelex DNA isolation method, the solution containing the biological sample is directly heated up to 98-100 °C in the presence of Chelex resin. The heating breaks open the cells, and partially denatures the DNA bound proteins resulting in the release of DNA in the solution. The Chelex resin binds to the cellular components and proteins leaving the DNA in the solution. The protein and cellular component bound Chelex resin is then removed by centrifugation. The DNA extracted using this method is single-stranded and therefore, is not suitable for RFLP analysis. However, can be successfully amplified and used in a PCR based analysis.

2.4 Magnetic Affinity Resin (DNA IQ™ system/ kits commercially available from Promega)

The DNA IQ™ system is commercially available as a kit from Promega and is specifically designed to isolate DNA for forensic and paternity testing applications. The DNA IQ™ extraction method uses novel magnetic resin particles to capture a consistent amount of DNA that is

highly purified for downstream forensic applications. The paramagnetic resin particles have a defined DNA binding capacity and therefore deliver a specific amount of DNA in the presence of excess biological sample and can be used to extract DNA from a variety of biological samples including liquid blood, dried blood stains, buccal or other biological sample swabs and variety of traditional and/or commercial blood sampling cards. To isolate DNA using this method the samples are predigested using a preferred method (buffers are supplied as part of the commercial kit) and then incubated with paramagnetic resin particles to facilitate the binding of DNA to paramagnetic resin particles. The DNA bound paramagnetic resin particles are separated from the solution using a magnetic stand and then washed with buffer formulations supplied in the kit. Finally, the purified DNA is eluted in a measured volume of elution buffer supplied in the kit. Since, paramagnetic resin particles binds specific quantity of the DNA, the kits are designed to isolate a measured quantity of DNA even if used with excess biological sample(s), this allows bypassing the quantification step typically necessary in other purification procedures. Furthermore, the DNA IQ system/kit does not require the use of harmful organic solvents such as phenol and chloroform and eliminates multiple centrifugation steps of most other DNA extraction procedures. However, DNA extracted using this method is single-stranded and therefore is not suitable for RFLP analysis. The method produces highly purified DNA sample preferred for PCR based forensic applications such VNTR and STR analysis.

2.5 Silica-Based Methods (after Hoss and Paabo 1993)

This method uses silica-gel membrane that selectively adsorb nucleic acids in the presence of high concentrations of chaotropic salts. The sample lysate is passed through a silica-gel membrane, which selectively binds to the DNA in the presence of high concentration of chaotropic salts present in the lysate buffer, while cellular proteins, and other metabolites remain in solution and are subsequently washed away. Then a low-salt buffer is used to elute purified ready-to-use DNA from the silica-gel membrane.

The silica-gel membrane-based method is implemented in many commercially available DNA extraction kits and comes with step by step instructions to perform DNA extraction.

The phenol-chloroform based organic extraction of DNA is one of the most widely used and cost-effective method and therefore, we included detailed step-by-step protocols for extracting DNA from various biological tissues/fluids using the organic extraction method in Table 1.

3. QUANTITY AND QUALITY ASSESSMENT OF PURIFIED DNA

3.1. DNA Concentration Measurement by Spectrophotometry

1. Dilute 10 µl of the DNA preparation in 990µl of ultrapure water (that is, 1:100 dilution), if expecting lower DNA concentration, make dilution 1:50 or 1: 20.
2. Blank a spectrophotometer with ultrapure water.
3. Measure optical density (OD) of the diluted DNA aliquot at 260 nm and 280 nm.

3.1.1 Quality Assessment

A ratio of measured OD values at 260 nm and 280 nm is used as an indicator of the extracted DNA purity. A ratio within the range of 1.6 to 2.0, is expected from purified DNA that is free from common salt and protein contaminants. An OD ratio below 1.6 may indicate residual protein or phenol contamination. Whereas a ratio higher than 2.0 may indicate RNA contamination.

3.1.2 Quantity Assessment

A DNA concentration of 50 µg/ml produce an OD measurement of 1.00 at 260 nm, in other words an OD measurement of 1.00 at 260 nm indicates 50 µg/ml DNA concentration in the solution.

Therefore, DNA concentration of an extracted sample can be calculated as follows.

DNA concentration = OD at 260nm*50* Dilution factor.

For Example: Sample DNA at 260 nm = 0.049 O.D.
1 OD = 50 µg/ml
0.049 OD*50 = 2.45 µg/ml
If diluted to 1: 100 (at the time of OD measurement)
then 2.45*100 = 245 µg/ml

Table 1: Detailed protocols for extracting DNA from various sources using organic extraction methods

Steps	*Fresh blood*	*Stored blood*	*Blood stains*	*Semen stains and swabs*
1.	To fresh blood sample, add 4 volumes of ***RBC lysis Buffer***[1] in a polypropylene tube. Mix gently until the solution becomes clear.	The DNA can be extracted from the intravenous blood specimens collected in EDTA or ACD anticoagulant and stored at -20 °C or -80 °C for long term preservation.	Cut the cloth or swab containing the bloodstains into small pieces in a sterile petri dish and then transfer into a sterile 15 ml tube.	Sock the swab- or stained fabric- sample in 1 ml of ultra-pure water in a sterile 1.5 ml microcentrifuge tube and incubate at room temperature for 30 minutes to hydrate the sample.
2.	Centrifuge at 2,500 rpm for 5 minutes and discard the supernatant containing lysed RBCs.	An equal volume of ***RBCs lysis buffer-I***[4] may be added to such specimens prior to their storage at ultra-low temperatures.	Add appropriate volume (sufficient to fully soak the stained material) of **1X *Phosphate Buffer Saline***[8] to the stain(s) and incubate for 2-4 hours at 56 °C	Swirl the swab/ fabric sample vigorously for 2 minutes with an autoclaved toothpick to release the cells.
3.	To obtain a white blood cells pellet that is free from RBCs, repeat RBC lysis steps to remove any residual RBCs from the cell pellet.	For DNA extraction, thaw the frozen sample tube in a water bath for 10-15 minutes. If the ***RBCs lysis buffer-I***[4] was not added prior to sample storage, add equal volume, and then mix the content by turning tube end to end. Centrifuge the tube at 10,000 rpm for 10 minutes at 15 °C.	Separate the fabric from elute by pressing it through a 10 ml syringe without needle and discard the fabric pieces.	Remove the swab/fabric substrate and toothpick. Do not discard the substrate until microscopic analysis is performed to detect the sperm and/ or cells. Store the substrate in sterile tube. If the microscopic analysis fails to detect sperm/cells in the extract agitate the substrate more vigorously.
4.	Disturb the cell pellet thoroughly and then add half volume (to that of the total blood sample) of ***Digestion buffer***[2]. Mix thoroughly and gently by inverting the tube 3-4 minutes or until the solution becomes viscous.	After centrifugation, discard the supernatant without disturbing the cell pallet. Alternatively, the entire content of the frozen sample may be digested directly as describe in next step, however, adjust the reagent volumes accordingly.	Spin the tube containing the eluted sample at 10,000 rpm for 10 minutes at 15°C and collect the pellet.	After positive microscopic results, centrifuge the sample extract at 10,000 rpm for 2-3 minutes at room temperature. Without disturbing the pellet, discard the supernatant.
5.	Add 1/4th volume to that of the digestion buffer, of ***5M sodium per chlorate solution*** [3] and then mix gently for 3-4 minutes.	Add equal volume (to that of blood) of ***Digestion buffer***[5] to the cell pellet, dislodge the cell pallet and then add 100 ì g/ml ***Proteinase-K***[6] and 2% ***SDS***[7] (final concentration). Mix thoroughly but gently and then incubate at 56 °C for 3-4 hours in a water bath.	Add equal volume of the ***Digestion buffer***[9] to the pellet, dislodge the pellet, and then and then add 100 ì g/ml ***Proteinase-K***[6] and 2% ***SDS***[7] (final concentration). Mix thoroughly but gently and then incubate at 56 °C for 3-4 hours in a water bath.	To the extracted pellet, add equal volume of the ***Digestion buffer***[9], dislodge the pellet, and then add 40 mM ***dithiothreitol,*** 100 ì g/ml ***Proteinase-K***[6] and 2% ***SDS***[7] (final concentration). Mix thoroughly but gently and then incubate at 56 °C for 3-4 hours in a water bath.
	Proceed to step 6-18 immediately.			

Table 1: Contd...

Steps	*Soft tissue*	*Bone marrow*	*Bone*	*Hairs*
1.	Cut the tissues into small pieces with a sterile scalpel and then separate out any adherent adipose tissues from it.	Fix or hold the bone firmly and brush the bone surface with sandpaper.	Cut the bones into small pieces and scrape the exterior with fresh sandpaper and wipe with 70% alcohol.	Wash each hair to be analyzed separately by immersing in fresh deionized water.
2.	Grind the tissues thoroughly with an autoclaved porcelain mortar and pestle by frequent additions of liquid nitrogen. Alternatively, a mechanical homogenizer may be used.	Using a handsaw or mechanical saw, open the bone longitudinally. (Make sure to clean the saw surface and teeth with 70% alcohol, before and after the use).	With the handheld mechanical driller, drill holes in the bone and collect the powder. Incubate the bone powder in ***0.5M EDTA (pH 8)*** [15b] at 56 °C for 48 hours.	Using a clean scalpel, cut the 1 cm portion of the hairs from root end and collect the root-ends in a sterile 1.5 ml microcentrifuge tube.
3.	Transfer the tissue homogenate into a sterile 15 ml tube and allow the liquid nitrogen to evaporate.	Scrape the interior portion of the bone (containing bone marrow) with a sterile spatula or blade and collect the scrapings in a sterile 15- or 50-ml tube.	Homogenize the bone powder and then centrifuge at 10,000 rpm for 15 minutes at 15 °C. Discard the supernatant and collect the pellet.	Add 0.5 ml of ***Digestion Buffer***[12], 20 ì1 of ***1 M dithiothreitol*** (final concentration of about 40mM) and 15 ì1 of 10 mg/ml ***Proteinase-K***[6].
4.	Add ***Digestion buffer***[10] (1.0 ml for every 500 mg of the tissue), 150 ì g/ml ***Proteinase-K***[6] *and* ***2% SDS***[7] (final concentration) into the tube.	Add appropriate volume of ***Digestion buffer***[11], mix thoroughly and then incubate at 56 °C for 2 hours in a water bath. Transfer the tube content into a glass homogenizer and then homogenize thoroughly.	Wash the pellet thrice with ***1X Phosphate Buffer Saline***[8] (to ensure complete removal of EDTA).	Vortex at high speed for 30 seconds and then incubate at 56 °C for 6-8 hours or overnight (until the hair strands are completely dissolved.
5.	Incubate the tissue digestion tube overnight at 37 °C in a water bath or until the tissue/ cells are completely digested.	Collect the homogenate in a sterile 15- or 50-ml tube and then add 100 ì g/ml ***Proteinase-K***[6] and 2% ***SDS***[7] (final concentration) and incubate at 37 °C for 4-6 hours or overnight (until the cells are fully digested).	Add appropriate volumes of the ***Digestion buffer***[5], ***Proteinase-K***[6], ***and SDS***[7] (2% final concentration) and then incubate overnight at 37 °C.	After digestion vortex the sample for 30 seconds and then centrifuge at 12,000 rpm for 1 minute at room temperature, to remove any pigment particles. Transfer the supernatant to a new sterile 1.5 ml microcentrifuge tube.
	Proceed to step 6-18 immediately.			

Steps	*Common for all sample types*
6.	Add equal volume of ***Tris-saturated phenol (pH 8.0)***[14] and mix gently for 10 minutes by inverting tube end to end.
7.	Centrifuge at 10,000 rpm for 15 minutes at room temperature.
8.	Collect the supernatant (aqueous phase) into a fresh sterile tube and then add equal volumes of ***Phenol+ Chloroform+ isoamyl alcohol***[14a] (25:24: 1), mix gently for 10 minutes by inverting tube end to end. Centrifuge at 10,000 rpm for 10 minutes at room temperature.
9.	Transfer the upper aqueous layer into a fresh sterile tube carefully using a broad mouth tip. Make sure the middle protein layer is not disturbed.
10.	Add equal volumes of ***Chloroform+ isoamyl alcohol*** (24: 1), mix gently for 2-3 minutes by inverting tube end to end. Centrifuge at 10,000 rpm for 10 minutes at room temperature.
11.	Transfer the upper aqueous layer into a fresh sterile tube carefully using a broad mouth tip.
12.	Add 1/20th volumes of ***3 M sodium acetate (pH 5.2)***[13] and equal volumes of chilled isopropyl/absolute alcohol, mix gently to precipitate the DNA.
13.	If DNA is not precipitated keep the tube at -20 °C for one hour.
14.	Spool the precipitated DNA into a fresh sterile tube using a pipette tip or collect by centrifugation. Discard the alcohol.
15.	Wash the extracted DNA with 70% ethanol.
16.	Air or vacuum dry the purified DNA pellet. Do not over dry the DNA pellet otherwise it will be difficult to resuspend into the solution.
17.	Resuspend/dissolve the DNA pellet in appropriate volumes (50 to 200 µl) of ***Tris EDTA (TE) buffer***[15] at 56 °C in a water bath for 30 to 40 minutes.
18.	Store the isolated DNA samples at 4 °C for short term for down stream applications or at -20 °C or -80 °C for a longer term.

3.2. Quality Check by Gel Electrophoresis

The extracted DNA should be of high molecular weight and not broken down in small fragments, particularly for RFLP analysis. This must be ensured for each DNA sample by running a small aliquot of DNA sample on an agarose gel.

1. For electrophoretic analysis of the extracted DNA, wash an appropriate size gel tray and an electrophoresis gel comb.
2. The edges of the gel plate are sealed with sticky tape so that it forms a wall 1 cm. high all around the plate.
3. Place it on a leveled surface preferably on a screw feet spirit leveler table.
4. Place a gel comb so that there is about 1mm of space between the base of the teeth and the gel tray.
5. To prepare ~1% agarose gel in a 7cm. x 7cm. gel tray, weigh ~ 0.4 g of agarose into a 250 ml conical flask.
6. Add 40 ml of 0.5X *TAE buffer*[16], mix gently and heat in a microwave oven or boiling water bath until agarose dissolves.
7. Let the agarose solution cool to about 65 ^{o}C and then add 2µl of 1% *ethidium bromide*[17] solution (~ 0.5µg/ml final concentration in the gel), mix it gently and pour it on to the gel tray and allow it to set for 20 – 30 minutes at room temperature.
8. Remove the sticky tape from the gel tray and place it in an electrophoresis tank.
9. Pour 0.5X *TAE buffer*[16] into the electrophoresis tank until the gel gets immersed.
10. Take a 2µl aliquot of the extracted DNA sample.
    Note: If the concentration of the extracted DNA sample is very high dilute the DNA with ultrapure water or TE and record the dilution factor.
11. Add 2µl of the *6X loading dye*[18] to the 2µl DNA sample aliquot.
12. Carefully remove the gel comb.
13. Connected the electrophoresis tank to an electrophoresis power supply, cathode towards the wells side, DNA will move towards the anodal side. Do not start the electrophoresis yet.
14. Load the prepared samples and an appropriate size molecular ladder into the wells.
15. Switch on power supply and electrophoresis at 50 to 80 volts for 30 to 60 minutes or until Bromophenol Blue dye (*loading dye*) move up to the middle to two-thirds of the gel.
16. Switch off the power supply and take out the gel from the tank and drain excess liquid.
17. Place the gel on to a UV transilluminator and document the quality and size of the sample DNA band with reference to the molecular ladder.

4. CLASSICAL DNA PROFILING

(DNA FINGERPRINTING USING DNA HYBRIDIZATION BASED TECHNIQUE)

The classical/early DNA profiling mainly relied on larger VNTR regions/loci and was carried out using Restriction Fragment Length Polymorphism (RFLP) analysis. In early 1970s the discovery of class II endonucleases that cut the DNA in a predictable and sequence specific manner paved the way for the development of RFLP techniques. In 1985, Jeffreys and his co-workers used the same technique to develop the first human DNA fingerprint. The class II endonucleases also commonly referred to as restriction enzymes cuts DNA into smaller molecules called as restriction fragments. When an individual's DNA carrying the autosomal VNTR region is treated with specific restriction enzyme, it usually results in restriction fragments of two different sizes, one inherited maternally and the other inherited paternally and these alleles of a VNTR locus are elements of genetic polymorphism. The lengths of the corresponding restriction fragments define the polymorphic alleles that were used in classical DNA profiling to develop individual's unique DNA fingerprint. (Panneerchelvam and Norazmi 2003; Snowdon and Langsdorf 1998).

Restriction Enzymes Fundamental Tools in DNA Analysis

As discussed above restriction enzymes are characterized by their ability to recognize a specific DNA sequence, usually comprised of 4-6 base pairs, and 'cut' the DNA double strands either within or in close proximity to the DNA sequence recognition site. Consequently, restriction enzymes are being used to cut the DNA molecules reproducibly and predictably in a se-

quence specific manner and therefore have become the biochemical tools of fundamental importance in both gene technology and DNA analysis.

4.1. RFLP Analysis Protocols

4.1.1. Restriction Enzyme Digestion

Set up the restriction enzyme reaction in a 1.5 ml microcentrifuge tube.

1. Add up to 2 µg of DNA, prepared as described in section 2 and 3 of this chapter. If the DNA solutions is viscous, a wide bore pipette tip (~ 2 mm) may be used for measurement of the DNA solution.
2. To the DNA sample, add 5 µl of the 10X Restriction Enzyme buffer.
3. Add a required amount of *Restriction Enzyme*[19] (up to 5 µl volume). The amount of enzyme required is calculated in units (1 unit of enzyme cuts 1 µg of DNA in 1 hour at 37°C in a volume of 50 µl reaction).
4. Bring the final reaction volume to 50 µl with ultrapure water.
   Note: A larger reaction volume may be used if more than 2 µg DNA is to be cut or if the enzyme is too dilute. But make sure the amounts of reagents are adjusted accordingly and the volume of enzyme used do not exceed one-tenth of the reaction volume.
5. Incubate the reaction mixture for 1 hour at appropriate temperature. Most enzymes work best at 37°C but some may require a different temperature, follow the restriction enzyme manufacturer's instructions. Mostly, 1-hour incubation is sufficient; however, it is often convenient and advantageous to incubate the reaction longer or even overnight.

A fast test gel should be run initially to assess if the DNA is well cut:

1. Using agarose gel electrophoresis technique described above, perform agarose gel electrophoresis of 2-5 µl of the reaction digest at 120 V (50 mA) for 1-2 hour(s).
2. Visualize the restriction fragments using an UV transilluminator. The gel may be photographed for documentation. If DNA is not cut to completion, re-digest the DNA using more restriction enzyme. If the DNA is cut as desired, the remaining reaction mix is separated by agarose electrophoresis as described below for Southern blotting.

4.1.2. Concentrating Restriction Digested DNA

If the volume of digested DNA exceeds the capacity of the agarose well or if same sample is to be digested with another restriction enzyme. The DNA may be concentrated using following steps:

1. Add 1/10 volume *3M NaAcetate*[13] to the restriction digest and mix well by inverting tube end to end several times.
2. Then add 2 to 2 ½ volume of ice cold 100% ethanol, mix by inverting the tube end to end several times.
3. Incubate the tube at -20 °C for 30 minutes to facilitate DNA precipitation. If do not wish to proceed, the reaction can be paused over night at this step.
4. After incubation centrifuge at 12,000 rpm and 4 °C for 15 to 20 minutes. Carefully discard the supernatant (the DNA pellets may be fragile and difficult to see).
5. Wash the DNA pellet with ice cold 70% ethanol. Centrifuge at 12,000 rpm and 4 °C for 5 to 10 minutes. Discard the supernatant without disturbing the pellet.
6. To remove the remaining liquid, centrifuge inverted at 180g for 30 seconds or air dry the DNA pallet.
7. Resuspend the DNA pellet in a volume of *TE Buffer*[15] sufficient to achieve 400-500 ng/µl DNA concentration. Resuspension of DNA may be aided by placing tube in a 37 °C water bath. The resuspended DNA may be stored frozen for a prolonged period.
8. Return to *restriction digestion protocol* for restriction digestion with another restriction enzyme or proceed to analysis by agarose gel electrophoresis. The DNA must be completely and uniformly resuspended prior to loading on an analytical gel.

4.1.3. Agarose Gel/Analytical Gel Electrophoresis[20]

1. Prepare a 20 x 20 cm gel tray and an appropriately sized gel comb using the method described in section 3.2. The teeth of the gel comb should be ~ 5 mm broad and 1-1.5 mm thick.
   Note: (If the gel is 9 mm thick, a 5 mm x 1 mm well will hold 40 µl of DNA sample and a 5 mm x 1.5 mm well will hold 60 µl).

2. Place the gel tray mounted with the gel comb on a leveled surface preferably on a screw feet spirit leveler table. The distance between the base of the comb teeth and the gel tray should be about 1 mm.
3. To prepare a 0.6% to 0.7% agarose gel, weigh 2.1 to 2.45 g of agarose in a 500ml conical flask and then add 350 ml of *Agarose Gel Running Buffer*[21].
4. Mix gently by swirling the flask and then heat in a microwave oven or a boiling water bath until agarose is completely dissolved.
5. Let the agarose solution cool to about 65 °C and then pour it in the gel tray. Allow the gel to set at room temperature for 20 to 30 minutes. The gel may be store overnight in a humidifying chamber at 4 °C.
6. Remove the tape used in preparing the gel tray boundary and place the gel tray into an appropriate size electrophoresis tank.
7. Fill the electrophoresis tank with *Agarose Gel Running Buffer*[21] to the level that the buffer is 1 mm above the gel surface.
8. Add agarose *Gel Loading Solution*[22] to the restriction digest (Final concentration 20% to 30%). *For example, for 30 µl of restriction digest add 9 µl of Agarose Gel Loading Solution.*
9. Prepare a Biotinylated DNA Molecular Weight Markers to be run in one well of the analytical gel. The volume and concentrations of the Molecular Weight Markers working solution should be same as the restriction digest/samples.
   For Example, a 40 µl working solution of the Molecular Weight Markers will contain 10 µl of Biotinylated DNA Molecular Weight Markers, 3 µl 10X Restriction Enzyme Assay Buffer, 17 µl of ultrapure water, and 10 µl Agarose Gel Loading solution.
10. Heat the Molecular Weight Markers working solution to 65 °C for 5 minutes and then immediately cool on ice.
11. Load the Molecular Weight Markers and restriction digested DNA samples into the gel and electrophoresis at 40 V for 20 hours or until bromophenol blue dye has just left the gel and at room temperature.
12. To stain the gel with *Ethidium bromide*[17], prepare an appropriate amount of 0.5 µg/ml Ethidium bromide working solution in *Agarose Gel Running Buffer*[21].
13. Place the gel in an appropriate tray/container and pour the Ethidium bromide working solution so that the gel is fully submersed.
14. Incubate at 37 °C with constant agitation for 30 minutes.
15. Place the gel on an UV transilluminator and examine the migration of digested DNA. The gel may also be photographed for documentation.

4.1.4. Southern Transfer

Denaturation and Blotting[23]

1. Place the gel in an appropriate tray/container and carefully trim away any unused portions of the gel.
2. Perform acid depurination to partially hydrolyze the gel. Submerge the gel in several volumes of *Southern Transfer Depurination Solution*[24] for 30 minutes. Due to the acid treatment Bromophenol blue dye will turn yellow. The Bromophenol blue colour shift may be used as a control/indicator of acid depurination process.
3. After acid depurination, denature the DNA by submersing the gel in several volumes of *Southern Transfer Denaturing and Transfer Solution*[25] and incubating at room temperature for 30 minutes under constant agitation.
4. The colour of bromophenol blue shifts back from yellow to blue due to alkaline *Southern Transfer Denaturing and Transfer Solution*[25].
5. Carefully invert the gel so that its original under side is uppermost and place it on a Whatman No. 3 filter paper wick that has been saturated with *Southern Transfer Denaturing and Transfer Solution*[25].
6. Place the gel on a glass plate, filter paper wick facing down and then place the glass plate in a tray/container filled with *Transfer Denaturing and Transfer Solution*[25] in such a way that only filter wicks touches the transfer solution. Squeeze out any bubbles between the gel and filter paper wick.
7. Carefully align one side of the nitro cellulose (marked side down) membrane to one edge to the gel and then gently place it on to the gel. Remove any air bubble forms between the gel and the nitro cellulose membrane.
8. Cut 4 pieces of Whatman No. 3 filter paper to the gel size. Saturate the two filter paper

pieces with distilled water and place them on the nitro cellulose sheet.

9. Place the remaining two dry pieces of filter paper on top of wet filter papers and remove air bubbles carefully.
10. Immediately place a stack of paper towels on top of filter papers followed by an appropriate size glass plate. On the top of the glass plate place a weight of 500 g, make sure the weight is distributed evenly over the entire area of the stack.
11. Allow the transfer to proceed at room temperature for 6 to 8 hours or overnight.
12. Using a pair of forceps dissemble the transfer apparatus/stack and remove the cellulose membrane.
13. Neutralize the membrane by submerging it in ***Southern Transfer Neutralization Solution***[26] for 5 minutes.
14. Transfer the membrane to a dish containing 2X ***SSC***[29a] and submerge for 2 minutes.
15. Place membrane between two sheets of Whatman No. 3 filter paper in a vacuum oven at 80 °C for 1 hour or until dry.
16. Membrane may be stored overnight by covering with a Whatman No. 3 filter paper and placing in dry place at room temperature.

4.1.5. DNA Hybridization[27]

1. Prepare 40 ml *Prehybridization Solution*[28] (~0.1 ml solution/cm^2 membrane).
2. Submerge the baked membrane in distilled water for 2 minutes.
3. Handle the baked membrane edges with forceps and transfer it in sealable plastic pouch.
4. Add Prehybridization Solution to the pouch, squeeze any air out and seal the pouch using a heat sealer (make double seal).
5. Place the sealed pouch in a container filled with water heated to 60 °C and then incubate in a shaking water bath at 60 °C for 2-4 hours.
6. Prepare 10 ml *Hybridization Solution*[28] (~0.025 ml solution/cm^2 membrane) and add 100 µl biotin labelled denatured DNA Probe (that is,10 µl probe/ml of Hybridization solution). *(For denaturation, aliquot a calculated volume of probe solution into a tube. Heat the tube to 95 °C for 5 minutes in a dry bath or in a thermal cycler and then immediately cool it on ice. Add hybridization Solution to the probe just prior to use).*
7. At the end of prehybridization, remove the pouch from the water bath, cut one side and remove the prehybridization solution.
8. Add *Hybridization Solution*[28] containing denatured probe to the pouch, squeeze out any air introduced during this process and re-seal the pouch with a heat sealer.
9. Place the sealed pouch in container filled with water pre-heated to 60 °C and incubate in a shacking water bath at 60 °C overnight with gentle shaking.
10. Prepare *Hybridization Wash Solutions*[29&30] and store in a closed container. Pre-warm the solutions to 60 °C before use.
11. At the end of hybridization remove the membrane pouch and cut one side to remove the membrane. Handle the membrane with forceps. Do not touch membrane with hand.
12. Wash the membrane in *Hybridization Wash Solution No. 1*[29] briefly (a 400 cm^2 membrane will require 300 ml Wash Solution).
13. Repeat the wash twice in Hybridization Wash Solution No. 1.
14. Transfer the membrane in *Hybridization Wash Solution No. 2*[30] using forceps, close the container and incubate at 60 °C in a shaking water bath for 5 minutes.
15. Repeat the wash twice in *Hybridization Wash solution No. 2*.

4.1.6. Colour Development and Autoradiography

Colour Development

1. Prepare Avidin - Horseradish Peroxidase - Complex solution by adding 50 µl of Biotinylated Horseradish-Peroxidase and 50 µl of Avidin probe to 40 ml of *Colour Development Buffer A*[31].
2. Incubate the Avidin-Horseradish Peroxidase-Complex Solution at room temperature for 30 minutes.
3. Using forceps transfer the membrane from *Hybridization Wash Solution No. 2* container to a container of *Colour Development Buffer A*[31], make sure the entire membrane is submersed in the buffer. Incubate at room temperature for 5 minutes with gentle shaking.
4. After 5 minutes discard the *Colour Development Buffer A*[31] and using forceps trans-

fer the membrane into a sealable plastic pouch.

5. Immediately pipette the Avidin-Horseradish Peroxidase-Complex solution into the plastic pouch and squeeze out any air entered during this process. Seal the pouch and incubate at room temperature for 40-60 minutes with gentle agitation.
7. Using forceps remove the membrane from the plastic pouch and transfer to a container filled with 300 ml of *Colour Development Buffer B*[32]. Incubate at room temperature for 5 minutes with gentle agitation.
8. Wash the membrane four additional times in *Colour Development Buffer B*[32]. A total of 5 washes.
9. Prepare a 400 ml 1:20 solution of reconstituted TMB (3,3', 5,5;-tetramethylbenzidine) Chromogen (2 mg/1 ml TMB in 100% ethanol) and *Colour Development Buffer C*[33]. Keep the solution on ice.
10. Transfer the membrane to a container filled with 300 ml of *Colour Development Buffer C* and incubate for 5 minutes at room temperature with gentle agitation.
11. Discard *Colour Development Buffer C* and pour half (200 ml) of the Chromogen/Buffer C solution (prepared in step 9) on the membrane. To avoid photo-oxidation of TMB, protect the container from light. Incubate at room temperature for 5 minutes with gentle agitation.
12. To the second half of the Chromogen/Buffer C solution add 90 μl of 3% *Hydrogen Peroxide*[34] (0.0014% final concentration).
13. Proceed immediately, using forceps transfer the membrane to a clean empty container and then add the Chromogen-Buffer C-Hydrogen Peroxide solution. Incubate at room temperature for about 30 minutes with gentle agitation and protected from light. Make sure the entire membrane is covered with solution.
14. When optimal contrast is achieved, stop the colour development reaction by draining the chromogen Buffer C- Hydrogen Peroxide solution from the membrane. Rinse the membrane and container with distilled water.
15. Add fresh distilled water to fill the container and incubate for 30 to 60 minutes at room temperature with gentle agitation.
16. Store the membrane dampened with distilled water in a sealed pouch in the dark.

(For long term storage, wash the membrane 5 times for 30 minutes in distilled water to remove remaining free (unreacted) TMB trapped in the membrane and then store in a sealed plastic pouch protected from light. Unremoved free TMB will photo-oxidize and darken the background.

Alternatively, membrane may be air-dried and stored in the dark. Photograph while wet or Photocopy the dried membrane for documentation.

Autoradiography

Alternatively, the DNA probe(s) may be radioactively labelled, often with ^{32}P for detection. The radioactively labelled probes are detected using X- ray film and the process is commonly referred to as autoradiography. Typically, an X-ray film is placed against the gel or blot carrying the hybridized radiolabeled DNA probe overnight. The beta particles emitted by radiolabeled probe penetrates the X- ray film emulsion to a depth proportional to the particle's energy. The beta particles passing through the film activates the silver halide crystals in the X- ray film emulsion and when the X- ray film is developed, the activated crystals are reduced to form black silver grains, which produces a permanent record of the positions and relative intensities of radiolabeled bands in a gel or blot.

1. Place the blot against an unexposed X-ray film in a film cassette. It is important that an intensifying screen is placed over the X-ray film, to enhance the signal strength.
2. Incubate the cassette at -70 °C, preferably overnight.
3. After the appropriate exposure allow cassette to warm to room temperature to prevent condensation upon opening the cassette.
4. In a dark room open the film cassette and place the film in developer solution for 5-10 minutes with gentle agitation.
5. Rinse briefly in water and then place in fixer solution for 10 minutes.
6. After fixing rinse the X- ray film in water and view under normal light.

4.1.7. Interpretation of Photograph or Autoradiographs

Interpret the results from the photograph, if chromogenic staining is used- or from autoradiograph if autoradiography is used- for probe

detection. A control DNA sample of known profile should always be included in the experiment for a comparative analysis, to determine that all the steps were performed precisely and accurately. The results on photograph or autoradiograph developed by multi-locus and/or locus specific probes are analyzed as per the objective of the DNA profiling test. The known and unknown samples are run in different lanes in parallel and the bands on the photograph or autoradiograph are examined to determine " the match" of unknown sample and the known sample(s) by visual inspection (size and location of the bands measured manually) or using a computer connected digitizer. A match is said to occur if the sizes and number of the detected RFLP bands in the known sample lane and unknown sample lane are indistinguishable or are within a permissible degree of error. If a match is found, it is important to determining the likelihood of the match by random chance or in other word the likelihood that the match is unique. The likelihood tests are performed by generating the probe specific allele frequency data in relevant population(s). The population studies to determine the allele frequencies should utilized the same methodology as used in the DNA profiling.

4.2. Advantages and Limitations

The greatest advantage of RFLP based DNA profiling is that the classical VNTR regions/loci are highly polymorphic and shows a very high degree of allelic variability. Therefore, identical DNA profile of two unrelated individuals are extremely unlikely. However, there are several inherent weakness of a RFLP based analysis, which includes (i) large restriction fragments with small variation in their size/molecular weight are often difficult to separate clearly on a electrophoresis gel, which makes correct identification of the variant(s) harder and error prone, especially if the samples were analyzed on separate gels; (ii) high molecular weight DNA is essential for successful RFLP-based DNA profiling; and (iii) large amount (at least 20 nanogram) of highly purified DNA is required for RFLP analysis. These limitations restrict the successful use of this methodology in medico-legal cases where the sample(s) are limited and are often degraded. For example, DNA extracted from blood stains, semen stains and stains of other body fluids collected from crime scenes, are often limited and degraded. Furthermore, the interaction of DNA molecules with co-extracts from the background, for example dyes from cloths or other contaminants lifted from the crime exhibits may alter the DNA's molecular configuration and/or molecular weight, which consequently will affect the migration rate of the restriction fragments on an analytical gel.

5. MODERN DNA PROFILING – TECHNOLOGY

Modern forensic DNA profiling methodologies uses short tandem repeat (STR) loci, also commonly referred as microsatellites, instead of larger VNTRs. The STR loci are highly polymorphic and contains a repeated nucleotide sequence, that is two to seven nucleotides in length. The numbers of nucleotides per repeat unit are mostly same in the entire length of the STR locus. However, the number of repeat units may differ and hence making the loci highly polymorphic in their lengths (Fan and Chu 2007; Moretti et al. 2017; Tautz 1989). In a STR based DNA profiling, the STR loci/region(s) are first amplified by polymerase chain reaction (PCR), that produces millions of copies of the desired DNA templet for downstream analysis of the allelic length(s). Under predefined conditions, the PCR technology allows simultaneous primer extension of the two complementary stands of DNA and thereby produce several million copies of a specific DNA sequence within a few hours.

5.1. Basic Component of Polymerase Chain Reaction

1. DNA sample(s) to be analyzed,
2. DNA polymerase (an enzyme to synthesize complementary copies of the DNA templet),
3. The four-deoxyribonucleoside triphosphates (dNTPs).
4. Two small oligonucleotides (single-stranded DNA molecules), commonly referred to as primers, usually 18-30 nucleotides long and are designed to hybridize the DNA templet on opposite strands, on each side of the region of interest in a sequence specific manner.

PCR amplification of the sample DNA typically involves:

1. ***Initial Preparation:*** (1) Design and synthesis of primer oligos that are comple-

mentary and specific to the regions flanking the DNA amplicon (DNA segment to be amplified). The designed primers may be obtained from a commercial source. (2) Quantified DNA sample isolated from biological evidence/source. It is also important to note that under special circumstances, partially purified or un-purified DNA may be successfully amplified using PCR technique. (3) Acquire other necessary reagents such as DNA polymerase enzyme, four dNTPs and PCR reaction buffer. All these reagents are commercially available.

2. ***Preparing the PCR Reaction Mix:*** To a suitable amount of purified DNA sample add a premixed volume of the reaction mixture containing measured quantities of complementary primer pair, four dNTPs (equal amounts of dATP, dCTP, dGTP, and dITP), reaction buffer, and DNA polymerase. Mix the reaction volume by gentle pipetting.
3. ***PCR Reaction:*** Mount the tubes or plate containing the PCR reaction mix on a thermal cycler (PCR machine), programmed with predefined suitable temperature conditions and number of cycles (usually 25-35 cycles). A typical PCR cycle involve three steps: (1) denaturation of the DNA templet, which is accomplished by heating the PCR reaction mix to about 94 °C for a brief period (0.5 to 1 minute; specific to each PCR reaction); (2) followed by primer annealing at a temperature and time specific to the primer pair used (usually range from 56 °C to 62 °C for 0.5 to 1 minute.); and (3) primer extension/DNA synthesis at about 72 °C. Usually an initial (one time) brief denaturation (before the PCR cycles) and a final DNA extension, after the completion of PCR cycles are also added. Theoretically, each PCR cycle doubles the quantity of targeted DNA.

5.2. Analysis of Amplified DNA

The STR's allelic size are determined by electrophoretic analysis of the PCR products/amplicon. Sophisticated genetic analyzers and computer-based programs are now available and being used in the analysis of PCR amplified polymorphic STR loci and while directly comparing them with DNA molecules of known size that co-migrate with the PCR products on the gel. The studies of dinucleotide STR loci has shown that the Taq DNA polymerase enzyme used in the PCR amplification of the dinucleotide STR loci, regularly 'slips' during the reaction and produce artefactual stutter bands 2 bp apart, same as the genuine allele of such locus. These stutter bands make unambiguous allele designation difficult and therefore severely limit the reliable interpretation of such loci. Within a forensic setting the integrity of the amplification and the clarity of the result are of paramount importance, hence, dinucleotide STRs are generally avoided in a forensic analysis. Tetranucleotide repeat loci, however, have wider allele spacing and are significantly less prone to enzyme slippage thus making them significantly more suitable for routine forensic identification. Some tetranucleotide repeat loci display not only alleles differing in size by 4 bp but also intermediate alleles differing in size by 2 bp. This increases the discrimination power of such loci, almost similar to the dinucleotide repeat loci but without the associated limitations. Like the single locus probe, single STR locus provide limited discriminatory power in identifying an individual. It is therefore a multiplex or a number of STR loci are analyzed to obtain a discrimination potential robust enough for forensic identification.

Multicolor dye technology and automation allows multiplexing of the STR loci, including loci that have alleles of overlapping size, and therefore multiple STR loci can be analyzed in a single lane of an electrophoresis gel or capillary injection. The alleles of the STR loci that are very similar or same in size are distinguished by labeling the locus-specific primers with different florescent dyes. In a STR multiplex reaction by experimental design only one primer in a pair is labeled, and therefore the automated DNA analyzer detects DNA fragments amplified from only one strand of the DNA, eliminating the doublets that may arise from the different mobility of complementary strands, that are often observed during the manual gel staining and detection procedure. Using automated DNA analyzers and locus specific florescent labelling, hundreds of loci can be analyzed in a single day. Therefore, automation and multiplex STR kits dramatic increase the productivity as compared

to a manual gel staining technique, which visualize all PCR products in the same color. Additionally, the use of an internal size standard eliminates the need to run multiple lanes of the allelic ladder, thus allowing analysis of more samples in a single run.

5.3. STR Multiplex Kit

There are a number of fluorescent labeled STR multiplex kits available commercially for different application. For example, ThermoFisher Scientific offers a number of such kits for forensic applications. (1) GlobalFiler kits, which includes GlobalFiler, GlobalFiler IQC and GlobalFiler Express and assays 24 STR markers in a single multiplex reaction using 6 fluorescent dyes. The GlobalFiler kits contain all markers recommended in the Combined DNA Index System (CODIS) by the Core Loci Working Group and those commonly used in Europe. (2) VeriFiler kits includes VeriFiler Plus and VeriFiler Express PCR Amplification kits and are designed for challenging forensic samples. These kits utilize 6-dye STR chemistry and includes 25 markers - 23 autosomal STRs, and two gender discrimination markers. (3) Yfiler Plus kit uses 6-Dye technology and contains 27 Y chromosome STR markers. (4) AmpFlSTR Identifiler kits, includes AmpFlSTR Identifiler Plus and AmpFlSTR Identifiler Direct kits and assays 15 STR (tetranucleotide repeat) loci and a gender-determining marker in a single PCR amplification reaction.

Here we describe a detailed STR multiplex analysis protocol using AmpFlSTR Identifiler Plus kit. The primer sequences in the AmpFlSTR Identifiler Plus kit are maintained to allow genotyping concordance with the samples typed previously using an older generation of STR multiplex kits such as Identifiler, Profiler Plus, COfiler, or SGM Plus kits, and therefore facilitate comparison of current samples with existing databases. The key features of this kit are detailed below:

1. All thirteen CODIS required loci, important for known offender databasing in the United States are included in this kit.
2. Additional two loci, D2S1338 and D19S433, consistent with the AmpFlSTR ® SGM Plus™ PCR Amplification Kit and ensure compatibility with additional global database requirements are also included in this kit.
3. The 15 STR loci included in AmpFlSTR Identifiler Plus kit are consistent with several worldwide database recommendations.

The locus information (Table 2) and detailed PCR amplification and analysis protocol using ABI Prism 3730 Genetic Analyzer is described below:

5.3.1. PCR Amplification of Targeted STR loci Using AmpFlSTR® Identifiler™ Kit

1. To prepare **AmpFlSTR® Identifiler plus** master mix, add the following quantities of regents in a 1.5 ml micro-centrifuge tube.

Reagents	*Volume per reaction*
AmpFlSTR® Identifiler® Plus Master Mix	10.0 µl
AmpFlSTR® Identifiler® Primer Set	5.0 µl

Note: Include additional volume to accommo date loss of reagent during pipetting.

2. Mix the reagents by vortexing at medium speed for 3 seconds and then centrifuge briefly.
3. Dispense 15 µl of the prepared master mix into each PCR tubes or reaction well of a 96 well PCR plate.
4. Add DNA samples as detailed below. The final volume of each PCR reaction should be 25 µl.

DNA sample	*Volume per reaction*
Negative control	10.0 µl of low TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0).
Test sample	10.0 µl of DNA sample diluted in low TE at 0.1 ng/µl (total 1 ng of DNA)
Positive control	10 µl of 9947A control DNA supplied with the kit (0.1 ng/µl).

5. Cap the PCR tubes or seal the 96 well PCR plate with an appropriate seal compatible for PCR.
6. Vortex the reaction tubes/PCR plate for 3 seconds at medium speed and then centrifuge at 3000 rpm for 20 seconds to re-

move any air bubbles introduced in the process.

PCR Cycling Condition

1. Place each reaction tube/PCR plate containing 25µl of final reaction volume in a thermal cycler and set up the cycling condition as follows:

Initial incubation step	*Denature*	*Anneal and extend step*	*Final extension*	*Final step*
HOLD	Cycle (28-29 cycles)		HOLD	HOLD
95 °C 11 min	94 °C 20 sec	59 °C 3 min	60 °C 10 min	4 °C Up to 24 h

2. Close the thermal cycler's heated lid and start the PCR run.
3. On completion, proceed to electrophoresis/analysis on an automated genetic analyzer such as 3730/3730xl, 3100/3100-Avant, 3130/3130xl or 3500/3500xl.
4. The amplified DNA may be stored protected from light at 2-8 °C up to 2 weeks or at -20 °C for more than 2 weeks.

5.3.2. Preparation of Amplified Samples and AmpFlSTR Identifiler Plus Allelic Ladder for Analysis on Automatic 3730 Genetic Analyzer

1. Add 8.7 µl of Hi-Di™ Formamide and 0.3 µl of GeneScan™-500 LIZ® Size Standard per reaction in a single microcentrifuge tube. Additional volume may be prepared to accommodate pipetting loss as shown below:
(Number of samples + 2) × 8.7 µl Hi-Di Formamide
(Number of samples + 2) × 0.3 µl GeneScan-500 LIZ Size Standard
2. Vortex the Hi-Di™ Formamide and GeneScan™-500 LIZ® Size Standard mix and then centrifuge briefly.
3. Dispense 9 µl of the Hi-Di Formamide and GeneScan-500 LIZ mix into each well of the Genetic Analyzer sample plate.
4. Add 1 µl of the PCR product or Amp*Fl-STR* Identifiler Plus Allelic Ladder to the plate well and mix by pipetting 4-6 times. *Note:* Make sure to include at least one injection of Amp*FlSTR*® Identifile® Plus Allelic Ladder per 16 samples.
5. Seal the sample plate with appropriate septa or heat seal (see analyzer heat seal options).
6. Vortex the sample plate for 3 seconds at medium speed and then centrifuge briefly.

Table 2: Locus information of AmpFlSTR® Identifiler® Plus PCR Amplification Kit

Locus name	*Chromosome location*	*Alleles included in Identifiler® Plus Allelic Ladder*	*Dye label*
D8S1179	8	8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	6-FAM™
D21S11	21q11.2-q21	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38	6-FAM™
D7S820	7q11.21-22	6, 7, 8, 9, 10, 11, 12, 13, 14, 15	6-FAM™
CSF1PO	5q33.3-34	6, 7, 8, 9, 10, 11, 12, 13, 14, 15	6-FAM™
D3S1358	3p	12, 13, 14, 15, 16, 17, 18, 19	VIC®
TH01	11p15.5	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3	VIC®
D13S317	13q22-31	8, 9, 10, 11, 12, 13, 14, 15	VIC®
D16S539	16q24-qter	5, 8, 9, 10, 11, 12,13, 14, 15	VIC®
D2S1338	2q35-37.1	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28	VIC®
D19S433	19q12-13.1	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16. 2, 17, 17.2	NED™
vWA	12p12-pter	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24	NED™
TPOX	2p23-2per	6, 7, 8, 9, 10, 11, 12, 13	NED™
D18S51	18q21.3	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27	NED™
Amelogenin	X: p22.1-22.3Y: p11.2	X, Y	PET®
D5S818	5q21-31	7, 8, 9, 10, 11, 12, 13, 14, 15, 16	PET®
FGA	4q28	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2	PET®

Source: AmpFlSTR® Identifiler® Plus PCR Amplification Kit user guide, ThermoFisher Scientific.

7. Denature the samples for 3 minutes at 95 °C in a thermal cycler and then chill on ice for at least 3 minutes. The samples are now ready for fragment length analysis on an automated genetic analyzer.

5.3.3. Loading Samples Plate to 3730 Genetic Analyzer for Analysis

Before loading the plate into the autosampler of the analyzer create a plate record for the analysis to be carried out. A general method of creating plate record on a 3730 analyzer is described below *(For different analyzer, please refer to the analyzer manual):*

Creating GeneMapper Plate Record for Auto-analysis

1. Launch the Data Collection Software and in the Tree pane, click GA Instruments > ga3730 > Plate Manager.
2. In the New Plate Dialog box, complete the following information and then select OK.

a) Plate ID
b) Plate name and description. The description is optional but may help adding the relevant information to the run.
c) In the Application drop-down list, select GeneMapper application.
d) In the Plate Type drop-down list, select appropriate plate type (96-well or 384-well).
e) If analyzing more than one plate, the relevant plate scheduling information is entered.
f) Select the relevant Heat Seal or Septa Seal option, enter owner and operator names.

3. This will open the GeneMapper Plate Editor dialogue box. Complete the following records in the GeneMapper Plate Editor table.

a) ***Sample Names:*** enter relevant sample ID/ names
b) ***Comments:*** any additional information or notations for the sample may be recorded in this column.
c) ***Sample Type Column:*** from the drop-down list, select the applicable sample type.
d) ***Size Standard Column:*** from the drop-down list, select an appropriate size standard.
e) ***Panel column:*** select an applicable panel.
f) ***Analysis Method Column:*** from the drop-down list, select an appropriate analysis method.
g) Enter relevant information in the User-Defined columns 1 to 3.
h) ***Results Group 1 Column:*** from the drop-down list, select an applicable result group.
i) ***Instrument Protocol 1 Column:*** from the drop-down list, select an appropriate protocol.

4. Complete the aforesaid records for each sample and then select "OK" to save and close the plate record.
5. Assemble the plate cassette using appropriate assembly components (specific for 96 well or 384 well plate). Each assembly will include, a Plate retainer, a Plate septum (if septa is used), the Sample plate and a Base plate.
6. Place the assembled plate cassette in the instrument stacker drawer. Make sure the plate is in the "In Stack tower".
7. For scheduling runs: Go to the Tree pane of the Data Collection Software and select the following sequence.
   GA Instruments > ga3730 > instrument name > Run Scheduler.
   (You may schedule a run or runs using either manual mode or auto mode. Both modes and their features are described below.)
8. To select a run mode, follow the command sequence: Run Scheduler > Instrument > Instrument Name > Run mode (Auto or Manual)

The Manual Mode

Features

a) Allows adding the sample plates in the plate stacker individually and at different times. Runs are scheduled in the order sample plates were added in the stack.
b) An inbuilt plate barcode reader is not required to link the sample plates to plate records in the local database.
c) Plates need not be barcoded.

1. To Schedule the run(s) in Manual Mode:

a) Click the Run Scheduler icon and follow the command sequence: Select Instrument > Instrument Name > Manual mode.
b) In the Run Schedular window, search the plate record(s) created before.

c) Select the desired plate record(s) and then add to the scheduled run(s). Make sure the order of the added plate records matches the order of plates in the In-Stack tower. The bottom plate runs first.
d) Select "done" to close the Add Plates dialog box.
Note: The order of the plate record must match the order of the plates in the In-Stack tower.

The Automatic Mode

Features

a) Plates must be barcoded.
b) Analyzer's inbuilt barcode reader is necessary to link the sample plates to the correct plate records in the local database.
c) Sample plates may be added to the In Stack in any order.
d) Sample plates can be added or removed at any time while the analyzer is in operation except when the autosampler is operational.

1. To schedule the run(s) in Auto mode:

a) Click the Run Scheduler icon and follow the command sequence: Select Instrument > Instrument Name > Auto mode.
b) You may notice that the Up, and Down search buttons are no longer available as they were in Manual mode. The Add Plate (Scan or Type Plate ID) option is also disabled.
c) Place the sample plate(s) in the In-Stack tower in any order. Remember that the bottom plate will run first, and the top plate will run last.
d) Press green button to start the run.
Notes: After the run completion, results are stored in a location specified in the result group. The genotyping analysis of the data is performed using GeneMapper software.

6. MITOCHONDRIAL DNA ANALYSIS FOR FORENSIC IDENTIFICATION

The analysis of mitochondrial DNA (mtDNA) variations is fast gaining ground; both in the judicial system, for personal identification, and in anthropological and archaeological phylogenetic investigations. Due to its high abundance in the cells, mtDNA sequencing has often proven successful in cases where biological evidence is degraded and/or available in very small quantity, not sufficient for an autosomal marker's analysis. Because of its maternal inheritance, mtDNA variations allows construction of matrilineal genealogies and are often used in missing person cases when skeletonized remains are recovered and compared to samples from the maternal relatives or personal effects of missing individuals. Similarly, in medico-legal cases where hairs, bones or teeth are the only evidence recovered, mtDNA variation analysis is often used to exclude suspects. Due to high mutation rate, mtDNA is highly variable particularly in its D-Loop region also known as hyper variable region I (HVR-I) and hyper variable region II (HVR-II), and therefore it is highly unlikely that two maternally unrelated individuals shares the same mtDNA variants profile. About half of the mtDNA variation is confined within its *two* hyper variable non-coding regions encompassing about just 750 base pairs and therefore these regions are of special interest in personal identification/DNA profiling analysis.

Here in this chapter we have discussed in detail a methodology of sequencing mtDNA HVR-I, including the interpretation of results, the phenomenon of heteroplasmy and mtDNA variation and population genetic analysis.

The protocol described earlier in this chapter for the extraction of DNA from various biological tissue sources, co extract both nuclear as well as mitochondrial DNA, which after quality and quantity check and proper dilution can be used for PCR amplification of mtDNA HVR-I or other regions of interest.

6.1. PCR Amplification of the mtDNA HVR-I (D-Loop) Region

Primer specific to mitochondrial HVR-I region
HVR 1F: 5'TCA TTG GAC AAG TAG CAT CC 3'
HVR 1R: 5'GAG TGG TTA ATA GGG TGA TAG 3'

1. Prepare the PCR master mix by adding the following quantities of PCR regents into a 1.5 ml micro-centrifuge tube. Alternatively, commercially available ready to use PCR master mix may be used. If using commercial ready to use master mix, follow the manufacturer protocol.

Reagents	Volume per reaction
10X PCR buffer	1.0 µl
dNTPs (2.5mM each)	0.5 µl
Forward Primer (10 pM/µl)	0.2 µl
Reverse Primer (10 pM/µl)	0.2 µl
Taq DNA Polymerase (5U/µl)	0.2 µl
Ultrapure Water	6.9 µl

2. Vortex the master mix at medium speed for 3 seconds and then centrifuge briefly.
3. Dispense 9 µl of the prepared master mix into each PCR tubes or reaction well of a 96 well PCR plate.
4. Add 1.0 µl (~ 10 ng/µl) of DNA sample to each PCR tube/well. The final volume of each PCR reaction should be 10 µl.

PCR Cycling Condition

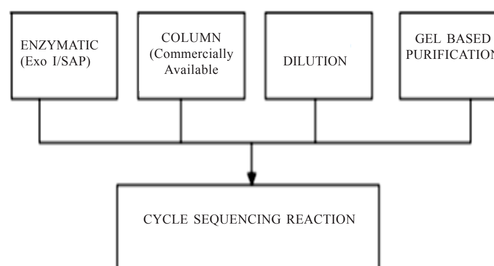
1. Place each reaction tube/plate containing 10 µl of PCR reaction mix/well in a thermal cycler and set up the PCR cycling conditions as detailed below:

Initial incubation step	*Denature*	*Anneal*	*Extend*	*Final extension*	*Final step*
HOLD	Cycle (28-29 cycles)			HOLD	HOLD
95 °C 1 min	94 °C 30 sec	60 °C 1 min	72 °C 2 min	72 °C 7 min	4 °C Up to 24 h

2. Close the thermal cycler's heated cover and start the PCR run.
3. On completion of the PCR cycles, check the quality of the amplicon by agarose gel electrophoresis as detailed below and then proceed to PCR clean-up and cycle sequencing reaction.
a) Aliquot 1 to 2 µl of the amplicon (sample from PCR product) in a tube(s) and mix with equal volume of the ***6X Loading dye***.
b) Perform DNA quality check electrophoresis using 2% agarose gel as described in section 3.2. A good amplicon will appear as a crisp single band on the test gel.
c) If the amplification was successful, proceed to PCR clean-up and then to cycle sequencing reaction.
4. The amplified DNA may be stored at 2-8 °C up to 2 weeks or at -20 °C for longer period.

6.2. PCR Cleanup

Before proceeding to the cycle sequencing reaction, it is essential to purify the PCR amplicon to remove any residual (unincorporated) PCR reagents. Any of the following strategies shown in the schematic diagram below can be used to purify the PCR amplicon for cycle sequencing reaction.



Each PCR cleanup method shown in the schematic above has their own advantages and disadvantages depending upon quality of the PCR amplicon and kind of application involved. We have extensively used enzymatic clean-up and simple dilution strategies in our work. Here we describe the Exo I/SAP enzymatic cleanup strategy in detail.

1. Prepare an enzymatic cleanup mix by adding following quantities of each reagents per sample into a 1.5 ml micro-centrifuge tube:

Reagents	Volume per reaction
Ultrapure Water	7.5 µl
Exonuclease I (10 U/µl)	0.5µl
Shrimp Alkaline Phosphatase (1 U/µl)	0.5 µl

2. Aliquot 8.5 µl of the enzymatic cleanup mix into new -tubes or -plate wells.
3. Add 8 µl of the PCR amplicon (PCR product) to the enzymatic cleanup reaction mix.
4. Incubate the reaction tube/plate at 37 °C for 30 minutes and then at 80 °C for 15 minutes in a thermal cycler.
5. After the Exo I/SAP enzymatic reaction, clean-up the amplicon by ethanol precipitation as detailed below.
a) Add 0.1 volumes of 3M Sodium Acetate (that is, 1.65µl in the 16.5 µl reaction) and then 2.0 volumes of pre-chilled (-20 °C)

95% ethanol (that is,33μl) to each reaction well.

b) Mix the tube content well and then centrifuge at 4000 rpm for 30 minutes in a refrigerated (4 °C) centrifuge.

c) After centrifugation, invert the tubes/plate to decant excess ethanol.

d) Gently blot the tubes/plate on an absorbent paper pad (for example, folded paper towels). Be very careful, not to disrupt the precipitated DNA pellet. The DNA pellet may not be visible.

e) Wash the DNA pallet with 100 μl of ice cold 70% ethanol (preferably, pre-chilled in a –20 °C freezer and removed just prior to use). Centrifuge at 4000 rpm for 10 minutes. Decant the excess ethanol and then briefly reverse spin at 500 rpm for 30 seconds to dry the DNA pallet.

6. Resuspend the dried DNA amplicon in 1 volume of Ultrapure water and determine the quality and quantity of the amplicon by gel electrophoresis.

6.3. Cycle Sequencing of the PCR Product

Set-up the Cycle Sequencing Reaction using commercially available Big Dye terminator kit.

Note: The kits described in the following steps of DNA sequencing are commercially available from ThermoFisher Scientific.

1. Thaw BigDye™ Terminator Cycle Sequencing Kit components and the primer(s) to be used for sequencing at room temperature, make sure the entire content of each tube is completely thawed and then immediately store on ice.
2. Vortex the tubes at medium speed for 3 seconds each, and then centrifuge briefly.
3. Prepare the Cycle Sequencing master mix by adding following quantities of each reagents into a 1.5 ml micro-centrifuge tube.

Reagents	*Volume per reaction*
BigDye™ Terminator	4.0 μl
Ready Reaction Mix	(~3.2 μM)
Forward or Reverse primer	1.0 μl
Ultrapure Deionized water	4.0 μl

*Note:*Only one primer (either forward or reverse) is to be used per cycle sequencing reaction.

4. Vortex the Cycle Sequencing master mix tube at medium speed for 3 seconds, and then centrifuge briefly.
5. Dispense 9.0 μl of the Cycle Sequencing master mix into each reaction well of a 96 well sequencing plate. Prepare one well for each cycle sequencing reaction.
6. Add 1.0 μl of the purified/cleaned PCR amplicon per sample (containing about 150 to 300 ng of the purified PCR amplicon), to a reaction well containing 9 μl of cycle sequencing master mix.
   Note: The concentration of the purified amplicon (template) may affect the cycle sequencing reaction volume, if the templet is too dilute and more than 1μl is to be used, adjust the volume of water to keep the total reaction volume to 10 μl.
7. Seal the plate with appropriate adhesive film or any other suitable plate sealer. Make sure the plate seal is PCR compatible.
8. Vortex the cycle sequencing reaction plate at medium speed for 3 seconds, then centrifuge briefly.
9. Mount the cycle sequencing plate containing 10 μl of the final reaction volume per well on a thermal cycler and set up the following cycling conditions:

Initial Incubation Step	*Denature*	*Anneal*	*Extend*	*Final Step*
HOLD	Cycle (28-29 cycles)		HOLD	HOLD
96 °C 1 min	96 °C 10 sec	50 °C 5 sec	60 °C 4 min	4 °C Until ready to purify

Notes: Set thermal cycler ramp rate to 1 °C/second. A shorter extension time may be used for short templet.

10. After completion of the cycle sequencing reaction, remove the plate from the thermal cycler and centrifuge briefly.
11. Proceed to purifying the cycle sequencing product.
    Note: Cycle sequencing product is to be purified to remove unincorporated primers, dNTPs/ddNTPs and primer dimers that may have formed during the reaction.

6.4 Purifying Cycle Sequencing Product

The following two methods are commonly used in purifying cycle sequencing product. The first method described here, uses a commercially available **BigDye XTerminator ™ Kit.** To perform cycle sequencing clean-up using the BigDye XTerminator ™ Kit follow the steps detailed below:

1. Vortex the bottle of BigDye XTerminator™ beads at full speed for 8 to 10 seconds. Make sure the beads are fully and uniformly resuspended in the solution.
2. Immediately prepare an appropriate amount of SAM and BigDye XTerminator™ beads working solution by mixing the beads and SAM solution in following proportions:

Reagents	*Volume per reaction*
SAM solution	45.0 μl
BigDye XTerminator™ Bead solution	10.0 μl

3. Keep the SAM/BigDye XTerminator™ bead working solution on ice and well mixed while pipetting.
4. Remove the Adhesive Film from the cycle sequencing plate and then add 55 μl of SAM/BigDye XTerminator™ bead working solution to each reaction well.
5. Mix by pipetting 3-4 times.
6. Seal the plate with Adhesive Film or a heat seal if the 3730 DNA analyzer is equipped with heat seal piercing.
7. Vortex the sequencing plate at 1,800 rpm for 20 minutes (Digital Vortex-Genie™ 2 is recommended).
8. Centrifuge the sequencing plate at 1,000 × g for 2 minutes in a swing bucket rotor. *Note:* The plate is now ready for sequencing on 3730 DNA analyzer. Alternatively, the sealed plate may be stored at 4°C for up to 10 days or at –20°C for longer period. The plates can be stored at room temperature for up to 48 hours which includes the time on the DNA analyzer.

Alternatively, the cycle sequencing reaction may be purified by **ethanol/EDTA precipitation** method. Please read the "notes" provided at the end of these steps before starting.

1. Prepare sufficient volume of solution I by diluting 125 mM EDTA in the ratio of 1:5 with ultrapure water.
2. Add 12 μl of solution I to each reaction well containing 10 μl of the cycle sequencing product. Ensure well content is mixed thoroughly.
3. Prepare sufficient volume of solution II by adding 3M sodium acetate pH 5.2 and absolute ethanol in 1:20 ratio, respectively.
4. Dispense 42 μl of the solution II into each reaction well and seal the plate with a clear adhesive seal.
5. Invert the plate a few times to mixes the contents and incubate at room temperature for 15 minutes.
6. Centrifuge the plate at 3000 x g for 30 minutes and at room temperature.
7. After centrifugation, decant the supernatant by inverting the plate on a stack of 3-4 paper towels and then centrifuge inverted up to 180 x g to remove residual supernatant.
8. Add 100 μl of 70% ethanol, seal the plate with clear adhesive seal and then centrifuge at 3000 x g for 10 minutes and at room temperature.
9. Again, decant the supernatant by inverting the plate on a paper towel stack and then centrifuge inverted up to 180 x g to remove residual supernatant.
10. Air or vacuum dry the purified cycle sequencing product pellet.
11. At this stage, a sealed plate may be stored protected from light at 4 ºC while preparing the DNA analyzer for sequencing analysis or at -20 ºC for a longer period. *Notes:* (1) Consider an opened bottle of absolute ethanol at 95% while preparing 70% ethanol (absolute ethanol absorbs enough moisture once exposed to open air). (2) Prepare 125 mM EDTA solution from sterile 0.5 M EDTA pH 8.0, on a regular basis (at least weekly). (3) To make 10 ml 70% ethanol, add 7.5 ml of stock ethanol (which is 95%) and 2.5 ml of ultrapure water. Prepare fresh 70% ethanol daily. The 70% ethanol should be at room temperature when added to the reaction.

6.5. DNA Sequencing Analysis on a 3730 DNA Analyzer

Before scheduling a sequencing run, verify the quality of the current matrix file and spectral

calibrations. A new matrix file or spectral calibrations may be generated by running appropriate matrix and/or sequencing standards that are specific to your instrument. This information may be found in your instrument's manual/guide. The existing mobility files can be used with the respective instruments.

If ethanol/EDTA precipitation-based purification was used, immediately before sequencing analysis, resuspend the purified and dried sequencing reactions in 10 µl of Hi-Di™ Formamide. Denature the DNA by heating the reaction at 96 °C for 3 minutes in a heat block or thermal cycler and then immediately chilling the plate on ice. Keep the plate on ice until loaded on the DNA analyzer for sequencing analysis.

Before loading the plate into the autosampler of the DNA analyzer create a plate record for the analysis to be carried out. A general method of creating plate record on a 3730 analyzer is described below *(For different analyzer, please refer to the analyzer manual):*

Creating a Sequencing Plate Record for auto-analysis:

1. In the Data Collection Software's Tree pane, select the following in a sequence detailed below.
   GA Instruments > ga3730 > Plate Manager.
2. Complete the following information in the New Plate Dialog box, and then select OK.

a) Plate ID
b) Plate name and description. The description is optional but may help adding the relevant information to the rum.
c) Select Sequence Analysis application from the Application drop-down list.
d) Select an appropriate 96-well or 384-well Plate Type from the drop-down list.
e) If analyzing more than one plate, the relevant plate scheduling information is entered.
f) Select the relevant Heat Seal or Septa Seal option, enter owner and operator names and then select OK.

3. This will open the Sequencing Analysis Plate Editor dialogue box. Record the following information in the Plate Editor table.

a) **Sample Names:** enter relevant sample ID/names
a) ***Comments:*** any additional information or notations for the sample may be recorded in this column.
b) ***Results Group 1 Column:*** select the relevant result group from the drop-down list.
c) ***Instrument Protocol 1 Column:*** select appropriate protocol from the drop-down list.
d) ***Analysis Method Column:*** select the appropriate analysis method from the drop-down list.
   Note: To create the desired Result Group, Instrument Protocol and Analysis Protocol/Method refer the instrument operation manual.

4. Once the aforesaid records are created for each sample, select OK to save and close the plate records.
5. Assemble the plate cassette using appropriate assembly components (specific for 96 well or 384 well plate). Each assembly will include, a Plate retainer, a Plate septum (if septa is used), the Sample plate and a Base plate.
6. Place the assembled plate cassette in the instrument stacker drawer. Make sure the plate is in the "In Stack tower". However, plates may be loaded in any order if Auto run mode is used and the analyzer is equipped with barcode reader. The onboard scanner can scan the plate barcode and associate the correct plate record based on the plate ID/bar-code.
7. For scheduling runs: Again, go to the Data Collection Software's Tree pane and select the following in sequence described below.
   GA Instruments > ga3730 > instrument name > Run Scheduler
   (Similar to the fragment analysis, the sequencing run may also be scheduled using either manual mode or auto mode as described in section 5.3.3.)
8. To select a run mode, follow the command sequence:
   Run Scheduler >Instrument > Instrument Name > Run mode (Auto or Manual).
9. Load the plates to the In-Stack tower of the analyzer.
   Note: Bottom plate will run first, and the top plate will run last.
10. Press the green button to start the run.
   Note: After the run(s) completion, the results will be store in the location specified in the result group. The sequence for each sample can be analyzed for quali-

ty and sequence length in the sequence analysis software.

6.6 Interpretation Guidelines

For analysis of the mtDNA variants, the generated mtDNA sequences can be aligned to the reference sequence, which may be Revised Cambridge Sequence, or Andersons reference sequence using the DNA sequence analysis software SeqScape or using any other third-party software. Once alignments are complete and the DNA sequence assemblies are generated, an experienced DNA examiner, examines and edit the individual sequences in the sequence assembly, which generally contains multiple DNA sequences from the same template, to obtain a final consensus sequence of the DNA templet. After the thorough examination of the sequence assembly, the difference(s) between the DNA templet consensus sequence and the reference sequence are recorded as sequence variants. The mtDNA sequence variants obtained from the evidence(s) of questioned identity are then compared to the mtDNA sequences of the known and/or reference samples to determine the personal identity. A complete sequence match between the evidence sample and the known/reference sample indicates a non-exclusion, whereas a difference at one or more base pair positions indicates a mismatch/exclusion.

There are trillions of cells in a human body, and each cell contains thousands of copies of mtDNA. Therefore, a complete homoplasmy (the same sequence of mtDNA) for each of these mtDNA molecules is unlikely because chances of a somatic mutation occurring in any of the immense amounts of mtDNA molecules are very high. Thus, some levels of heteroplasmy (i.e. the occurrence of more than one base at a particular position in the DNA sequence of an individual) in the mtDNA sequences of an individual is expected. Heteroplasmy can be subdivided into two categories, (1) DNA sequence heteroplasmy or point heteroplasmy, is the occurrence of more than one base at a particular position or positions in the mtDNA sequence of an individual, and (2) the length heteroplasmy, is the occurrence of more than one length of a stretch of same bases in the mtDNA sequence of an individual. A low level of mtDNA heteroplasmy may not be detected by traditional Sanger sequencing methodology described here, however, can be detected with deep next generation sequencing, which allows sequencing of multiple DNA templets simultaneously.

7. RECIPES

Buffers/solutions recipes (as per the superscript numbers in the manuscript text)

1. RBC Lysis Buffer

Sucrose	320 mM
MgCl2	5.00 mM
Triton X 100	17.0 mM
Tris-Hcl (pH 8.0)	10.0 mM

2. Digestion Buffer

Tris-Hcl (pH 8.0)	40.0 mM
NaCl	15.0 mM
EDTA (Na salt)	5.0 mM

Autoclave and then add SDS 1% w/v final concentration.

3. 5M Sodium per chlorate solution

100 g Sodium per chlorate dissolved in 142 ml of ultrapure water, **do not autoclave**

4. RBC Lysis Buffer – I

Tris-Hcl (pH 8.0)	30.0 mM
EDTA	5.0 mM
NaCl	50.0 mM

5. Digestion Buffer

EDTA (pH 8.0)	2.0 mM
NaCl	75.0 mM

6. Proteinase K

Dissolve 10 mg of Proteinase-K in 1.0 ml of ultrapure water.

7. 20% SDS

Dissolve 20 g of SDS in 80 ml of ultrapure water by heating the solution to 65 ^{o}C make up the volume to 100ml.

8. 10X PBS (pH 7.2)

NaCl	1.37 M
KCl	0.27 M
Na_2HPO_4	0.08 M
NH_2PO4	0.02 M

9. Digestion Buffer

Sucrose	0.32 mM
$MgCl_2$	10.0 mM
Tris HCl	10.0 mM
EDTA	50.0 mM
NaCl	100.0 mM

10. Digestion Buffer

Tris-HCL (pH 8.0)	50.0 mM
EDTA (pH 8.0)	10.0 mM
NaCl	100.0 mM

11. Digestion Buffer

Tris-HCl (pH 8.0)	100.0 mM
EDTA	10.0 mM
NaCl	100.0 mM

12. Digestion Buffer

Tris-HCl (pH 7.5)	9.2 mM
EDTA	9.2 mM
NaCl	0.46 M
SDS (w/v)	1.8 %

Do not autoclave, store at 4 °C.

13. 3 M NaAcetate

Dissolve 40.82 g of NaAcetate-3H_2O in 80 ml ultrapure water. Adjust pH to 5.2 with glacial acetic acid and then makeup the final volume to 100 ml with ultrapure water. Sterilize by autoclaving and store at room temperature.

14. Tris-saturated phenol (pH 8.0)

Caution: Phenol can cause severe burns. Appropriate personal protective equipment (PPE) including safety glasses and gloves should be worn when working with phenol or chloroform. If phenol solutions come in contact with skin or eyes, wash immediately with large volumes of water.

Commercial, liquified phenol can be used without re-distillation if phenol is colourless. Crystalline phenol can be used if the crystals are white. Liquified phenol solutions that are pink or yellow, or coloured crystalline phenol must be redistilled prior to use. Liquified or re-distilled phenol should be stored at -20 °C in aliquots until needed.

As needed, remove phenol aliquots from freezer, warm to room temperature, and liquify by immersing in a water bath at 68 °C and then add 8-hydroxyquinoline to a final concentration of 0.1% (0.1 g/100 ml). The 8-hydroxyquinoline is an antioxidant and the yellow colour provides a convenient way to identify the phenolic phase during DNA extraction. Extract the liquified phenol once by adding an equal volume of 1.0 M Tris-HCl, pH 8.0 (Reagent # 15a), and mixing. After separation of phases discard the aqueous (upper) phase into organic waste container. Stepwise add 1 M Tris Base Solution (Reagent # 14b) and mix until the pH of the aqueous phase is between 7.0 and 8.0. Discard the aqueous phase into organic waste containers. Repeat extraction with 1 M Tris- HCl, pH 8.0, once more. Discard the aqueous phase into organic waste containers. Add an equal volume of fresh TE-buffer (Reagent #15) to the phenol. Add 2-Mercaptoethanol to a final concentration of 0.1% and store the equilibrated phenol at 2-8 °C and protected from light in a brown glass bottle. Alternatively, Tris-saturated phenol (pH 8.0) can be commercially obtained.

Chloroform-Isoamyl alcohol

Chloroform in this protocol refers to a mixture of 24 volumes chloroform and 1 volume Isoamyl Alcohol, the later reduces foaming and facilitates separation of aqueous and organic phases during DNA extraction.

14a. Phenol-Chloroform-Isoamyl alcohol

To prepare phenol-chloroform for DNA extractions, mix 1 volume of Tris buffered phenol and 1 volume of chloroform and isoamyl alcohol (24:1), and then centrifuge for 2 minutes to separate phases. Use only the organic phase for extraction; discard the aqueous phase into organic waste containers.

14b. 1 M Tris Base Solution

Dissolve 121.1 g Tris Base in 800 ml of ultrapure water. Adjust the final volume to 1 liter with ultrapure water. Autoclave and store at room temperature.

15. TE-Buffer (Tris-EDTA buffer)

Tris-HCl pH 8.0	10.0 mM
EDTA, pH 8.0	1.0 mM

Store at room temperature.

15a. 1 M Tris-HCl, pH 8.0

Dissolve 121.1 g of Tris Base in 800 ml of ultrapure water. Adjust the pH to 8.0 with concentrated HCl (approximately 42 ml). Make-up the final volume to 1 liter with ultrapure water. Autoclave and store at room temperature.

15b. 0.5 M EDTA, pH 8.0

Dissolve 186.1 g of disodium Ethylene-Diamine-Tetra-Acetate-$2H_2O$ in 800 ml of ultrapure water by stirring vigorously on a magnetic stirrer. Adjust pH to 8.0 by adding NaOH pellets (approximately 20 g)

Note: EDTA will not dissolve completely without pH adjustment. Bring final volume to 1 liter with ultrapure water.

16. 10X TAE (Tris acetate) buffer (pH 8.0)

Tris Base	0.40 M
Glacial acetic acid	0.20 M
EDTA (pH 8)	10.0 mM

To make 0.5X TAE working solution dilute 10X TAE 20 times with ultrapure water.

17. 1 mg/ml Ethidium Bromide (100 ml)

Caution: Ethidium Bromide is a mutagen. Wear gloves and mask while handing Ethidium Bromide and do not mouth pipette.

Dissolve 0.1 g of Ethidium Bromide in 100 ml of ultrapure water. Stir on a magnetic stirrer for several hours to dissolve the Ethidium Bromide dye completely. Store at 2-8 °C protected from light in a dark bottle or bottle wrapped in aluminum foil. The solution does not require sterilization.

18. 6X Gel Loading Dye

Dissolve 0.25 % (w/v) Bromophenol blue, 0.25 % (w/v) Xylene cyanol FF and 40 % (w/v) Sucrose in ultrapure water and store at 4 °C.

19. Restriction Enzymes

The digestion of DNA into small fragments is done by special enzymes, which cut the DNA at specific sites. These are known as *restriction enzymes* and are usually named, after the bacterium from which they are derived *(e.g.* Bam – *Bacillus amyloliquefaciens*; Eco – E. *Coli)*. A comprehensive list of restriction enzyme recognition sites may be found in (Cornish-Bowden 1985; Kessler, et al. 1985). Restriction fragment lengths particularly in the repetitive region of the genome may vary from individual to individual due the differences in individual's DNA. Since these fragments are produced by *restriction enzymes,* the variations have been called *restriction fragment length polymorphisms* (RFLPs) *or DNA sequence polymorphisms.* The majority of the RFLP site occur in the non-coding regions of DNA and can be divided into two principal classes:

1. Polymorphisms resulting from the presence or absence of a specific restriction site, when the site is present, the enzyme will cut the DNA, producing a shorter fragment than when it is absent. Such polymorphisms usually only have two alleles, unless the same site occurs more than once in a short length.
2. Variable number of tandem repeat (VNTR) polymorphisms. These polymorphic sites have a series of short repeated sequences, the number of which may vary between individuals. The length of the restriction fragment depends on the number of these repeat units present. Therefore, a VNTR may have multiple alleles and are especially important in forensic DNA profiling.

20. Agarose Gel (RFLP Electrophoresis)

Once the DNA has been digested, it must be separated and sorted to identify RFLP(s). This is accomplished by gel electrophoresis. In this process, an agarose gel, is placed under a weak electric field, positive at one end and negative at the other. The restriction fragments are then loaded into the negative end of the gel. Since DNA is negatively charged the fragments flow toward the positive end of the gel. The agarose gel is composed of holes of varying sizes and as a result, the DNA fragments are separated based on their size, while they move from negative to positive end of the agarose gel. The DNA fragments separated by size are documented and analyzed to identify the RFLP alleles.

21. Agarose Gel Running Buffer

89 mM Tris-Borate – 2.5 mM EDTA, pH 8.0.

22. 10X Agarose Gel Loading Solution

Dissolve 0.25% (w/v) Bromophenol blue, 0.25 % (w/v) Xylene cyanole and 25 % (w/v) Ficoll - 400 in ultrapure water and store at 4 °C.

23. Denaturation and Blotting

After the restriction fragments are separated on a gel, the DNA is denatured to splits the two strands of the DNA molecule as shown in the following example.

A C T G
* * * *
T G A C

the DNA under examination now looks like:

A C T G

T G A C

The denatured DNA is then transferred from the gel to a firm surface of nylon membrane. Where DNA fragments are permanently fixed in their respective positions.

24. Southern Transfer Depurination Solution

0.3 M HCl

25. Southern Transfer Denaturing and Transfer Solution

0.4 M NaOH
Store at room temperature.

26. Southern Transfer Neutralizing Solution

A solution of 0.5 M Tris-HCl and 1.5 M NaCl, pH 8.0

27. Hybridization

Hybridization is the process in which a known probe that recognize a specific DNA sequence is utilized to identify an unknown restriction fragment. The membrane carrying the denatured and separated restriction fragments is transferred into a solution of known labelled probes. The labelled probes hybridize to the complimentary strand of the restriction fragment and enable the identification of the RFLP alleles.

28. Hybridization/Prehybridization Solutions

5X SSC, 10 mM Tris - HCl (pH 8.0), 2 mM EDTA (pH 8.0) 1% (w/v) SDS, and 5 µg/ml t-RNA.

To prepare hybridization solution add 100 mg/ml B.E.S.T. and probe MZ1. Prepare the solution just before use.

For prehybridization reaction, use 1 ml prehybridization solution/cm^2 of the membrane.

For hybridization reaction, use 0.25 ml hybridization solution per cm^2 of the membrane.

29. Hybridization Wash Solution No. 1

2X SSC and 0.5% Tween 20
Store at room temperature.

29a. 20X SSC Buffer

20X SSC buffer contains 3.0 M NaCl and 0.3 M Sodium Citrate $C_6H_5Na_3O_7$ in ultrapure water.

To make 2X SSC buffer dilute 20X Stock buffer 1: 9 with ultrapure water and store at room temperature.

30. Hybridization Wash Solution No. 2

0.6X SSC and 0.5% Tween 20
Store at room temperature. Preheat to 60 °C before use.

31. Colour Development Buffer A

Dissolve 0.1 M NaCl, 5% (v/v) Triton X-100 in PBS adjust pH to 7.4 and store at 2-8 °C.

32. Colour Development Buffer B

To Colour Development Buffer A add 1 M urea, 1% Dextran Sulphate (500,000

M.W.). Store at 2-8 °C. Before use prewarm to room temperature.

33. Colour Development Buffer C

Prepare 0.01 M Sodium Citrate and 0.01 M EDTA, solution and adjust pH to 5.0 by adding citric acid (approximately 0.75 in 1 liter). Store at 2-8 °C. Prewarm to room temperature just before use.

34. 3% Hydrogen Peroxide

Just prior to use, mix one volume of 30% H_2O_2 with nine volumes of ultrapure water.

REFERENCES

Aljanabi SM, Martinez I 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-

based techniques. *Nucleic Acids Res*, 25(22): 4692-4693. Epub 1998/02/12. doi: 10.1093/nar/25.22.4692. PubMed PMID: 9358185; PMCID: PMC147078.

Chambers GK, Curtis C, Millar CD, Huynen L, Lambert DM 2014. DNA fingerprinting in zoology: Past, present, future. *Investig Genet*, 5(1): 3. Epub 2014/02/05. doi: 10.1186/2041-2223-5-3. PubMed PMID: 24490906; PMCID: PMC3909909.

Cornish-Bowden A 1985. Nomenclature for incompletely specified bases in nucleic acid sequences: Recommendations 1984. *Nucleic Acids Res*, 13(9): 3021-3030. Epub 1985/05/10. doi: 10.1093/nar/13.9.3021. PubMed PMID: 2582368; PMCID: PMC341218.

Fan H, Chu JY 2007. A brief review of short tandem repeat mutation. *Genomics Proteomics* B*ioinformatics*, 5(1): 7-14. Epub 2007/06/19. doi: 10.1016/S1672-0229(07)60009-6. PubMed PMID: 17572359; PMCID: PMC5054066.

Genomes Project C, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR 2015. A global reference for human genetic variation. *Nature*, 526(7571): 68-74. Epub 2015/10/04. doi: 10.1038/nature15393. PubMed PMID: 26432245; PMCID: PMC4750478.

Hoss M, Paabo S 1993. DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic Acids Res*, 21(16): 3913-3914. Epub 1993/08/11. doi: 10.1093/nar/21.16.3913. PubMed PMID: 8396242; PMCID: PMC309938.

Jamieson A 2000. Forensic biology: its past, present and future. *Biologist (London)*, 47(2): 69-73. Epub 2001/02/24. PubMed PMID: 11190231.

Jeffreys AJ, Wilson V, Thein SL 1985. Hypervariable 'minisatellite' regions in human DNA. *Nature*, 314(6006): 67-73. Epub 1985/03/07. doi: 10.1038/314067a0. PubMed PMID: 3856104.

Jeffreys AJ, Wilson V, Thein SL 1985. Individual-specific 'fingerprints' of human DNA. *Nature*, 316(6023): 76-79. Epub 1985/07/04. doi: 10.1038/316076a0. PubMed PMID: 2989708.

Kessler C, Neumaier PS, Wolf W 1985. Recognition sequences of restriction endonucleases and methylases- A review. *Gene*, 33(1): 1-102. Epub 1985/01/01. doi: 10.1016/0378-1119(85)90119-2.PubMedPMID: 2985469.

Moretti TR, Just RS, Kehl SC, Willis LE, Buckleton JS, Bright JA, Taylor DA, Onorato AJ 2017. Internal validation of STRmix for the interpretation of single source and mixed DNA profiles. *Forensic Sci Int Genet*, 29: 126-144. Epub 2017/05/16. doi: 10.1016/j.fsigen.2017.04.004. PubMed PMID: 28504203.

Panneerchelvam S, Norazmi MN 2003. Forensic DNA profiling and database. *Malays J Med Sci*, 10(2): 20-26. Epub 2003/07/01. PubMed PMID: 23386793; PMCID: PMC3561883

Roewer L 2013. DNA fingerprinting in forensics: past, present, future. *Investig Genet*, 4(1): 22. Epub 2013/11/20. doi: 10.1186/2041-2223-4-22. PubMed PMID: 24245688; PMCID: PMC3831584.

Sambrook J, Fritsh EF, Maniatis T 1989. *Molecular Cloning: A Laboratory Manual*. New York, NY: Cold Spring Harbor Laboratory Press.

Snowdon RJ, Langsdorf A 1998. An Introduction to DNA Fingerprinting Using RFLP and RAPD Techniques. In: D Tietz (Ed.): *Nucleic Acid Electrophoresis: Springer Lab Manual.* Berlin, Heidelberg: Springer.

Sunnucks P, Hales DF 1996. Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus Sitobion (Hemiptera: Aphididae). *Mol Biol Evol*, 13(3): 510-524. Epub 1996/03/01.doi:10.1093/oxfordjournals. molbev.a 025612. PubMed PMID: 8742640.

Tautz D 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res*,17(16): 6463-6471. Epub 1989/08/25. doi: 10.1093/nar/17.16.6463. PubMed PMID: 2780284; PMCID: PMC318341.

Walsh PS, Metzger DA, Higuchi R 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques,*10(4): 506-513. Epub 1991/04/01. PubMed PMID: 1867860.

Watson JD, Crick FH 1953. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*, 171(4356): 737-738. Epub 1953/04/25. doi: 10.1038/171737a0. PubMed PMID: 13054692.

Paper received for publication in February, 2018
Paper accepted for publication in November, 2018