Forensic Biology: A Passport for Biological Evidence

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Abstract

Though acts of crime committed today may have evolved to be part of the cyberspace, most criminal activities originate from the space that all living creatures live in: Earth. From harming fellow human beings to causing disarray in the environment, mankind has been the cause of and victim of criminal activity. From the very first crime to have ever occurred on planet Earth, to the petty theft that occurred in the neighbourhood store, biological evidence has always been a beacon in the run to serve justice.

As time evolved (and so did the means of committing crime), the means of testing biological evidence found has evolved too. Perpetrators who may have gotten away with, say murder, prior to DNA testing are being held accountable now. Forensic biology, in all its glory, is a frontrunner in proving the theory that "no crime can be perfect".

This chapter delves into the field of forensic biology and how it serves as not only a passport for biological evidence but also a seemingly one-way ticket to crime solving and justice.

6.1 Introduction

The first crime to have occurred on Earth (according to most discussions) is that of the murder of Abel by his brother Cain. As detailed in the Bible, Cain struck Abel's head with a rock, smashing his skull and leaving him bleeding to death. Even in this rendition of a crime that occurred when perhaps the concept of crime wasn't even

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born will you find *biological evidence*: entities of biological origin that prove or disprove a theory. The rock smeared with blood and blood on the ground show how and where the crime happened. An injured Abel proves who the victim is, and his blood on Cain's hands tells us in which direction we ought to be looking while searching for a perpetrator. Of course, there are many other questions to be answered, but to a layperson, it is as clear as day—Cain killed Abel.

This was a story of centuries ago, when the world first seemingly came into existence. Today, with studies specific to forensic science and biology, we now have more questions that we can ask (and seek the answers to) and more equipment and scientific method to reach a conclusion.

The biosphere consists of biotic and abiotic components, of which the biotic content far surpasses the abiotic one. Hence, it should come as no surprise that the scope of forensic biology is unfathomable. Every living entity that crosses paths with a crime can be tested as evidence. Forensic biology is one of the strong pillars on which the foundation of the justice system is set. This chapter discusses the evolution of forensic biology, what constitutes a biological evidence, how different types of biological evidence are tested and analysed, and what are some of the issues faced in the field of forensic biology.

6.2 History of Forensic Biology

While mummification, a process Egyptians used to preserve mortal remains of the dead, is the earliest possible record of autopsy studies (say around the year 400 BC) [1], it was in 1853 when Ludwik K Teichmann discovered that the haemin protein found in blood can be crystallised. This discovery let to the description of the Teichmann test, one of two microcrystalline assays run to confirm the presence of blood in a particular sample [2]. In 1903, the popular colour test for blood—the Kastle-Meyer test—was described. Joseph H Kastle made the rough draft for the test in 1901, and Erich Meyer modified it in 1903 and wrote down the three-step procedure, as followed today [3]. The Adler/Benzidine colour test for blood was then introduced in 1904, by Adler and Adler [4]. Eight years later in 1912, Takayama described the procedure to confirm the presence of blood in a sample, by the formation of ferroprotoporphyrin crystals of the haemin protein [5]. In 1930, Hans Fischer received the Nobel Prize in chemistry for artificially synthesising the haemin protein [6]. These are all ground-breaking discoveries and inventions related to confirming or negating the presence of blood.

The most popular method to identify the morphology or "group" of red blood cells in the human body is the ABO blood grouping system, which was prescribed by Karl Landsteiner in 1901 in the now Medical University of Vienna. He formulated the system after finding out that red blood cells would agglutinate if the source varied, as in, if the blood of two persons was mixed. This is the first recorded proof that the make-up of blood was not constant throughout the entire human population.

While all the available tests for blood in the twentieth century were helpful, there were still hurdles that needed to be crossed. For instance, there was no provision to

check for the presence of blood that had already been wiped out from a surface. In a parallel setting in 1928, H O Albrecht realised that the presence blood increased the luminance of luminol in the presence of an alkaline medium. It was only nearly a decade later, in 1936, that Karl Gleu and Karl Pfaanstiel attributed this increased shine to haematin, a component of blood. These findings are what crime scene investigators use to perform the luminol test in crime scenes, today, where the liquid is sprayed over an area suspected to have housed blood and viewed under ultraviolet (UV) light [7, 8].

Towards the end of the twentieth century, specifically in the 1980s, there was a lot of scientific research done in order to make use of DNA as an individualising mark. In 1983, Sir Alec Jeffrys, in collaboration with the Forensic Science Services (FSS) of the United Kingdom, invented DNA profiling, or DNA fingerprinting, wherein short tandem repeats (STRs) in a DNA sequence are used to determine individual characteristics in a person. This was first used in 1983 and 1986 to bring about convictions in the rape and murder of two teenagers in Leicestershire, England [9]. In 1983, Kary Mullis invented the polymerase chain reaction (PCR) technique to make multiple copies of the same sequence of DNA, a step which is now very pivotal in DNA profiling.

In present time, the twenty-first century, we have reached an era where entire genomes have been mapped and the data interpreted and saved (The Human Genome Project 2003) and where all the textbook steps of analysing blood are fast-forwarded to DNA analysis. As of today, there has been research on new software that gives the result of DNA testing based on statistics and algorithmic actions of a computer, that aid in providing the weightage, or percentage of contributions in a mixed or degraded DNA samples.

6.3 Sources and Types of Biological Evidence

There are many situations in a forensic setting in which evidence originates from a biological/natural source, the most crucial and common of which is when a crime is committed against another living being—human or otherwise. In cases of violent acts of crime like murder and rape/sexual assault, it is very imperative to identify and collect samples of body fluids, the most common of which is blood. Other seemingly transparent or non-coloured body fluids like saliva, sweat, and genital discharge may take a keen eye to locate. All body fluids are the store house for DNA samples, the proper testing and identification of which can lead a forensic investigator to identify victims and culprits. Blood and sweat may also be found at the scene of burglaries, where the criminal may get scathed against a broken glass or may wipe sweat on a surface without meaning to do so.

Another common biological evidence found in a violent crime is that of skin and residue under fingernails. In cases of struggle—commonly seen in assaults—the victim may try to break free from the perpetrator, often scratching them enough to pull skin, and (in some cases) draw blood. Skin cells being nucleated also house genetic material, which can lead to making an identification.

Any sample that can provide DNA—toothbrushes, hair brushes with strands of hair, and cigarette butts with traces of saliva—are commonplace in cases of determining blood relations. These articles became most useful in identifying human remains of the September 11 attacks in 2001, after the biggest fragment of human remains measured barely a few inches, as a piece of bone. For maternity disputes, it is considered more helpful to submit samples of hair, as hair strands contain mitochondrial DNA (mtDNA) which is always passed down the maternal line of a family. For paternity identification cases, usually blood or a swab of inner cheek cells is taken as samples.

As with the definition of biological evidence, the origin isn't necessarily limited to human beings. Diatoms play a huge role in determining the cause of death in suspected cases of drowning. The location of the diatoms in the body determines whether drowning occurred anti or post-mortem, and the types and morphology help one assess in what water body drowning occurred. Many types of diatoms can be indicative of the dead individual drifting across water bodies with the flow of water currents.

Most animal parts are crucial evidence in wildlife-related crimes. Claws, fur, hide, teeth/fangs/tusks, horns, antlers, and hooves prove useful in handling cases of poaching, hunting, and illegal trafficking of animals. Certain samples of caviar can help a wildlife forensic practitioner determine the species of sturgeon they were derived from.

Plant parts too are considered evidence of biological origin. The poppy flowers and leaves of *cannabis* are evidence samples in cases of illicit drug production and trafficking. Destruction of growing endangered species of plants is considered a crime, and hence, these plant parts can be used as evidence. There have been many cases of smuggling of sandalwood from the forests of India, the most common targets being in the states of Kerala and Karnataka.

The possibilities for finding evidence that is also of biological origin are endless.

6.4 Biological Evidence as Evidence for Other Forensic Fields

This, however, does not mean that all evidence that originated from a biological source shall be tested as such. Since forensic science is a multidisciplinary field, it is very common to have multiple connections among the various subfields within the study of forensic science. It is often through collaborative effort and teamwork that evidence is analysed and cases are solved.

The examination of human remains post-mortem—an autopsy—is considered to be a study under *forensic pathology*, which also focuses on microbial activity and stages of degradation of human/animal remains at different time intervals.

All body fluids become what is known as "serological evidence" under the subfield of *forensic serology*. It is under forensic serology that all colour and microcrystalline tests for blood are conducted. Other fluids like genital discharge (vaginal fluids and semen), saliva, sweat, and even vomit are tested to confirm their presence or absence in a sample in question.

Organ remains like stomach, section of intestine, brain, and liver are tested in the sub-discipline of *forensic toxicology*, where concentration levels of certain toxins in the viscera are determined through various chemical-related tests.

Bone specimens are studied carefully under the field of *forensic anthropology*, and the marrow of such bone may be extracted for DNA testing and identification. It was mainly through anthropological studies that the remains of the last Russian Romanov family were identified and the causes of their deaths in 1918 was ascertained. Teeth samples fall under the sub-specialty of *forensic odontology*. While the pulp (if available) is extracted for DNA testing, the morphology and positioning of teeth provide information of age, time since death, and presence of dental disease (if any), among other details. Upon examination, bones can relay information like age, height, race, presence of fractures, and cause of death (a crack in the skull can be indicative of a blow to the head).

Often it happens that fine particles participate in cross-transfer of evidence that help provide the link between the scene of crime, the victim, and the perpetrator of the crime in question. A common example of such fine particles, apart from sand, is pollen. Due to its microscopic size and somewhat sticky exterior, pollen is quick to stick on any surface it comes in contact with. While Mother Nature has made the provision for pollen to stick to the stigma of a female flower, pollen is also known to stick to clothing, shoes, skin, and other surfaces that may help establish a link to an outdoor crime scene or an outdoor secondary crime scene. This study of the microscopic morphology of pollen is called *forensic palynology*.

Forensic botany deals with the legal implication of plant parts as evidence of a crime. The most common cases of this instance are when plant parts are used to manufacture illicit drugs, like the *datura* flower and various parts of *cannabis* that are used to make different drug products. Plants also become evidence when endangered species are smuggled or destroyed, or even illegally grown, as in the case of when endangered wood species are used to make guitars and other musical instruments.

Any type of evidence that is found in the wild naturally is analysed by the subdivision of *wildlife forensics*. In case of dead animals, the rules of *forensic veterinary pathology* are nearly the same as that for human remains.

6.5 Testing and Assessment of Biological Evidence

Like all articles that are considered evidence, proper collection, packaging, transportation, and testing are crucial for them to be presentable in a court of law. These practices when done properly, followed by maintaining a proper *chain of custody* (a chronological record of the journey of evidence, from its discovery at the crime scene to the court where it is presented) solidifies the authenticity of what is being presented before the magistrate. This convinces the judge and/or the jury to rely on the evidence put forth in order to reach a decision without hesitation and worry of error.

Wherever possible, samples are tested following the following path in the exact order:

- Presumptive tests—those tests that are highly sensitive, but not specific to one type of substance, that serve as an indicator for what the sample *may* be
- Confirmatory tests—a highly specific test that gives a positive result for one particular substance only, thereby *confirming* the contents of the sample
- Individualising tests—tests that help pinpoint the source of the sample, as in the case of DNA testing

6.5.1 Analysis of Major Body Fluids Analysis of Blood

The most common of all serological evidence is blood, a specialised connective tissue that, by virtue of circulation, indirectly connects all parts of the body to each other via the heart. It consists of three types of cells, or *corpuscles*—the *erythrocytes* (or red blood corpuscles, RBCs) that are responsible for oxygen transport by the use of haemoglobin, the *leucocytes* (or white blood corpuscles, WBCs) that serve the immune system and fights infection, and the *thrombocytes* (or blood platelets) that help clot blood and prevent haemorrhage. All these cells are suspended in what is known as *plasma*—a straw coloured medium that is made up of 90% water and 10% of miscellaneous substances like proteins, urea, fats, and hormones, among others [10].

It is the erythrocytes and leucocytes that have the most forensic relevance in the testing of blood. RBCs have haemoglobin, which houses the haemin—a porphyrin that contains iron and chlorine. This haeme (the complex that helps bind haemoglobin to oxygen) group is what causes a colour change, luminescence, and microcrystal formation in testing for the presence of blood. However, since RBCs are enucleated (lack a nucleus), they cannot be used for DNA testing. This is why WBCs become the source of genetic testing with respect to blood.

Presumptive tests for blood include all colour-based tests and chemiluminescent tests. In the former, a change in colour due to oxidation is a presumptive positive, and in the latter, the emission of a light of certain wavelength is an indication of haemoglobin presence [10].

The most reliable and sensitive of all colour tests is the *Kastle-Meyer Test* or the phenolphthalein test and is considered so because it can detect blood from a sample that is diluted down to a part in ten million and also because old stains of blood can be detected by use of this test [10].

To perform the test, a cotton swab (Q tip) is dipped in distilled water and rubbed over the surface or stain that is suspected to house blood. A drop of phenolphthalein is added, followed by a drop of 3% hydrogen peroxide. The swab goes from colourless to pink when the peroxide is added, if blood is supposedly present. This is because phenolphthalin (colourless)—a reduced form of phenolphthalein (pink) that is formed so by boiling the substance with zinc in an alkaline medium—becomes oxidised in the presence of hydrogen peroxide to form the pink coloured

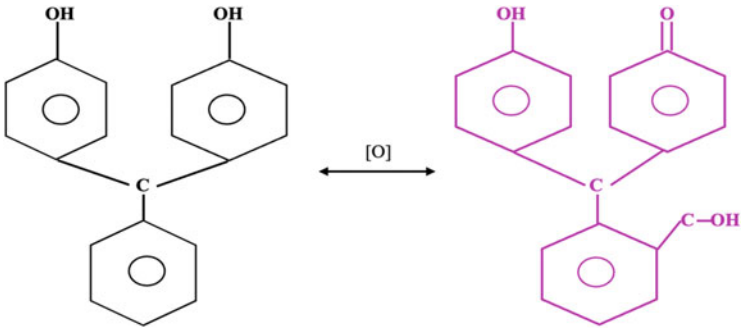

Fig. 6.1 The Kastle Meyer test: Phenolphthalin (reduced, colourless) reacts with hydrogen peroxide to form phenolphthalein (oxidised, pink)

phenolphthalein [3]. The colour change must be observed rapidly, as the pink colouration forms naturally as time progresses. If no colour change is observed, it can be inferred that the given sample either does not contain blood or does not have blood in the minimum quantity required for a positive result (Fig. 6.1).

The Kastle Meyer test gives a positive result for substances like fruits, vegetables, and food stuffs made from these (like tomato ketchup) as these contain peroxides as well.

Other reagents used in the colour testing for blood is *tetramethyl benzedine (TMB)* and *leucomalachite green*. These reagents are used in the same three-step process as the Kastle Meyer test, but the colour change varies. While TMB changes a colourless swab to a blue—green colour, leucomalachite green gives a greenish-yellow tint upon oxidation. Not only are these two reagents less sensitive to blood, but they also are not very cost effective and are known to be carcinogens.

Most crimes where blood is found to be an evidence are violent in nature, and finding just a few drops of blood is rare. Blood is often found covering a large area of the surface it rests on, and while cleaning may be a time-staking process, blood pattern analysts require that the blood pattern remain undisturbed in order to properly study spatter [10]. Hence, in these cases, rapid colour tests are not feasible, and so chemiluminescent tests are used to determine the possible presence of blood on a given surface. Once sprayed, the emitted light is observed under an alternate light source (ALS), as the output may be faint [10]. Such practice, however, is not encouraged if the blood stain is visible, and a sample of it can be collected (Robert Spaulding 2002) [10]. The common reagents for chemiluminescent tests are *luminol* and *fluorescein*.

Luminol (3-aminophthalhydrazide) works the same way as does phenolphthalein in the Kastle-Meyer test; only this time, the colour of light emitted varies from a blue-white to yellow-green. A solution of luminol, an oxidiser, and water is made and sprayed upon the surface. The resultant light emission is viewed in darkness and in the presence of a light source. This reagent is extremely sensitive to haemoglobin and can detect blood diluted up to a part in five hundred thousand. The pattern must be photographed immediately, as the light is best emitted for half a minute only,

following which additional treatment must be done to increase duration of emittance. This, unfortunately, comes at a cost as minute details are lost on multiple treatments.

Fluorescein is also used in the same way, with the additional thickening substance in the preparation, which is used in order to increase the staying capacity of the solution on vertical surfaces, thereby preventing its running off [10]. The solution, however, emits fluorescence and must be lit up with the help of ALS with a wavelength of 450 nm.

While both reagents are not carcinogenic, they cause severe irritation if brought in direct contact with skin, and so, proper care must be exercised while handling them. Studies about whether or not a chemiluminescent test destroys the chances of a DNA test being done vary.

Confirmatory tests for blood include microcrystalline assays and the Vibert's fluid test. In these tests, the microscopic nature of complexes formed with haemoglobin are studied. These tests are specific to blood and can hence confirm its presence in a given sample.

The *Takayama Test*/haemochromogen test is one of two confirmatory tests for blood that checks for the formation of crystals of a haeme complex. In this test, a drop of sample containing the stain presumed to be of blood is placed on a glass slide and gently hated after having a coverslip on top. A drop of pyridine (in an alkaline medium, in the presence of a reducing sugar) is placed over the sample drop [10]. The formation of light-pink coloured crystals in the shape of needles confirm the presence of blood. These crystals are called pyridine ferroprotoporphyrin.

The Takayama test is said to be very sensitive and can give a positive result from very old bloodstains as well. However, proper care should be taken while heating the sample, as improper heating may lead to a false negative result.

Prior to the Takayama test, the *Teichmann test* was widely used as microcrystalline test. While the principle of both tests are the same, the reagents and crystal morphology vary. For the Teichmann test, a potassium halide is dissolved in glacial acetic acid, a drop of which is placed over the sample drop on a slide and warmed. Microscopic inspection will yield brown-coloured rhombic crystals, which are of ferriporphyrin halide.

Another way of confirming the presence of blood is by observing the sample under a microscope, after it has been treated with *Vibert's fluid*, a solution containing sodium chloride and mercury chloride in distilled water. This procedure extracts the red blood corpuscles and can be viewed as red dots/blobs under the microscope [11].

In real-life situations, it is very rare to find laboratories perform presumptive and confirmatory tests as mentioned above. This is because when a stain is suspected to be of blood, it is directly sent in for DNA analysis, where a DNA profile is generated. The DNA profile will not only confirm the presence of blood but also provide information related to species and the source of the blood themselves. Bypassing the traditional analytical steps not only saves on time and money, but the end result will be more accurate, given the nature of DNA testing. This, unfortunately, puts a lot of strain on the DNA laboratories and creates a tremendous backlog of testing and report filing.

6.6 Semen Analysis

Semen is a viscous, white fluid that is secreted by males who have crossed puberty, via the urinogenital tract, upon sexual stimulation. It contains the male gamete, spermatozoa/sperm, along with a complex mixture of sugars, amino acids, and salts to ensure the viability of sperm once it leaves the male body (and preferably enters the female reproductive tract, where fertilisation is due to take place). Each ejaculate from a healthy male is known to hold around 125 million sperm cells, all of which have genetic material of the male source. Hence, finding and testing semen traces are crucial in solving cases of sexual assault.

For **presumptive analysis**, the key component of forensic relevance is *acid phosphatase*, an enzyme secreted by the prostate gland, which is tested by use of Brentamine Fast Blue B. For the test, a buffer of anhydrous sodium acetate is prepared, in which alpha-naphthyl phosphate is dissolved. Another solution of the buffer with the Fast Blue B reagent is also prepared. Next, a portion of the sample stain is cut, and both the reagents are placed on it, one after the other, a minute apart from each other. A rapid purple colouration is indicative of a presumptive presence of semen on the stain. Older stains may not stain purple, due to decreased acid phosphatase activity, [10] but that does not mean that semen is absent from the stain. Another downside to the test is the fact that other body fluids, like vaginal secretions, may test positive as these contain acid phosphatase too.

Semen stains can also be views under alternate light sources at 450 nm, under amber goggles, and give a blue-white fluorescence [10]. This method of identification can be used on light and dark surfaces and covers a large area. However, fluids like saliva and urine will also fluoresce, so care must be taken to look for the exact colour of emitted light. It is noteworthy that an absence of fluorescence does not imply absence of semen.

A long-time popular method of **confirming the presence of semen** is through staining the spermatozoa and viewing under microscope. This is due to the abundance of these cells in semen (an exception would obviously be the semen from men who are aspermic/unable to produce sperm in semen). The *Christmas tree stain* does exactly as the name suggests: light a sperm cell up, like a Christmas tree: the tip of the head stains pink, the bottom becomes dark-red, the idle portion becomes blue, and the tail becomes yellow-green. The reagents involve nuclear fast red (a solution of ammonium sulfate and nuclear fast red in deionised water), and picro indigo carmine (picric acid and indigo carmine in water) [10]. After a drop of the extract is placed on a slide, the nuclear fast red solution is added and allowed to sit for ten minutes. After the excess is washed off with water, picro indigo carmine is added and let to sit for half a minute, following which it is washed off with absolute ethanol. Once complete, the slide is viewed under the microscope to view the stainings [10].

Using the enzyme-linked immunosorbent assay (ELISA) test, a *prostate specific antigen* by the name of p30 is detected to confirm the presence of semen. ELISA is based on interaction between antigens and antibody, and when the reagent is added

to the sample swab, an intense purple colour is observed. Deeper the colouration, more is the quantity of p30 in the sample [10].

The *time since intercourse* not only helps draw a time sheet of events but also helps locate the position of sperm cells in the case of rape (where protection is not used). Motile sperm survives for the shortest period in the vagina (3 h, due to the surrounding acidity), followed by the mouth (6 h) and the longest in the rectum (6–65 h, or until defecation). P30 levels are used to estimate the time since intercourse, as most p30 content becomes obsolete a day after the act [10].

As with the case of blood, most semen samples are sent straight for DNA testing, apart from the p30 test for determining time since intercourse.

6.7 Saliva Analysis

Analysing saliva is often tricky, mainly because it is rarely found as an evidence to begin with. In the cases of bite marks during violent crimes, movement of the victim may cause accidental wiping off of any saliva residue. Items that come in contact with the mouth—licked articles (as an adhesive), cigarette butts, beverage cups, and cans—all produce trace amounts of saliva. Spitting may yield scope for DNA testing, due to its quantity.

Common **presumptive tests** for saliva check for amylase activity (such as the one with starch water and iodine: a blue-black coloration if amylase is absent, no colour change if amylase is present). However, these are not selective at all, as amylase is a component in other body fluids too. Hence, a presumptive positive for saliva via these tests is unreliable.

The best shot at getting a confirmation of the presence of saliva is through DNA analysis. This is possible due to the fact that saliva often contains epithelial cells of the cheek and, in case of oral injury, blood too.

6.8 Urine Analysis

Urine can be presumptively tested by checking the presence of urea (through urease enzyme), by its foul odour on heating or by testing for creatinine with picric acid (yellow-orange colouration). Though DNA typing for urine is rare, it may be possible if the sample is very concentrated [10].

6.8.1 Bloodstain Pattern Analysis (BPA)

Bloodstain pattern analysis (BPA) is the study and interpretation of patterns formed by bloodstains that form when a violent crime has occurred. The practice uses biology, fluid dynamics, and mathematics (and logic) in order to *reconstruct* the crime and piece together the chain of events that are assumed to have occurred during the commission of the crime in question.

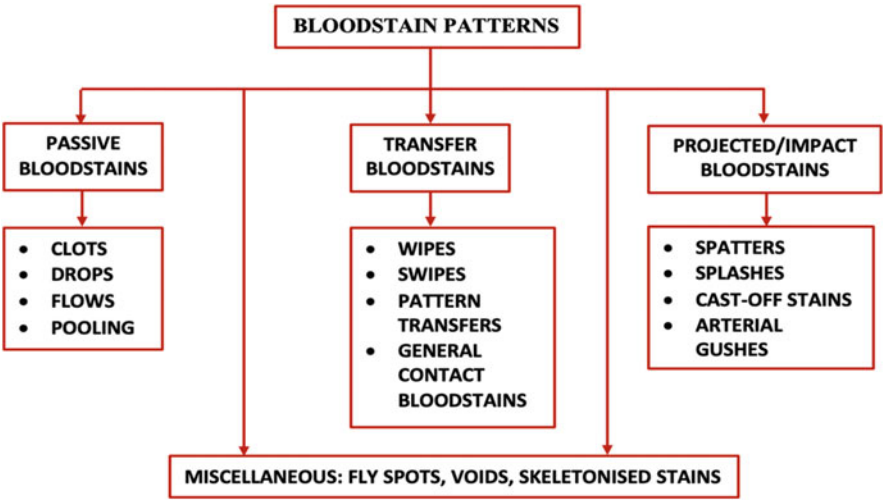

Fig. 6.2 Categories and types of bloodstain patterns [10]

BPA can be defined as "analysis and interpretation of the dispersion, shape characteristics, volume, pattern, number, and relationship of bloodstains at a crime scene to reconstruct a process of events" *(Bloodstain Pattern Analysis by Bevel and Gardener 2002)*. A great amount of work and experience is required to be a bloodstain pattern analyst. Even then, this procedure is not entirely reliable, and many accused individuals convicted by this form of evidence have been acquitted in subsequent trials due to the information not being convincing enough. Hence, BPA is mostly treated as a corroborative evidence, instead of direct.

There are many types of bloodstain patterns an analyst will come across, often from the same scene of crime. Rather than being one big picture, bloodstain patterns are considered to be many minute details that make up a whole, larger pattern. The classification of bloodstain patterns is as follows (Fig. 6.2):

Passive bloodstains form when blood flows due to its viscosity, as well as due to gravitational pull (if blood falls on a vertical surface, like a wall). As a result, passive bloodstains can be observed as a *clot* (when blood becomes jelly-like after being left unabsorbed), *drops* (solitary blood drops that aren't connected to any other pattern part), *flows* (blood dripping due to gravity), and *pooling* (an area filled with blood).

Transfer bloodstains are created when an object comes in contact with blood, thereby creating a pattern by disturbance, and include *wipes* (when an unstained object in moved through blood; like a shoe being pressed in blood, leaving a pattern) and *swipes* (when an object stained with blood is rubbed across a clean surface; like a blood-coated hand leaving a mark on a door frame).

When a pattern is made after an object is struck against blood, a *projected pattern* or *impact pattern* is formed. The types of this include *spatters* (that can be *forward*—when blood droplets move in the direction opposite to the object causing it—or *backward*—when blood flows in the same direction of the spatter causing object),

splashes (when an object is thrown in a pool of blood, resulting in a splash of blood droplets), *cast-off stains* (when an object that bears blood is repeatedly shaken so as to get rid of the blood on the surface, like a hammer being shaken back-and forth), and *arterial gushes/expirations* (when blood sprays out of a cut artery or vein). Expirations can also occur when a person with a lung injury coughs blood out.

Whilst these are the main categories of bloodstain patterns, there are many bloodstain patterns that do not fit in any of these brackets. Such patterns include *fly spots* (that are created by activity of flies, including flying, sitting on, and eating and defecating blood), *voids* (empty spaces in bloodstain patterns that are caused due to an object blocking the area from the blood projectiles, and are in the outline of the obstructing object), and *skeletonised stains* (old stains with cracking edges as the blood dries) among others.

Mathematics—especially geometry—plays a major role in bloodstain pattern analysis, as it is used to determine what is known as *point of origin*, the area where blood first emanated from before hitting the target surface. In order to determine the point of origin, the basic measurements must first be understood. The *angle of impact* is the acute angle formed in between the intercept of the target and the vector of the bloodstain [10], while the *direction angle* is what comes in between the long axis of the blood stain and a zero-degree vertical that is taken as the reference point [10].

To find the point of origin in a three-dimensional space, analysts first find the *area of convergence*, which is the area on the surface where blood first struck before dispersing into smaller patterns. This area is found by unfurling thread along the long axis of many bloodstains, such that they appear to converge at a similar area [10]. The point, or area of origin, is then assumed by drawing strings in the z-axis and may help determine the probable position of the victim when first injured.

Many tools—from basic strings, pencils, and geometric apparatus to the most sophisticated software—are used in aid of visualising the pattern better. The process is also made easier by documentation, which when done properly can help reach a solution better. Photography is a must in order to re-assess the bloodstain pattern again in the future when needed. It is imperative to first click photographs relative to a stationary point, in order to establish where the pattern was found in the crime scene. Individual photographs of fragile patterns must be taken first, and all photographs must be accompanied with a scale to measure. Photographs must also be taken in close-up and far ranges. These photographs can then be analysed as individual parts of a grid or as a whole [10].

Case Study: Sam Sheppard—The Wrong Man [12]

On the morning of 4th of July, 1954, Marilyn Sheppard was brutally murdered in the bedroom of her home in Ohio, USA, having been bludgeoned by an object. As a result, there was blood all over the room, and some drops of blood were found on the floors all over the house. When her husband, Sam Sheppard (a neurosurgeon), was questioned, he told the police that he fell asleep on the couch in the living room the previous night, during a movie watch with their guests. Marilyn bid the guests farewell and went to her room to retire for the night. Sometime later, Sam was

awoken by the cries from his wife, and when he ran upstairs to the bedroom, he saw a "figure" before being knocked unconscious. By the time he regained his bearings, the assailant was downstairs, so he chased the person to the beach further down where they scuffled and he was knocked out again. This inconsistent story, backed with a lack of murder weapon, immediately made Sam Sheppard a suspect. In his first trial, the prosecution argued that Sam killed his wife as a means to legitimise an extra marital affair with a nurse in his clinic. The prosecution also led the jury to believe that the weapon in question was a surgical scalpel, based on a blood print made by a supposed scalpel on the pillow on which Marilyn's head was placed. The defence was denied access to any physical evidence, and hence could not make any assertions with respect to injuries, blood patterns, even the surgical scalpel blood print in question. The trial ended on December 21, 1954, where Sam Sheppard was found guilty of second-degree murder and was awarded a life sentence. After serving 10 years of his sentence, he was released in 1964 and began the process of re-trial, which began in 1966. It was here that the jury realised that the prosecution back at the first trial put forward statements with no concrete evidence whatsoever and went with the flow based on mere assumptions. It was during this time that renowned criminologist Paul Kirk presented the bloodstain pattern analysis report based on analysis of the spatter in the bedroom, which showed that the killer was left-handed, when Sam was right-handed. This evidence, coupled with the now baseless argument of the prosecution, resulted in Sam Sheppard to be exonerated and proven not guilty. The experience, however, took a toll on his mental health, and Sam Sheppard died of alcoholism-related complications four years later.

6.8.2 DNA Fingerprinting

The method of DNA fingerprinting/profiling that has become popular today was first put forth by Sir Alec Jeffrys in 1985, when he discovered that certain parts in a sequence of DNA kept repeating over and over at close proximity to each other and also that these repeating sequences vary from person to person. These repeated sequences came to be known as *variable number of tandem repeats (VNTRs)*, and the process was termed *restriction fragment length polymorphism (RFLP)* as it used restriction enzymes to cut the sequence that held the VNTR. A smaller form of the VNTRs, the *short tandem repeats (STRs),* is commonly tested in most cases. Since its inception, the profiling process has become almost indispensable, as almost every situation in life—ranging from paternity testing to identifying victims of a mass disaster—requires a DNA profile to be made.

When a sample is first found at the scene of crime, it is collected and packaged carefully to prevent damage that could lead to incorrect results. Once at the laboratory, it undergoes three phases of processing:

In the *biology* phase, the sample is processed so as to extract the genetic material, lysing the cells that hold it. It is then measured to check for the amount of DNA recovered. Once all this is done, the DNA strands are cut at the STR points by the use

of restriction enzymes, and the STRs so formed are amplified by the use of the PCR process [13].

Next comes the *technology* phase, where the products of PCR are separated by means of electrophoresis, and the STRs are detected in order to characterise them. The strands are then fluoresced in order to measure them, and these are later assessed to determine the sequence of the tandem repeats, in a process called sample genotyping. The DNA profile bands are then prepared at the discretion of the practice that varies among laboratories [13].

Finally, in the *genetics* phase, the DNA is profile is matched to the profiles of other samples, including that of the reference sample. Should there be no match found, it is inferred that the reference and questioned samples have originated from different sources. A no match is called exclusion. In the eventuality of a match, or an inclusion, the profile is matched with a database bearing profile information of samples of the same demographics. At the end, a test report is generated which includes the probability that a random match may have occurred (as in, a chance that a random person from a demographic can have an STR profile identical to the markers used for the sample in question) [13] (Fig. 6.3).

Case Study: The First Case Solved by DNA Profiling [13]

In November 1983, Lynda Mann—a 15-year-old schoolgirl in Leicestershire, England—was found raped and strangled to death after being missing for an entire day. She was found in a deserted area, which was part of the shortcut between her school and home. With the forensic technique prevalent back then, the police found a semen stain and found it to match a person with blood type A, along with an enzyme profile that was common in only 10% of males. The case ran cold soon after, due to lack of evidence and further leads.

In July 1986, another girl, Dawn Ashwood (also 15 years of age) was found in the same way as Leslie Mann—beaten, raped, and strangled to death in a deserted area. She was found two days after she went missing, having never returned home after going to visit a friend. The modus operandi, along with the genetic details of the semen sample found, were the exact same as the case three years prior. Richard Buckland, a 17-year-old boy with learning difficulties, confessed to the crime against Dawn Ashwood but denied having anything to do with the murder of Leslie Mann.

By now, it had been a year since the DNA fingerprinting technique was formulated. Dr Jeffrys compared the semen samples found in both cases to each other, and they matched. However, the DNA type did not match Richard Buckland's, proving that he had lied. So began a countywide hunt across three villages, where samples of 4000 men were collected and compared to the profile of the semen sample found. It was like finding a needle in very big haystack, and the police were beginning to lose hope at the lack of a breakthrough.

And then, the breakthrough came. Not as a match, but as an eyewitness account, where a person testified to having seen and heard a person brag about how he "provided a sample on behalf of Mr. Pitchfork". The impersonator, Ian Kelly, informed the police that his colleague, Colin Pitchfork, had told him that he had already impersonated his friend and given a sample in the friend's name, who was

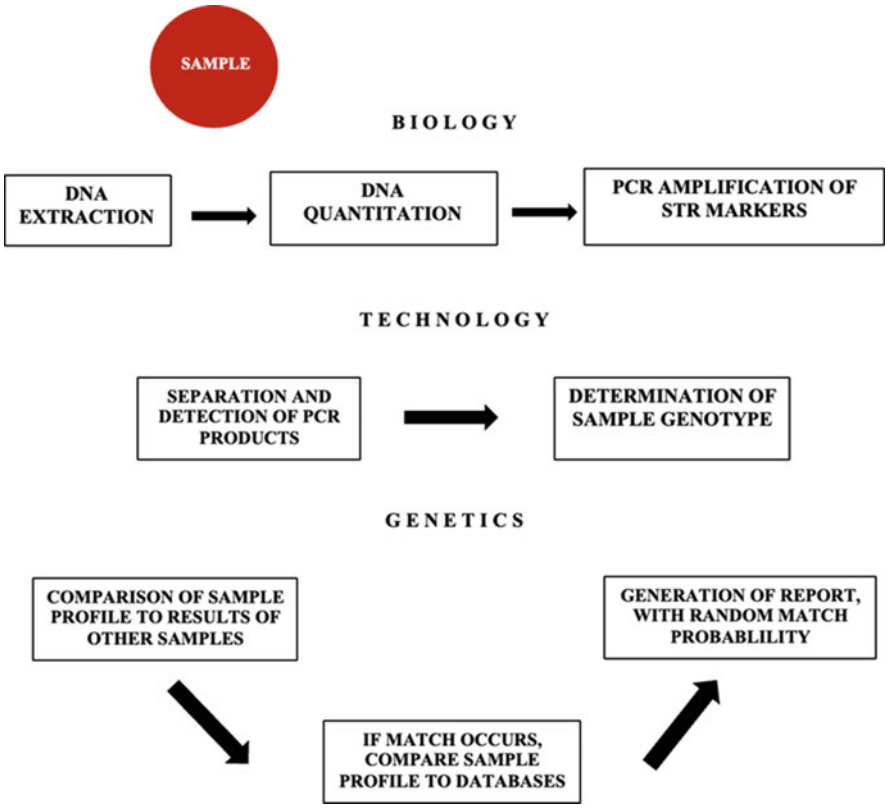

Fig. 6.3 The DNA Fingerprinting Process [13]

being harassed over a sexual assault case. As a result, Colin could no longer give his own sample under his name, so he asked Ian Kelly to do so.

Colin Pitchfork was caught in 1987, and his blood was drawn for testing, where his DNA matched that found in the semen stains on both crime scenes. He pleaded guilty to both rapes and murders, as well as another case of sexual assault, and was sentenced to life imprisonment.

It is only in an ideal, textbook situation where one would find evidence with enough genetic material to be tested that is not only fresh but also free from contamination. While this is true when a reference sample is collected from a person for paternity testing, this is not the case for material collected from the scene of crime. Even if, supposing, a good sample is collected, the time delay from sample collection to sample testing (sometimes even years, in an ill-equipped laboratory) ensures that a mostly degraded sample is available for testing. Perpetrators, too, refuse to cooperate and hand over a reference sample for testing immediately, being

protected by the requirement of a warrant being issued first, which may not be granted if there is insufficient evidence that a warrant is required to begin with.

Thankfully, all these problems become miniscule in the eyes of the *polymerase chain reaction (PCR)* technique, which was developed by Kary Mullis in 1985. The PCR technique makes multiple (in the order of millions) copies of the specified sequence of DNA, which has made it possible to easily genetic material from a given sample, no matter the size. This invention won Mullis the Nobel Prize in Chemistry in 1993.

In the process of polymerase chain reaction, an enzyme is used to replicate a specific region of the DNA strand, by repeated heating and cooling at every cycle (usually, 30 cycles make up the entire process). As a cycle is completed, the target DNA sequence is replicated at every molecule where the said sequence exists. The end result, known as an *amplicon*, is then used for testing by various methods, as it contains enough genetic material to be detected by the equipment used in the testing process [13].

Most handbooks for PCR mandate that the sample be anywhere in the 20–50 μL volume range. This is because an optimal weight is required to get a good quality of amplicon: too small a sample may be lost to evaporation from high temperatures, and a heavier sample may cause an issue with thermal equilibrium; it takes more time for a temperature change on the outside to be transmitted on the inside of a sample with large volume [13].

As the years have passed since the inception of PCR, it has become more easy to perform the technique. Most forensic laboratories add a template DNA sequence to a pre-made PCR kit that contains all the components needed for the process to be carried out. These kits simply require the user to add in a small portion of the template DNA to the sample DNA that needs to be amplified and is best used when the volume of template DNA added roughly matches that of the concentration range for which the kit was designed [13] (Fig. 6.4).

The most important components of PCR include the primers that anneal at the 3' end of the sequence and is what precede the sequence that has to be multiplied, the DNA template itself (along with some information about the actual sequence, so that appropriate primers are selected), building blocks that contain nucleotides, and a polymerase that will help the nucleotides attach in the correct sequence. The most commonly used polymerase is the *Taq*, which is developed from the *Thermus aquaticus* bacteria found in hot springs, as these are most thermostable at the high temperatures at which the PCR process is carried out.

The sample is first incubated at 95 °C for 11 min, and the cycle count on the machine is set to 28–30. In the main process, the DNA strands are *denatured* or undone from its helix, at a high temperature of 94 °C for about a minute. Next, the primers (oligonucleotides) are *annealed* to the 3′ end of each strand at 59 °C for a minute. Finally, the strands are *extended* by the use of the Taq polymerase that will help the nucleotide blocks to get attached to each other in the correct sequence (complimentary to the template strand). This process occurs at 72 °C for a minute. In the final extension, the machine is run for 45 min at a lower temperature of 60 °C,

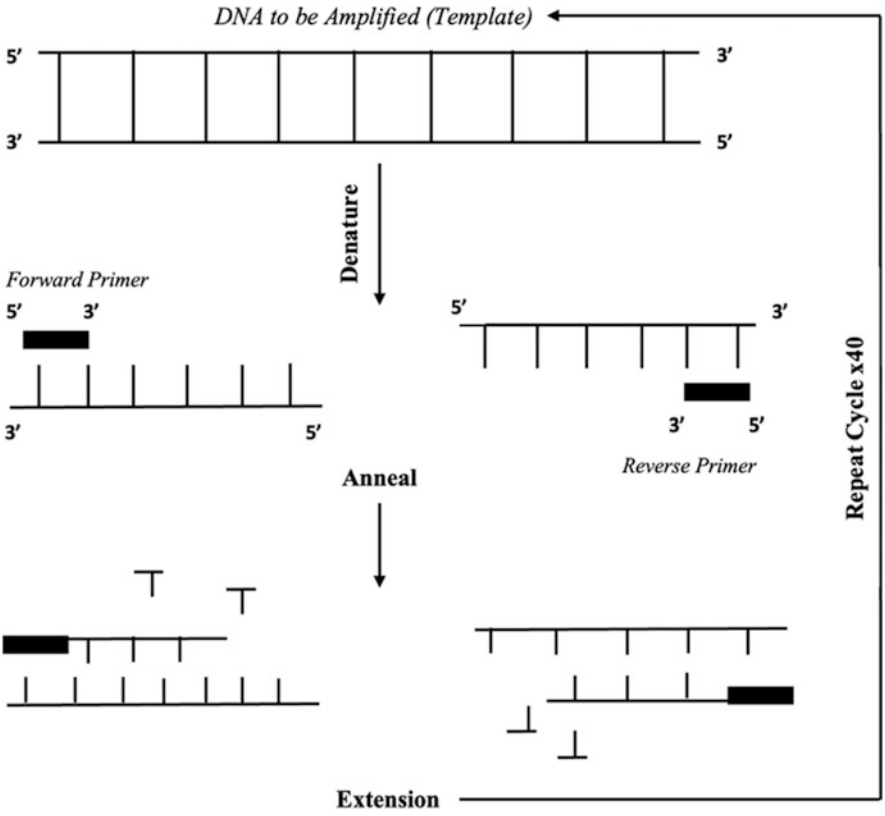

Fig. 6.4 The polymerase chain reaction (PCR) [13]

and the final soak requires that the temperatures be reduced to 25 °C until the samples are removed [13].

The polymerase chain reaction can be conducted in such a way that many copies of multiple regions within the same strand may be made, as long as more than one primer pair is added to the mix. When two or more regions of DNA are replicated simultaneously, the process is called *multiplex PCR*. For this to work without a hitch, the primers used must be compatible with each other, the annealing temperatures must be within the same range, and the excessive complementarity between primers must be minimised; else the primers will anneal to each other, and not the DNA template. The optimisation process, however, will be much more complicated here as compared to when a single region is copied [13] (Fig. 6.5)

Another popular PCR method that came about with new instruments is the *real-time PCR*, which analyses the changes that occur per cycle from the amplification of the template DNA sequence, by monitoring the change in fluorescence signals emitted. The most common of the approaches studied is the use of *5′ nuclease*

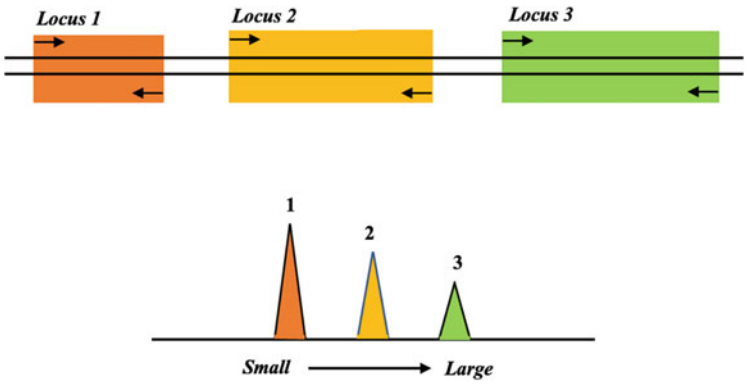

Fig. 6.5 Multiplex PCR with three loci. Primer size varied per loci, such that amplicons could be separated by size [13]

assay (TAQMAN) or by using an intercalating dye like *SYBR Green*, which is highly specific for a double-stranded molecule of DNA.

The TaqMan probes are labelled with two fluorescent dyes—the *reporter (R)* dye at the 5′ end and the *quencher (Q)* dye at the 3′ end—each emitting a different wavelength, which is made to hybridise specifically in the region of sequence between the two primers. The probe usually has a higher annealing temperature as compared to the primers so that the detection starts as soon as the annealing process begins. When the probe is intact and the R and Q dyes are close to each other, there is barely (if any) fluorescence that is emitted due to energy transfer between the two. When polymerisation (extension) begins, the strand being extended pushes aside any TaqMan probes that have hybridised to the target sequence. 5′ exonuclease activity in the Taq polymerase will eat out any probes that have annealed to the sequence. This causes the R dye to be released from the probe, and now that two dyes have separated, fluorescence signals begin emission. The signals increase if the TaqMan probe and the template sequence complement each other [13].

Each PCR process is characterised by three separate phases: *geometric/exponential amplification*, *linear amplification*, and the *plateau region (Bloch 1991)*. These are observed in a plot of fluorescence signals versus PCR cycle count [13].

When the exponential amplification is on-going, there is immense precision in which the amplicons are formed. When the process takes place at near perfect efficiency, the number of amplicons formed double with each complete cycle. A plot of cycle count versus the log scale shows a linear relationship during this phase. This is the optimal place to check for the relation between fluorescence and cycle count, since this is where there will be a consistency between input and output DNA [13].

The linear phase of amplification follows the exponential phase, as amplification efficiency slows down to an arithmetic increase instead of a geometric one, due to components falling below the critical concentration. Given that some components

like the primers may be used up at varied rates in the course of the reaction, the linear phase is not useful for comparison as this phase varies among samples [13].

Finally, the plateau region forms when the accumulation of PCR products becomes stagnant as multiple of the components reach their efficiency limit [13].

During the polymerase chain reaction of short tandem repeats (STR), there are many artefacts formed that can interfere with the process of interpreting and genotyping the alleles present in the template DNA. These artefacts are as follows:

Stutter products are characterised as small peaks near the STR allele peak, which are several base pairs smaller, and are formed when STR loci are copied by a DNA polymerase. These are also known as *shadow band* or *DNA slippage product* and have been found ever since STRs were first described. Analysis of stutter products from a locus has shown that these are usually one repeat unit short than an allelic peak. Stutter products that are a repeat unit bigger than an allelic peak are very rarely observed in commonly used tetranucleotide repeat STR loci. The most common way stutter products are formed is when a region of the primer-template complex becomes unpaired during the extension phase of PCR, thereby allowing a slippage of either primer or template strand such that one repeat unit falls short and forms a non-base-pair loop [13].

Stutter products are roughly the same size as PCR products of alleles, and so it is often challenging to determine if a small peak is actually a stutter product, or an allelic peak formed from a minor contributor in a mixed DNA sample. Laboratories often quantify the percentage of stutter products, as the ratio between stutter peak height and corresponding allele peak height. On studying the alleles from the standard thirteen loci in the CODIS, it was found that while each locus has a different amount of stutter product formation, the longer the allele for an STR is, the greater is the degree of stutter formed. Finally, it was also noted that stutter percentage for tetranucleotide repeats is less than 15% for standard conditions of amplification [13].

Stutter formation may be reduced by using STR markers that have longer repeat units (which have variations on the common repeating strand), with alleles that have imperfect repeat units, and by using polymerases that process faster, as a faster polymerase can copy both the strands of DNA before they could come apart and re-anneal out of turn during extension [13].

Non-template additions form PCR products that are one base pair longer than the actual template strand. This happens when the DNA polymerases add an extra nucleotide to the 3′ end of the amplicon as the template is being copied. For example, in the case of Taq polymerase, an extra adenosine is added in what is called *adenylation*. In partial adenylation, the amplicon does not have an extra adenine at the start of the sequence. These differences contribute to a peak broadening if the resolution capacity of the system is poor. Furthermore, varying adenylation across sample can cause an inaccurate sizing and genotyping of potential microvariants. For these reasons, it is preferred to have all amplicons either with or without an extra adenine, and not a combination of both [13].

Microvariants are those alleles which have a sequence variation of any kind, as compared to alleles that are commonly observed. These may differ at DNA markers by one or more base pairs and are called so because they only slightly vary from full

repeat alleles. Since microvariants do not size the same way as alleles present in the reference allelic ladder, they are also called ***off-ladder*** alleles [13].

Suspected microvariants are easy to spot: while observing a heterozygous sample, one allele will line up with the respective fragment size on the allelic ladder, and one will not. The relative size difference between the questioned sample and an allelic ladder marker run in the exact same conditions (reference) will then confirm if the allele is actually a microvariant or not [13].

When a DNA template exists for a particular allele but fails to replicate in the amplicon during PCR, a ***null allele*** or ***allele dropout*** occurs. Sequence changes are known to occur either within the repeat sequence, in the region that flanks the repeat sequence, or where the primer binds. If a base pair change occurs in the template while the PCR process is on-going, primer hybridisation can be hindered and amplification fails. This causes a failure to detect and replicate an allele in the template DNA. This, however, is extremely rare as the flanking sequence surrounding the STR repeat is known to be quite stable [13].

6.9 Y-Chromosome Testing

The evidentiary value of the Y-chromosome lies in the fact that it is found only in males, as maleness is determined by the *SRY (sex-determining region in Y chromosome) gene*. Given that most of the reported cases of sexual assaults involve men as perpetrators, Y-STR testing becomes handy in testing DNA samples in cases wherein autosomal DNA testing has limited use. This can be seen in cases where the perpetrators are aspermic or have had a vasectomy (thereby nulling sperm content) or in the cases of gang rapes, where the number of perpetrators (and thereby the number of contributors in a sample of mixed DNA) are more than two. Primers specific to the Y chromosome when used in PCR improve the chances of detecting trace amounts of the criminal's DNA and can also be used to verify men deficient in the amelogenin gene [13].

As much as it is a boon in forensic science, the degree of maleness is also a bane for the field. This is because the Y chromosome (the bulk of it, at least) is directly passed down the male line in generations, without variation or recombination. The only source of a change comes from mutations, which occur rarely. Thus, while the Y chromosome can match a suspect to the scene of a crime, it can only point towards blood-related men, and not just one individual. This means that the sample that originated from the suspect will also match his father, brothers, cousins, uncles, and so on. This is why while the Y-STR testing can be a useful tool, autosomal tests for DNA typing always take first priority. And while there is always a sense of uncertainty with regard to finding one person involved in a crime, many relatives having the same Y chromosomes increases the reference sample pool when it comes to identifying a victim of a mass disaster. It also helps when a paternity test needs to be carried out, but the father in question is missing or deceased [13].

There are two main categories of DNA markers that are adopted in use of looking for diversity/variation in Y chromosomes. The first is of *bi-allelic markers* that

include single nucleotide polymorphisms (Y-SNPs) and an *Alu element insertion* (a short strand of DNA that is characterised by the action of *Arthrobacter luteus*), of which the latter was discovered first. These are also called *unique event polymorphisms* since they have a very low mutation rate [13].

The second category is of the *multi-allelic markers* which consist of two minisatellites and over 200 Y-STR markers. Since they have a high mutation rate, these are used to differentiate Y-chromosome haplotypes with a higher resolution. Some Y-STR loci occur more than once on the chromosome, due to some regions being palindromic. When these loci are amplified during PCR, the primers end up producing more than one type of product, which may lead to confusing while determining the number of loci present. This may be mistankenfor the presence of two loci types on the Y chromosomes, when in fact there is just one [13].

Forensic scientists rely mainly on the presence of kits that are commercially available, in order to conduct tests. This is one of the reasons why most forensic science laboratories were apprehensive of testing Y-STRs during the PCR process. Kits available today amplifies not only the loci but also the amelogenin marker. This is essential as the presence of amelogenin confirms that the test has not failed on DNA samples from females since it will produce just one amplicon of the X chromosome. Added to this is the possibility of finding the concentration levels of male and female DNA from a mixed sample, which is sought from studying the amelogenin X and Y peak ratios. However, a quick consumption of PCR components occur when there is a high amount of female DNA, as the amelogenin primers will now have extra sites to attach to [13].

As with all DNA testing, a database is essential, as it serves the purpose of drawing an estimation at how rare the Y-STR profile in question really is. A lack of recombination between the Y-chromosome markers implies that the profile must be combined into a *haplotype* (a set of markers on a single chromosome) in order to search a database and determining rarity [13].

A Y-STR profile can be interpreted in three ways: there can be an *exclusion* as the Y-STRs do not match, and therefore do not have the same origin, an *inconclusive result*, because the data is insufficient to reach a conclusion, or it the results are not clear, or an *inclusion*, where the results from the questioned and reference sample have ample similarities to be considered to have originated from the same source.

Case Study: The Boston Strangler's Guilt Proved Half a Century Later [14]
It was the 1960s, and women across the capital of Massachusetts, Boston, became targets for a serial killer and rapist. By the time the spree was complete, eleven women were raped and strangled with the nylon stockings they wore during the attacks. The perpetrator was not identified for a long time and was soon nicknamed the Boston Strangler. The crimes had started five District Attorney's offices investigating the cases—such was how the locations of all eleven crimes were spread across the city.

In October of 1964, a woman was sexually assaulted by a person posing as a detective. The description given by this victim to the police led them to her assailant, a man by the name of Albert DeSalvo, and when his sketch was published, many

women came forward, claiming he attacked them as well. By this point, the police had yet to make a connection of the serial crimes to the assaulter.

While incarcerated, DeSalvo confessed to being the Boston Strangler to his fellow inmate, and once he was charged with rape, he gave detailed descriptions of the eleven crimes, reducing the mistaken count from 13. The police believed him, as though some parts of his confessions were inconsistent; he had divulged details of the crimes that had been kept away from the public. However, no physical evidence ever proved that he indeed was the Boston Strangler, and so he was given a life sentence for multiple rapes and thefts in 1967. After escaping from his hold at a hospital for the criminally insane, he was caught and placed in a high-security prison, where he was found stabbed to death in 1973. His killer/s were never found.

The last victim of the Boston Strangler, 19-year-old Mary Sullivan, was found brutally raped and then strangled in her apartment in 1964. Among the evidence was a maroon blanket her body was wrapped in, which bore traces of seminal fluid. This remains to be the only DNA evidence in this case of serial crime—six samples that were preserved by the Boston Police Crime Laboratory's lead forensic scientist, in hopes that a future scientific advancement could enable DNA to be matched to a suspect.

In 2013, members of the Boston Police tailed DeSalvo's nephew, Tim, to his workplace at a construction site, where they collected a water bottle used by him and left aside. Once the DNA sample was extracted from the water bottle, a Y-STR profile was generated and matched to the profile of the Y-STR from the seminal fluid found at the scene of Mary Sullivan's murder fifty years prior. The two profiles matched, and that was considered evidence enough to get a warrant to exhume Albert DeSalvo's remains from his resting place, in order to conduct proper autosomal testing. Since he was confirmed to have raped and killed Mary Sullivan, it is now widely assumed that this is evidence that DeSalvo killed the other women as well and is most likely the Boston Strangler everyone wanted to see the face of.

6.10 Mitochondrial DNA (mtDNA) Testing

Mitochondrial DNA (mtDNA) is the DNA that is found in the mitochondrion, an organelle where chemical energy from food is broken down into adenosine triphosphates. The human mtDNA was first sequenced by Fredrick Sanger in England in 1981. This sequence known as the *Cambridge Reference Sequence (CRS)* or *Anderson sequence* gave rise to the revised CRS version, which is now used as a standard reference to which all mtDNA profile reports are compared to [13].

For forensic relevance, human mtDNA is said to be passed down generations strictly from the maternal side of a family. This is because during fertilisation, the cellular components of the zygote are contributed to only by the egg, which is much larger than the sperm cell, that passes on just its nucleus. In the eventuality that sperm mitochondria is passed to the zygote, it is destroyed by the ubiquitin tag added

during spermatogenesis, which highlights sperm mitochondrion for destruction by the zygote's cellular machinery. Thus, a mother passes her mtDNA to her children and is therefore common in siblings and maternal relatives. This however, is not unique to an individual [13].

Like in the case of Y-chromosomal testing, mtDNA is helpful in solving cases of missing persons and identifying victims of mass disaster and can also help in crime solving, when autosomal DNA samples cannot be recovered.

An advantage of mtDNA typing over nuclear DNA typing is the fact that mtDNA has a high copy number per cell, which in turn increases its sensitivity. This proves important in cases where the nuclear DNA cannot be quantified due to its minute quantity, or is severely degraded [13].

When an mtDNA sample is extracted, care must be taken to perform the extraction in a clean environment, as this DNA type is extremely sensitive to contaminations. mtDNA is often extracted from material with less DNA to begin with, like hair, bones, and teeth. Since bones also undergo anthropological study, it is essential that the bone sample is taken in such a way that it does not hamper the main structure of the entire bone to be studied. For example, an analyst may cut the bone in the middle, instead of all the way through, in order to maintain the length of the bone. In the same way, physical examination of the hair strand sampled must be done prior to extraction of mtDNA, as this will destroy the hair strand in its entirety. Comparisons showed that hair from the head gives best results, as compared to that from pubic and axillary hair regions. [13]

Once extraction is complete, the HV1 and HV2 regions of mtDNA (hypervariable regions) are amplified by use of PCR and are the amplicons are then sequenced. The profile report is then compared to the revised Cambridge Reference Sequence, where dissimilarities are noted. The same procedure is performed for a reference sample, following which the dissimilarities of the samples with the revised CRS are compared to each other. Upon comparison, the results can be either of an inclusion, exclusion, or inconclusive (Fig. 6.6).

Case Study: The State of Tennessee Versus Paul Ware—Where mtDNA Was First Used as Evidence

In 1996, 27-year-old Paul Ware was suspected to have murdered a four-year-old child after having raped her. While all circumstantial evidence pointed to him, the defence counsel argued that the babysitter, another man in the house, committed the crime in drunkenness. He was found sleeping next to the body of the victim.

The girl's blood was not found anywhere on the suspect, nor was any seminal secretion found on her. But a small strand of red hair was found in the girl's throat during the autopsy, similar strands of which were also found on the bed at the scene of crime.

Mitochondrial DNA was sequenced and profiled from four sources—the hair strand found in the victim's throat, the strands found in the bedroom, saliva swabs taken from Paul Ware, and the victim's blood. Comparisons showed that both hair samples not only matched each other, but their mtDNA was found to be the same as Paul Ware's. No match was found between the hair strands and the girl's blood.

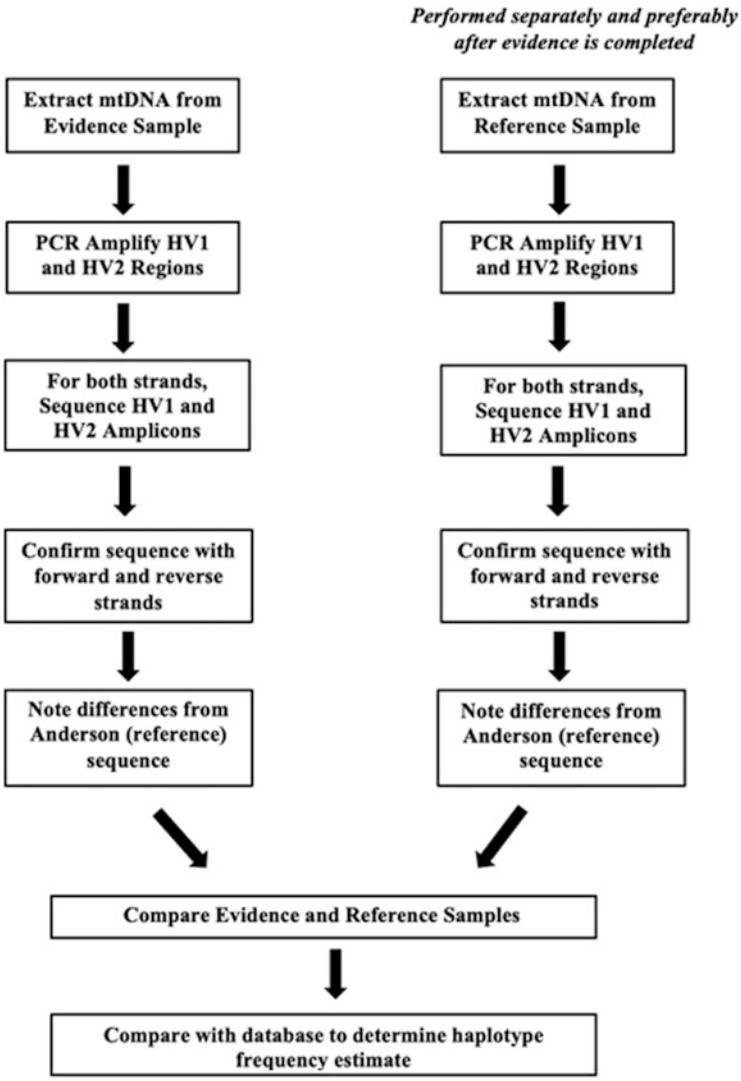

Fig. 6.6 Evaluation a sample of Mitochondrial DNA [13]

Currently, the FBI database has mtDNA sequences from 742 individuals. The sequence obtained from Paul Ware and his hair strands had never been seen before.

6.10.1 Non-Human DNA Testing

While most cases requiring a DNA analysis report is human-based, there are times at which DNA samples taken from non-human sources prove to be the most crucial in

an investigation. Pet animals are almost always near humans, and trace evidence transfer is inevitable. Plant DNA can link a deceased to a crime scene or a living person to a drug syndicate. In more serious situations, the now prevalent threat of bioterrorism needs DNA analysts to be vigilant over microbial DNA from time-to-time.

Animal DNA evidence can be found in such situations where the animal is a victim, where the animal is a perpetrator, or when the animal is a witness [13]. When a pet animal is abducted or abused, the victim's DNA sample can help locate where the pet is being held hostage. In cases of animal bites, saliva traces on the bite mark may prove sufficient to pinpoint which animal bit the human [13].

Case Study: How the Family Cat Helped Incriminate a Killer [15]
In 1994, Shirley Duguay went missing. She supposedly left her husband and three children and ran away. Nearly a year later, she was found dead in a shallow grave near her home in Prince Edward Island, Canada. Along with her remains was a jacket with her blood and many strands of feline (cat) hair scattered across the jacket. While interviewing her husband, Douglas Beamish (whom she had separated from), the investigators found out that the victim lived alone with her cat, Snowball, who had white fur. On a hunch, they confiscated Snowball and drew blood from it in order to have its DNA type identified. The investigators faced an issue, though: no crime lab ever tested animal DNA before. So, they got in touch with the Laboratory of Genomic Diversity, a laboratory that specialised in genetic diseases, and were able to devise a method to test the cat's DNA. They further solidified the testing by putting the same method to use on twenty random stray cats found on the island. This was done in order to rule out the chances of a common ancestor or relative of Snowball having deposited its fur on the jacket and to check if all cats on the island had the same ancestor. Eventually, the DNA of the fur on the jacket matched Snowball's DNA. Douglas Beamish was eventually convicted of the murder and sentenced to life imprisonment. This case was the very first to have tested for animal DNA to solve a crime against a human being.

When it comes to plants, they prove to be important when a cross transfer during a crime may have occurred or when illicit drugs need to be tested for. In the first scenario, if the plant part/grass is very rare, pinpointing the crime scene becomes crucial but was an easy task. This was the case in 1993, when two seedpods from the Arizonian Palo Verde tree was found in the back of a pickup truck and used to place a murder suspect at the scene of crime [13]. Upon DNA testing of the seedpods, it was found that out of twelve trees tested in the area, only one tree's DNA provided a match: the one under which the victim's body was found. Thus, this evidence was heavily used against the accused to get a conviction.

A sensitive test for DNA of *Cannabis sativa* (marijuana) was developed by the Connecticut State Forensic Science Laboratory [13], given that marijuana is the most commonly abused drug in the USA. This test can help link an individual to a sample, which in turn can help nab illegal cultivators and bring down a supply network. Unfortunately, this has limitations too, as if the marijuana is cultivated by means of

"cloning" (wherein a plant part is rooted directly in soil to propagate faster) instead of by seed, many samples will have identical DNA profiles [13].

Marijuana DNA is tested either by *randomly amplified polymorphic DNA markers (RAPDs)*, *amplified fragment length polymorphisms (AFLPs)*, or *short tandem repeats (STRs)*.

RAPD analysis uses short PCR primers which have random sequences that are roughly 8–15 nucleotides. These primers anneal too many regions in the given sample genome, thereby creating complex products of PCR. This prevents interpretation of mixtures that kill the reproducibility quality of this method of marijuana DNA testing. It also is tedious to perform, as the amplification conditions require thermal cycler ramp speeds [13].

In comparison, patterns formed from AFLP analysis can reproduce faster. AFLPs are generated by first cutting the double helix with more than one restriction enzyme, following which specific adapter sequences are ligated to the cut sites. PCR amplifiers that recognise the adapters are used to amplify the fragments of DNA that are variably sized, which are then sifted through by electrophoresis. As a result, one can observe a series of peaks in the 50–400 base pair range that can be scored and compared to other sample results by help of computer software. An advantage to this method is that even highly in-bred plants can be distinguished through their AFLP patterns [13].

Just like human STR markers, marijuana STR markers are also highly polymorphic, specific to unique sites on the genome, and can undo mixtures. There are many STR markers developed for marijuana analysis *(Gilmore et al. 2003)*. In 108 samples tested, a hexanucleotide repeat marker showed repeat units, and amplifying primers did not produce ant cross-reactive amplicons in 20 other samples tested *(Hsieh et al. 2003)* [13].

While all these methods do what is seemingly impossible, there is a lack of concrete databases to relate these findings to. These would sufficiently help law enforcement in making better comparisons, and delivering justice faster.

6.10.2 Microbial Forensics and Bioterrorism

With the onset of bioterrorism, a type of terrorist activity of hazardous substances of biological origin (mainly infectious microbes), there is a new-found urgency to equip DNA and forensic science laboratories to battle with a new era of crime solving. It is crucial to have a method of testing that is highly sensitive and specific that can measure components fast, that has portable apparatus, and that, obviously, give reliable results [13].

The challenges faced are humongous: one has to first identify the organism/agent, gather evidence, and trace the source of the agent, all the while being at a risk to contact the disease while doing the aforementioned tasks, while striving to maintain the chain of custody without contaminating the evidence and environment [13]. There also is a need for well-equipped databases that house information of the species studied and their virulent strains, along with reference material needed to

draw a comparison. Validation testing is required to rule out false positives and negatives as well [13].

Comparative genome sequencing looks to be a great tool for investigating outbreaks as was used in the whole genome testing of anthrax and will most likely be the case in the latest COVID-19 outbreak as well.

Case Study: The 2001 Anthrax Attack [13]
Merely a month after the horrifying September 11 Attacks in the USA, the country was faced with another threat. In October 2001, various news agencies and government offices received anonymous letters with the deadly Anthrax spores laced on the front side of the envelopes. These letters came mainly from the centralised postal service. The end result included 22 positive cases of Anthrax, five deaths from the disease, and a nation full of fear to do a seemingly mundane task: open their mail. In the years that followed, more than 125,000 samples were collected and processed by the FBI in an effort to get to the perpetrators. Yet, no charges were ever pressed, and in the ensuing chaos of nursing to health a post-9/11 America, the Anthrax attack case ran cold.

As is the case with most novel means of evidence testing, most courts do not readily accept evidence from non-human DNA testing. This is coupled with the issues of checking the validity of the scientific theory, validity of statistics used, along with the approval of the relevant scientific community prove that a long distance is yet to be covered in the practice. It also is a problem when finding a reference sample to match findings with take time, and a means to make a match may not even be readily available at the time of investigation. Finding appropriate experts to verify and cross-check the application may also be a big hurdle to cross [13].

6.11 Issues with Forensic DNA Evidence

A forensic science laboratory always has to deal with evidence that are less than ideal—body fluids that are contaminated, samples with degraded DNA, and so on. In most cases, a small taskforce ensures that cases are opened months or even years, after the samples were first collected. Improper collection of evidence often results in wet surfaces getting mouldy, thereby destroying the chances of proper DNA testing of the body fluid in question. Mostly, improper evidence results in problems for DNA sampling and analysis. There are also issues related to features of the testing instrument that may inhibit proper results. Some of the issues faced while handling a DNA based-evidence involves the following:

6.12 Degraded DNA

When left in the environment, DNA rapidly degrades to smaller fragments. While moisture causes decomposition to occur faster, presence of nuclease enzymes will cause natural chewing down of DNA strands. Older DNA profiling methods were not able to detect small fragments of DNA properly, as the molecules must have high molecular weight to detect, say VNTRs by use of the RFLP technique. While high-quality DNA typically has a molecular weight of 20,000 base pairs, degraded fragments of DNA appear as a mere smear, that clearly weigh much, much less. This is why PCR (especially multiplex PCR) is absolutely essential for the DNA profiling process, as very minute fragments can be amplified and brought to the required molecular weight. However, for the process to occur smoothly, the DNA strand surrounding the STR region must be entirely intact, so as to facilitate proper annealing of the primers. Should there be a break in the middle of the strand, the extension process will also end at that particular beak [13].

It is, therefore, best to use STR markers in PCR as it can easily be amplified with small size of product, along with the fact that there is a higher chance that the primers will find an intact strand for multiplication. Furthermore, a limited range of STR alleles are beneficial for degraded DNA samples because both alleles in a pair are identical in size, thereby preventing preferential amplification to occur. [13]

Another way to solve the issue of degraded DNA sampling is by the use of mini STRs. According to *Wiegand and Keliber (2001)*, highly degraded/low amount of DNA can be successfully multiplied by using STR primers that are very close in space to the repeated region, such that smaller but more concise products may be generated. While this is beneficial in its own right, there are many disadvantages as well, the major of which being that multiplex PCR may not proceed at optimal capacity, since small primers limit the loci that can be simultaneously amplified. Also, since different primers are used with the mini STRs, there is a chance of allele dropout from the primer binding site. Yet again, in rare occurrences, a point mutation may occur outside the region flanked by the primer, causing an undetectable change in the PCR products. Regardless, with the help of proper study, experimenting, and care, the use of mini STRs will be very beneficial in forensic science laboratories in the near future. [13]

6.13 PCR Inhibition

Another obstacle faced in processing DNA evidence is the fact that PCR amplification may be hindered by certain inhibitors present in the samples. Often it happens that a sample of blood or semen is found in soil, or on wood, or on wilted leaves if the scene of crime is outdoors. These contaminants may also get extracted along with the genetic material in the evidence. Non-DNA-containing items, like dyes, leather, fibres, and so on may, contain DNA polymerase inhibitors.

These inhibitors can either interfere with the cell lysis process that is essential for DNA extraction, interfere with degrading the nucleic acid present in the sample, or

hamper polymerase activity that in turn will hamper enzyme-based amplification of the DNA sequence. As a result, either some alleles from the loci are lost, or the entire loci may fail in replication altogether, which in turn causes production of partial DNA profiles which look as though they are of degraded DNA samples. This can be corrected by the use of mini STRs as smaller sequences can be amplified much more efficiently than larger ones [13].

As a solution to the inhibition problem, the sample may be diluted in order to dilute the inhibitors (in turn minutely reamplifying the DNA), or excess of Taq polymerase may be added, so that it can bind to the inhibiting molecules and eat them out, while the excess molecules can aid in the amplification process as usual. Another solution consists of adding additives, like bovine serum albumin or sodium hydroxide that can neutralise inhibitors of the polymerase. Lastly, a separation step may be introduced before PCR to separate DNA from inhibitors with the use of agarose gel plugs [13].

6.14 Issues Related to Contamination

To *contaminate* is to accidentally transfer DNA. There are three possible ways in which contamination during PCR may occur; one is when the sample itself is contaminated with genomic DNA from the environment (which is entirely dependent on how the sample is collected, and how much care is exercised to prevent contamination), second is when samples get contaminated with each other in the preparation phases, and lastly when a sample is contaminated with amplicons of a previously performed polymerase chain reaction. The latter two can easily be avoided with proper laboratory procedures being adopted [13].

Laboratory-based contamination can be cross-checked by running a negative control/blank sample in parallel with the evidence, keeping all reagent parameters across both samples the same. Presence of PCR products in the blank sample is indicative of contamination, and the source must be found and omitted [13].

Contamination can adversely affect a case and its end results, though very rarely seen. In practice, most laboratories are very careful when it comes to processing evidence; so much so that all pre- and post-PCR formalities are performed as far away from each other as possible. It is usually during the collection process where carelessness is observed. Supposing a police officer has handled evidence without the use of gloves, his/her DNA gets mixed in the sample and may mask the DNA of the actual culprit, which may lead to a wrongful declaration of innocence. This adds to the pressure in laboratories as the sample is now categorised as “mixed” and will be analysed as such [13].

6.15 Mixed Samples and Their Analyses

A *mixture* occurs when there are more than two contributors of DNA in a single sample. These can be observed in cases of gang rapes, where the DNA of multiple assailants gets mixed with that of the victim, or when an injury causes a murder accused to bleed onto an already bleeding victim. As discussed above, a seemingly simple sample may become a mixture if not handled with care by police officials.

Mixtures—as the name suggests—are complicated to analyse and undo without experience and training. With progress in technology, PCR sensitivity has increased and along with the help of fluorescence detection can help spot minor components in the DNA profile. Statistical calculations to interpret mixtures have been thoroughly studied and are now in use in various laboratories across the USA [13].

In order to increase the chances of mixtures being detected, more loci and genetic markers with high count of heterozygotes are used. The degree of detection of varied DNA sources is directly related to the contributing percentage of DNA from each source, the specific combinations of genotypes, and the total amount of amplified DNA. Due to these parameters, all mixtures cannot be assessed using the same scale, as these vary from one to the other. By using highly polymorphic STR markers with more alleles, there is a greater resolution between two DNA sources. The more markers are examined in multiplex PCR, the higher is the chance of seeing multiple components in a mixture. The quantity, too, vastly affects detection. There is a higher chance of differentiating between two components that have a roughly equal ratio, as compared to if one is fairly larger than the other. Usually, components below 5% concentration go undetected. Peak heights from electropherograms or fluorescence scanners are studied to quantify the components of a mixed sample, as well as to determine the possible genotypes of the sources of the mixed sample [13].In order to determine whether a sample in question is a mixture or not, the answers to the following questions are sought after:

- Do any of the loci show two or more peaks in the allele size range?
- Is there a severe imbalance of peak heights between heterozygous alleles in a locus?
- Does the “stutter product” appear unusually high?

Should the answer to any of these be in the affirmative, it can be deduced that the DNA profile being studied has come from a mixed sample. Usually, a mixture is first identified by the presence of more than two peaks at one or more loci. At the same locus, sample with DNA from multiple sources can exhibit one to four peaks due to varied genotype combinations. When contributors share more than one allele, they become masked and the genotypes may not be distinguished easily. However, by examining the profile at loci where there are no shared alleles, it becomes easier to find a contrast between the contributors [13].

Once it has been determined that the sample being examined is a mixture, the next step is to designate the allele peaks, a process that becomes complicated with the presence of stutter products and other artefacts that emerge on an electropherogram.

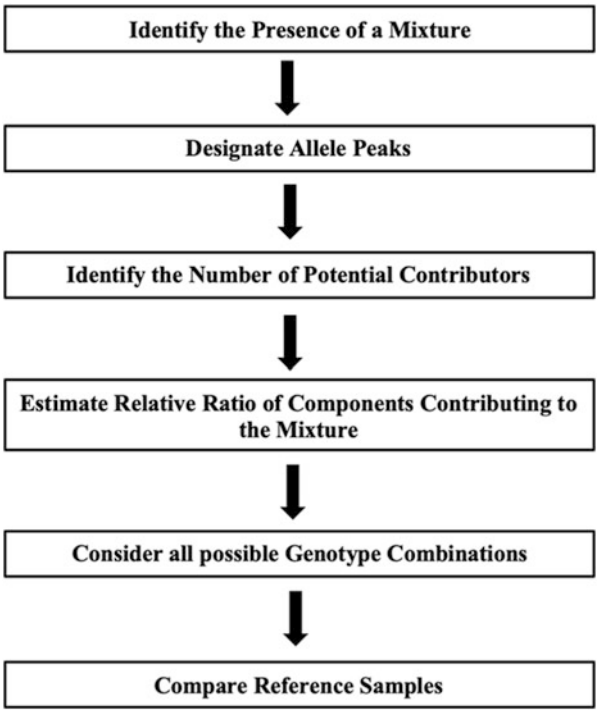

Fig. 6.7 Interpreting a mixture [13]

It is not always possible to exclude stutters since they are allelic products that differ by just one repeat unit. Generally, a stutter product is identified as being one repeat unit less and smaller than 15% of the area of the allelic peak. Still, confusion surrounding whether to consider a peak as a stutter product or an allelic peak gives rise to a bias within the examiner which may lead to an incorrect interpretation [13].

Next in line is to identify the number of contributors, as well as figuring out the ratio of contribution. The more the contributors in varying amounts, the more complicated the deciphering process. Studies have shown that the contributing ratios do not get affected by the PCR process *(Gill et al. 1998; Perlin and Szabady. 2001)*. In this way, peak areas and heights in an electropherogram can be related directly to the amount of DNA of each contributor in the mixed sample [13] (Fig. 6.7).

Once the ratio is estimated, genotype combinations have to be considered and drawn. Peaks that represent an allele are named alphabetically, starting from "A". Using the peak areas as reference, the genotype pairs are formed. Using the ratio estimate, along with the possible allelic pairing, the major and minor contributors can be estimated [13].

Finally, the genotype profiles are compared for possible contributors with the genotypes of reference samples provided. References can come from the victim or the accused. Once a match is found, the person cannot be excluded as a contributor to the mixed sample [13].

6.16 Conclusion

Biological evidence can be found in a plethora of crimes that are committed, and the use of forensic biology—study and testing—to analyse these evidence found and solve cases has proven to be a huge boon for the criminal justice system. However, the evidence found is only as reliable as the methods one would use to analyse it. If these procedures are not precise, or are done in a contaminated environment, it not only wastes a chance at analysing a minute quantity of sample but also wastes away kits and material that are quite expensive.

An improper collection, preservation, and handling of evidence has often caused a court to put forward a sentence that is contrary to facts put forward—innocent people have been wrongfully convicted, while culprits have gotten away scot free. The most famous of these cases, by far, is the OJ Simpson trial, where the defendant was given an acquittal solely on the fact that the police messed up the protocol that had to be followed while collecting a sample of his blood. The defence counsel argued that an entire vial of blood was collected, which could have been used to plant numerous evidence found at the scene of crime.

The onset of DNA analysis has helped many wrongfully incarcerated persons to be exonerated for the crimes they were held accountable for in an era where DNA testing did not exist. As many as 143 people (as of May 2004), most of whom were on death row, were proven to be free of any wrongdoing by help of advanced technology in DNA analysis. Cold cases, too, have been solved. Such was the case of Melanie Road, a 17-year-old who was raped and murdered in 1984. Her killer, Christopher Hampton, was caught and tried only in 2015—30 years after the crime—when his daughter got involved in a minor assault and her DNA profile made it to the CODIS (Combined DNA Index System), from where it partially matched with the DNA taken from Melanie's clothing, thereby proving that no crime can be perfect.

Despite these rays of hope in a pitch-black sky, there are many cases and evidence that are yet to see the light of a DNA analysis laboratory. Many sexual assault kits that are collected from a victim of rape have not been tested for DNA evidence, and are just tossed in a corner where the samples continue to degrade. There is an increased problem of funding as well, as most of the funds are put on high profile cases, leaving no equipment or funding to analyse the rape kits that keep coming in to police precincts. The same is also true for other cases which have run cold, have no record on the CODIS, nor have enough evidentiary samples, as those collected in the wake of the crime have severely degraded.

However, the biggest issue forensic science faces as a whole is what is known as the *CSI effect* that has stemmed from crime procedural shows like CSI: Miami, Criminal Minds, and Castle. The over-exaggeration of scenes portraying testing of evidence, such that a "100% match" is found by the databases, has made the courts—judges and jury—to incorrectly believe that anything less than a cent percent is not a match. This has caused jurors to ask for more evidence or rather higher matching evidence, thereby raising the standards to an extent that is just impossible to achieve. The credibility of circumstantial evidence too has lowered

due to the CSI Effect. In practice, it is common knowledge that there is always a high chance of a match with reference material in a database, and there is always room and scope for error. The portrayal of perfect matches that are obtained by typing a few keys on a computer are just facades of a good dramatic element in television.

As of today, *forensic genetic genealogy* has gained a lot of traction. The study stems from multiple companies running genealogical tests that can help people find their ancestry and pinpoint the start of their family trees. Most of the information stored in the databases of such companies have no scientific backing, except for the colour of hair, skin, and eyes. Though only recently emerging to be forensically relevant, a DNA profile uploaded on personal genomics website GEDMatch help nab Joseph DeAngelo, the Golden State Killer in 2018. He is known to have committed 13 murders, 50 rapes, and 120 burglaries between 1973 and 1986. Access to these databases is limited, given that these are run by private firms, and not by law enforcement.

As crime continues to increase and criminals become more aware of not leaving evidence behind, forensic science will evolve in order to continue putting justice at the heart of every scientific method followed. In a sense, forensic biology is a type of passport for biological evidence. The destination, however—either a courtroom as evidence or in a dull and dusty room while losing potency—depends on law enforcement officials at the crime scene and scientists at the crime laboratory.

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