Non-STR DNA Markers: SNPs, Y-STRs, LCN and mtDNA

This course is provided free of charge and is part of a series designed to teach about DNA and forensic DNA use and analysis.

Find this course live, online at:
http://dna.gov/training/markers

Updated: October 8, 2008

DNA INITIATIVE

www.DNA.gov

About this Course

This PDF file has been created from the free, self-paced online course "Crime Scene and DNA Basics for Forensic Analysts." To learn more and take this and other courses online, go to http://www.dna.gov/training/online-training/. Most courses are free but you must first register at http://register.dna.gov.

If you already are registered for any course on DNA.gov, you may login directly at the course URL, e.g., http://letraining.dna.gov or you can reach the courses by using the URL http://www.dna.gov/training and selecting the "Login and view your courses" link.

Questions? If you have any questions about this file or any of the courses or content on DNA.gov, visit us online at http://www.dna.gov/more/contactus/.

Links in this File

Most courses from DNA.Gov contain animations, videos, downloadable documents and/or links to other useful Web sites. If you are using a printed, paper version of this course, you will not have access to those features. If you are viewing the course as a PDF file online, you may be able to use some of these features if you are connected to the Internet.

Animations, Audio and Video. Throughout this course, there may be links to animation, audio or video files. To listen to or view these files, you need to be connected to the Internet and have the requisite plug-in applications installed on your computer.

Links to other Web Sites. To listen to or view any animation, audio or video files, you need to be connected to the Internet and have the requisite plug-in applications installed on your computer.

Legal Policies and Disclaimers
See Legal Policies and Disclaimers for information on Links to Other Web Sites, Copyright Status and Citation and Disclaimer of Liability and Endorsement.

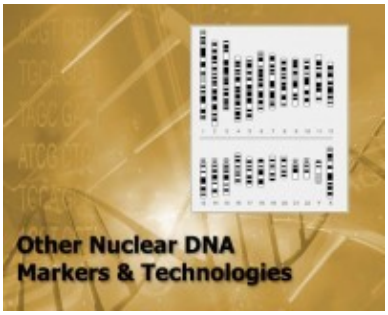
Non-STR DNA Markers: SNPs, Y-STRs, LCN and mtDNA

This course provides information in the two lessons.

Other Nuclear DNA Markers and Technologies. Learn the basis of single nucleotide polymorphisms, including the advantages and disadvantages of their use in forensic genetics; the molecular genetics of the Y-chromosome and the applicability of Y-STR analysis in casework; the strategies used to analyze low copy number DNA; and the range of species and genetic markers used in non-human DNA analysis.

Mitochondrial DNA—Non-Nuclear DNA Markers. Learn to recognize the biological functions and contributions of mitochondria, the different features of nDNA and mtDNA, the different test techniques for mtDNA, the significance of mtDNA results for forensic and identity casework, and the current and future mtDNA testing technologies.

Introduction



It is a routine for the forensic scientist to obtain a genetic profile of an individual from nuclear DNA recovered from a biological stain deposited at the crime scene. Potential contributors of the stain must either be known to investigators and compared to a known profile or searched against a database of DNA profiles, such as those maintained in the Combined DNA Index System (CODIS).

This module describes various techniques, including single nucleotide polymorphisms (SNPs), Y-STRs, low copy number (LCN), and non-human DNA. These methods can be used to obtain more probative investigative information and may be used as an adjunct to standard autosomal short tandem repeat (STR) analysis. In some cases, information is provided that otherwise could not be obtained by standard techniques.

Certain factors may preclude the development of genetic profiles despite the presence of probative biological material in a crime stain because:

- The quantity of DNA present is below the detection limit

- and/or -

- The DNA is partially degraded, such that the number average molecular weight is less than the STR amplimer sizes

Potential remedies include:

- Low copy number (LCN) analysis techniques for minute quantities of DNA
- SNP typing for partially degraded DNA

In mixed male/female specimens, Y-chromosome specific systems are invaluable for the identification of the genetic profile of the male component in the following instances:

- The female portion is present in overwhelming quantities relative to the male

- The standard autosomal STR analysis fails to yield the male donor profile

In cases where there is no developed suspect and no match with a database sample as yet, the DNA profile presently provides no meaningful information to investigators, with the notable exception of gender determination. In these situations, it would be advantageous to law enforcement investigators if additional information could be obtained from the biological stain. SNP analysis using specially selected markers can be predictive of ethno-geographic ancestry; this is expected to play an increasing role in the investigation of crime.

Another area that will contribute to the crime scene investigation is non-human DNA analysis. As techniques are developed they will become more commonplace in forensic biology.

Objectives

Upon successful completion of this unit of instruction, the student shall be able to:

- Understand the basis of single nucleotide polymorphisms, including the advantages and disadvantages of their use in forensic genetics
- Understand the molecular genetics of the Y-chromosome and the applicability of Y-STR analysis in casework
- Know the strategies used to analyze low copy number DNA
- Describe the range of species and genetic markers used in non-human DNA analysis

Single Nucleotide Polymorphisms (SNPs)

The most common form of genetic variation in the human genome (approximately 90%) is a class of genetic marker known as a single nucleotide polymorphism (SNP). As its name suggests, this class of variant is characterized by alternative deoxyribonucleotides at specific chromosomal sites.

For example, a particular SNP could be represented as ATTGCG[C/T]GATTCG in which the SNP is characterized by a C and a T allele embedded in non-variant flanking sequence. SNPs can be present in coding, regulatory, and intronic sequences within genes or in intergenic regions.

Population studies from various populations indicate that SNP sites (defined as sites of variation present in at least 1% of individuals) occur every 300-500 base pairs along the genome.01 It has been estimated that approximately seven million SNPs, exist, with a minimum allele frequency (MAF) of 5% across the genome, and an additional approximately four million occurring with a MAF of 1%.01 There are also innumerable single base variants that exist within a single individual. A number of studies indicate that the amount of variation present in the human genome is somewhat reduced due to substantial linkage disequilibrium between closely linked SNP markers, in effect creating haplotype blocks separated from one another by recombination hotspots.02

Read more about contamination in the DNA Amplification for Forensic Analysts PDF file.

SNPs initially arise via rare spontaneous mutations (approximately 10^{-8} per base pair per generation) and attain an appreciable population frequency by genetic drift and other evolutionary forces, such as selection. The spontaneous mutation rate is so low that the chances of a further mutation at the same site on the same

individual chromosome (either reversal to the original base or conversion to any of the other two) are negligible. Thus, most SNPs are *de facto* unique event polymorphisms (UEP) and are biallelic in nature with modest levels of heterozygosity in the population (50% maximum).

Read more about contamination in the DNA Amplification for Forensic Analysts PDF file.

Approximately 50-100 SNPs with a MAF between 20-50% would be required to provide a similar discriminating power to that afforded by the standard 13 CODIS STR loci, which have greater than 5 alleles per locus.03

SNPs in Forensic Genetics

Theoretically, the advantages of a SNP-based (Single Nucleotide Polymorphism) system include:

- Potential for automation
- Ability to analyze degraded DNA (with a number average molecular weight equivalent to the sum of the lengths of the two primers)
- Direct interrogation of genes whose variants have predictive consequences for a phenotypic trait

Disadvantages include:

- Difficulties with body fluid mixture detection and analysis
- Requirement for large numbers of individual SNPs
- Uncertainties about the availability and robustness of appropriate multiplex-capable analytical platforms

It is not likely that SNP typing will replace STRs (Short Tandem Repeats) as the principal method for human identification, but there are specialized instances in which this typing is warranted.

SNPs were the basis of the variation of the ABO and isoenzyme classic genetic markers used prior to the DNA revolution in forensic biology; the pre-DNA detection technology was based upon antigens and proteins.

Read more about contamination in the DNA Amplification for Forensic Analysts PDF file.

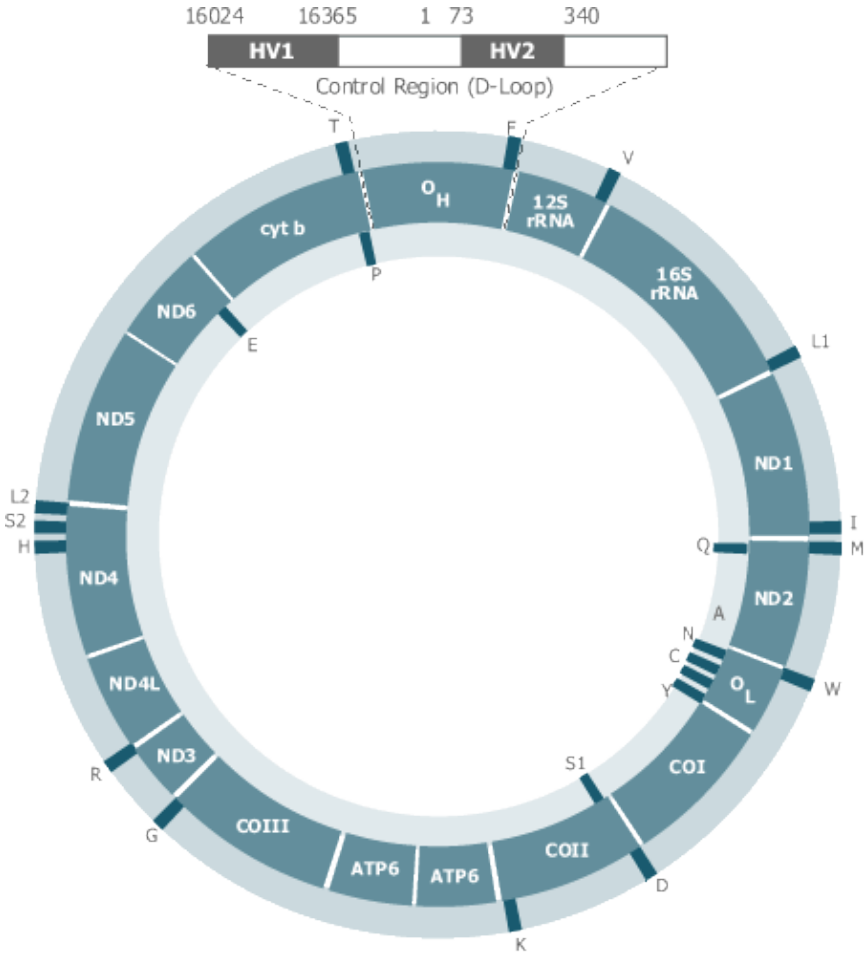
The first generation of PCR-based (Polymerase Chain Reaction) forensic kits employed the HLA DQAlpha1 and PM systems, which comprised a series of SNP markers detected by allele specific oligonucleotide hybridization using a reverse dot blot format.

Read more about contamination in the DNA Amplification for Forensic Analysts PDF file.

Applications

Mitochondrial DNA (mtDNA)

Typically, mtDNA is used to type samples such as teeth, bones, and hair that are normally intractable to standard STR (Short Tandem Repeats) analysis. The hypervariable control region of mtDNA used for typing contains a large number of linked SNPs (Single Nucleotide Polymorphisms) that are simultaneously determined by standard Sanger sequencing.



The coding region also possesses SNPs that can be useful in differentiating between samples that otherwise exhibit the most common Caucasian control region mtDNA haplotype. These coding region SNPs are less dense than in the control region and are typically typed using minisequencing/primer extension methods.

Physical Characteristics

Ethno-geographic ancestry can be predicted using a set of approximately 70 autosomal SNP markers.04 These ancestry informative markers (AIMs) use proprietary algorithms to partition an individual's AIM-SNP profile into sub-Saharan African, Caucasian, East Asian, and Native American components.04 Y-chromosome SNPs and *Alu* insertion polymorphisms (that are *de facto* biallelic UEP markers and can be analyzed by SNP methods) are also promising candidate markers for bio-geographic ancestry prediction.05,06

Eye color, contrary to popular genetics textbook explanations, is a complex trait determined by a number of interacting genes.07 The analysis of SNPs from these interacting genes or regions in close linkage with them can predict the eye color of a DNA donor.07

Degraded DNA

Identification of human remains from the World Trade Center disaster was aided by using a panel of approximately 70 autosomal SNPs whose amplimer lengths were greater than 100 bp and therefore suitable for degraded DNA specimens.08

SNP Typing Technology

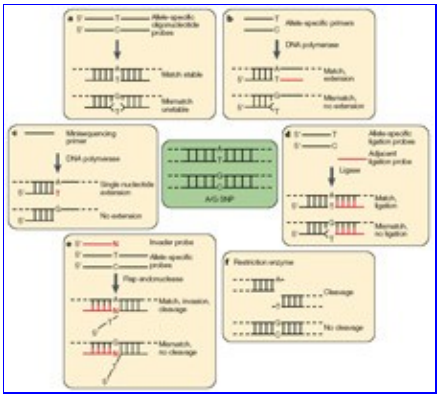
Spurred on by the need to type thousands of SNPs (Single Nucleotide Polymorphisms) for human disease association studies, a variety of technologies have been developed for SNP analysis. Most methods are amplification based and the SNP is subsequently detected by primer extension, oligonucleotide ligation, or hybridization of a probe to the amplified product (see figure below). In some ways, the best SNP-typing system is that of standard DNA Sanger sequencing, in which the SNP site and flanking sequences are simultaneously interrogated. However, such an approach is limited to densely packed SNPs, such as those present in the mtDNA control region. For the other SNP applications in which the SNP sites are separated by thousands (often hundreds of thousands) of bases, Sanger sequencing is neither practical nor cost effective.

The SNP analysis technology that has been used most productively for the coding region mtDNA, physical characteristics, and degraded DNA applications is based upon primer extension (or mini-sequencing) followed by capillary electrophoresis (CE) or solid phase capture.09 The SNaP shot™ assay (AB), which has been used for the mtDNA coding region analysis, uses a primer that abuts the SNP site, genomic DNA template, DNA polymerase, and fluorescently labeled terminator ddNTPs. The SNP site on the genomic template will determine, by complementary Watson-Crick base pairing, which ddNTP will be extended and incorporated into the primer. The now fluorescently labeled extended primer is separated by capillary electrophoresis, and the allelic status determined by the nature of the fluorescent dye detected. Multiplexing is achieved by designing primers of different lengths (or containing mobility modifiers) at the primers' 5' ends, such that each separate SNP in the multiplex has a characteristic mobility.

Read more on the SNap shot™ assay on Applied Biosystems website.

The SNPstream® UHT (Orchid Cellmark) is a primer extension methodology that has been used to predict bio-geographical ancestry and to analyze degraded DNA. This platform uses SNP extension primers that are modified at the 5' ends with oligonucleotide tags that are complementary to oligonucleotides that are bound to a solid microplate support. Each well of the microplate has 16 anti-tag sequences. Hybridization to it of a SNP primer extension reaction, previously carried out in solution, permits multiplex typing by location (determines the SNP locus) and bound dye (determines the allelic status).

Read more on the SNPstream® UHT on the Orchid Cellmark website.



Adapted by permission from Macmillan Publishers Ltd: Nature, copyright Vol. 2 December, 2001

SNP Analysis Case Example

The investigation of a series of five unsolved serial murders in southern Louisiana between September 2001 and March 2003 was aided by the use of AIM-SNPs. Prior to their use, psychological profiling had indicated the likelihood that a Caucasian male was the culprit. However, AIM-SNP analysis revealed that the killer was likely to be of African-American ancestry. Acting upon this lead, investigators eventually arrested an African-American suspect, Derek Todd Lee and tried him for the murder of Charlotte Murray Pace. Lee was subsequently linked by DNA evidence to seven other homicides from 1998 to 2003.

Y-STRs

Males commit the majority of violent crimes. For example, the U.S. Bureau of Justice Statistics reports that males commit about 80% of all violent crimes and 95% of sexual offenses in the United States. Many times autosomal STR (Short Tandem Repeats) markers are able to fully discriminate between unrelated individuals, but there are several circumstances in which Y-chromosome polymorphisms are useful in forensic analysis.

Molecular Genetics of the Y-Chromosome

The human Y-chromosome has often been considered an evolutionary relic of the X-chromosome. The Y-chromosome has retained the ability to dictate gender but has little other functional significance. Recent studies have demonstrated that it possesses numerous functional genes, including some that appear to be critical for normal male development.

Approximately 300 million years ago, the X- and Y-chromosomes were true homologues, comparable in size and genetic content.10 Through the passage of time, the Y-chromosome underwent a series of deletion mutations reducing it to its present size of approximately 50 megabases (Mg). This notwithstanding, significant X-chromosome sequence homology still persists.

The chromatin of the Y-chromosome exists in at least three functionally different forms including:

- Pseudoautosomal regions (PARs)
- Euchromatin
- Heterochromatin

The PARs, located in the telomeric regions of the chromosome, pair and recombine with the X-chromosome during male meiosis. The euchromatin (containing the functional genes) and the transcriptionally inert heterochromatin form the non-recombining region (NRY) of the Y-chromosome. Sequencing of the

euchromatic region has revealed a patchwork of three distinct sequence classes (see Figure 1).11

The X degenerate sequences are surviving relics from common autosomes from which the X and Y both arose and comprise 39% of contemporary euchromatin. Sequence similarity with the X-chromosome ranges from 60-96%. The X-transposed sequence comprises approximately 15% of the euchromatin sequence and consists of two blocks that are 99% identical to Xq21 sequences. This represents an ancient, massive single X to Y transposition event that occurred 3-4 million years ago after the divergence of the human/chimpanzee lineages. Approximately 46% of the euchromatin contains ampliconic sequences, which occur in seven segments that have as much as 99.9% sequence similarity to other sequences in the euchromatin. Amplicons are characterized by eight palindromic sequences, and the extreme sequence conservation has been attributed to intra-chromosomal gene conversion events. Thus, the Y-chromosome shares significant sequence homology with the X-chromosome, a factor that needs to be considered when designing Y-chromosome specific assays.12

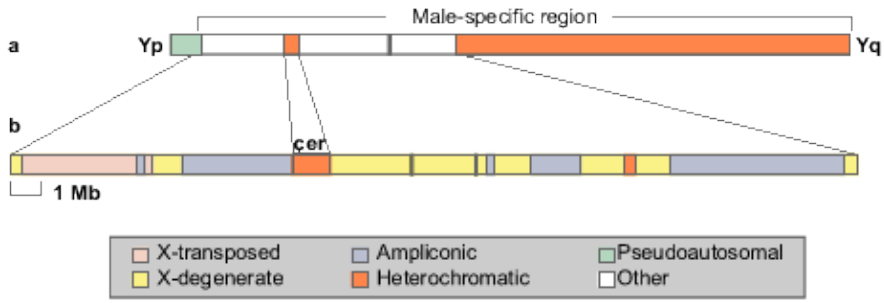


Figure 1
Adapted by permission from Macmillan Publishers Ltd: NATURE, Vol. 423, June 2003

The NRY region of the Y-chromosome is inherited in a patrilineal manner in which a haplotype of physically linked genetic markers is transmitted unchanged, barring the occasional rare mutation, from father to son (see Figure 2).

Reduced genetic variability results from:

- Non-independent segregation of genetic markers on the Y-chromosome
- Enhanced genetic drift potential (due to the smaller effective population size of the Y-chromosome – one-fourth that of autosomes)

Thus, significantly more Y-chromosome markers would be required to provide the same ability to discriminate individuals (the discriminating power) as that obtained by autosomal STR markers. It has even been suggested that this mode of inheritance could enable a particular Y-haplotype recovered at a crime scene to be equated with a surname, although the confounding effects of non-paternity and multiple independent origins of surnames would need to be taken into account.13,14 Notwithstanding the above, it is possible, and in certain circumstances advantageous, to make use of the unique biology of the Y-chromosome for forensic purposes.

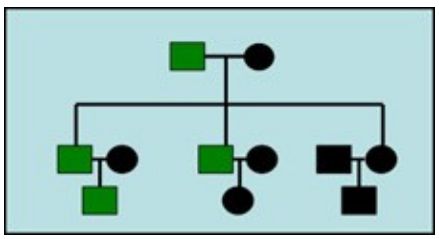


Figure 2
Adapted by permission from Macmillan Publishers Ltd: NATURE, Vol. 423, June 2003

Y-STRs in Forensics

Y-chromosome specific systems may prove invaluable for the identification of the genetic profile of the male component in mixed male/female specimens, specifically in those instances in which the female portion is present in overwhelming quantities, relative to the male.

Other instances where Y-STRs (Y-Single Tandem Repeats) may be useful are:

- Deposition of semen by an azoospermic or oligospermic males
- Cases of oral sodomy where only trace amounts of male buccal epithelial cells may be present
- Normal post-coital degradative and semen sample loss processes that occur with the passage of time
- Determination of the presence of the number of semen donors in cases of multiple perpetrator rape
- Criminal paternity analysis
- Disaster victim identification and/or missing person (male individual identified by typing a male relative such as a son, brother, father, nephew, or uncle)

In addition, the ability to specifically detect a male profile could obviate the need for the time-consuming and frequently inefficient differential extraction procedure for the separation of sperm and non-sperm fractions. Male specific systems may also aid in the investigation of cases involving mixtures or degraded DNA specimens (displaying partial autosomal STR profiles) by providing additional statistical discriminating power.

Y-STR Markers

A variety of polymorphic genetic markers have been identified in the euchromatin portion of the Y-chromosome, including a number of STR (Short Tandem Repeats) and SNP (Single Nucleotide Polymorphism) loci and a single hypervariable minisatellite locus. A number of candidate Y-SNP loci have been identified, but they suffer from a limited discrimination potential, and their implementation in forensic casework is dependent upon the development of additional markers and appropriately validated detection technologies. The hypervariable minisatellite, MSY1, is the most polymorphic single locus system found on the Y-chromosome but difficulties with the required minisatellite variant repeat (MVR) technique have discouraged its operational use. Y-STR loci, on the other hand, offer a number of advantages, including good discrimination potential, ease of analysis, and a number of available candidate loci amenable to multiplex analysis.

Although more than three hundred STR loci have been described on the Y-chromosome, a much more limited number have been appropriately evaluated for forensic casework use and some of these have presented a particular challenge for assay design. The Y-STR loci comprise di-, tri-, tetra-, and penta-nucleotide repeats with the di-nucleotides exhibiting the most polymorphism but an excessively high level of stutter artifacts.[15] Due to their evolutionary relatedness, many homologous sequences are found on both the X- and Y-chromosomes, which can confound the analysis of mixed male/female specimens. Additionally, some of the loci are bi-local in the sense that one or both of the primer sites and associated tandem repeats are duplicated upstream or downstream of the parent sequence. In these particular cases, two alleles are co-amplified, and there is some, but not complete, correlation between the alleles at both loci. Importantly, sample quantity limitations with forensic specimens require that candidate Y-STR loci be analyzed together in a parallel fashion by incorporating them into a multiplex PCR (Polymerase Chain Reaction) assay format, the design of which can be complicated by some of the aforementioned factors.

A major international multicenter study of 13 candidate Y-STR markers in 1997 resulted in recommendations for the use of nine core loci for standard forensic haplotyping (designated the minimal haplotype loci, MHL or minHt).16 The nine MHL loci include:

- DYS19
- DYS 385 (a)
- DYS 385 (b)
- DYS389 I
- DYS389 II
- DYS390
- DYS391
- DYS392
- DYS393

Subsequent to the development of the MHL loci, additional microsatellite loci were described that proved to have utility in forensic genetics. In January 2003 the Scientific Working Group on DNA Analysis Methods (SWGDAM) recommended for US forensic casework use a set of eleven core loci that included the MHL loci plus DYS 438 and DYS 439.

Read about the SWGDAM recommended core loci in the *Forensic Science Communications*, July 2004 publication.

Commercial kit vendors in the US have incorporated the SWGDAM core loci into their Y-STR multiplex systems (ABI, Promega and Reliagene), which comprise eleven (Reliagene's Y-PLEX-12TM), twelve (Promega's PowerPlex YTM) or seventeen (Applied Biosystems' Y-FilerTM) Y-STR loci (see Table). These kits have been fully validated for forensic use according to the SWGDAM developmental validation guidelines. The kits have been designed to perform with a high degree of specificity for Y-chromosome sequences in the presence of a vast excess of X-chromosomal DNA from a female, despite the previously described extensive sequence homology between both chromosomes.

Read the Revised Validation Guildelines by SWGDAM in July 2004.

Loci Comprising the MHL, SWGDAM Core Sets and Commercial Kits

Minimal Haplotype Loci	SWGDAM Core Loci	Reliagene's Y-PLEX-12™ AMEL	Promega's **PowerPlex Y™**	Applied Biosystems' Y-Filer™
DYS 19	DYS 19	DYS 19	DYS 19	DYS 19
DYS 385 a,b	DYS 385 a,b	DYS 385 a,b	DYS 385 a,b	DYS 385 a,b
DYS 389I	DYS 389I	DYS 389I	DYS 389I	DYS 389I

DYS 389II	DYS 389II	DYS 389II	DYS 389I	DYS 389II
DYS 390	DYS 390	DYS 390	DYS 390	DYS 390
DYS 391	DYS 391	DYS 391	DYS 391	DYS 391
DYS 392	DYS 392	DYS 392	DYS 392	DYS 392
DYS 393	DYS 393	DYS 393	DYS 393	DYS 393
	DYS 438	DYS 438	DYS 438	DYS 438
	DYS 439	DYS 439	DYS 439	DYS 439
			DYS 437	DYS 437
				DYS 448
				DYS 456
				DYS 458
				DYS 635
				Y-GATA-H4

Statistical Interpretation of a Y-STR Match

Since the Y-STR (Y-Short Tandem Repeats) loci are located on the NRY part of the Y-chromosome and are inherited as a block of linked haplotypes, estimates of the multilocus frequency cannot proceed by the product rule. Instead an estimate of the frequency of occurrence of a particular haplotype requires the counting method, which is based upon how many times a particular multilocus haplotype is observed in a particular database. This is akin to the procedure used to estimate mtDNA haplotype frequencies. The ability to obtain a reliable estimate thus depends upon the nature of the databases that are used. Specifically, the ethnogeographic composition of the database, the number of individuals deposited therein, and the number of searchable Y-STR loci are factors that should be maximized to increase confidence in the accuracy of a frequency estimate.

Read more about contamination in the DNA Amplification for Forensic Analysts PDF file.

A number of web-accessible, searchable databases exist. A Y-STR haplotype reference database (YHRD) was established by the Institute of Legal Medicine, Berlin in 2000 to generate reliable Y-STR haplotype frequency estimates for MHL Y-STR haplotypes to be used in the quantitative assessment of matches in forensic casework and the assessment of male population stratification among world-wide populations. This worldwide database extends to US populations and permits MHL and the SWGDAM loci searches. The principal commercial vendors of Y-STR kits support Y-STR databases that permit public access and searching using their particular Y-STR marker sets (See Table Below).

Visit the website for the YHRD.

Commercial Y-STR Databases

Company	Database Name
Applied Biosystems	YFiler Haplotype Database
Promega Corporation	PowerPlex® Y Haplotype Database
Reliagene Technologies Inc	Y-STR Haplotype Reference Database for U.S. Populations

Since the basis for the haplotype frequency estimation is the counting method, the application of a confidence interval is recommended to correct for database size and sampling variation. Reporting a haplotype count without a confidence interval may still be acceptable as a factual statement regarding observations in a particular database. If a confidence interval is applied, the following example calculation could be used (SWGDAM Y-STR Interpretation Guidelines). If the haplotype has been observed in the database, the formula for calculation of the upper 95% confidence limit in this case would be:

$$p + 1.96\sqrt{\frac{(p)(1-p)}{n}}$$

where p is x/n, n=database size, and x=number of observations in database. (Due to the enhanced population substructure effects of the Y-chromosome compared to autosomes, it may be necessary to apply an F_{ST} -like correction factor to this estimate, similar to the National Research Council-recommended for homozygotes. The appropriate studies to determine this are in progress.) If the haplotype has not been previously observed in the database, the formula for calculation of the upper 95% confidence limit in this case would be:

$1-(0.05)^{1/n}$

where n is the size of the database. Alternatively a simplified calculation could be 3/n. This value will be close to above formula, and for 95% of the time the real frequency will be less than that estimate.

Empirical studies have confirmed the non-linkage of Y-STR haplotypes and autosomal STR profiles. Therefore for those cases in which both autosomal and Y-STR profiles exist, it is possible to multiply the autosomal STR profile frequency with the upper confidence limit bounded Y-STR haplotype frequency to give an overall frequency estimate of the genetic profile obtained.

Read more about contamination in the DNA Amplification for Forensic Analysts PDF file.

Y-STR Analysis Case Example

Eight years after he requested postconviction DNA testing, Wilton Dedge was exonerated and released from Florida prison. In 1982, Dedge was convicted in Brevard County, Florida, of sexual battery, aggravated battery, and burglary. He was sentenced to two concurrent life sentences. The victim was attacked in her home on December 8, 1981. After coming home in the afternoon, she heard a sound while changing clothes and turned to find a man armed with a blade. He cut off her clothes and raped her vaginally and anally. During the assault, the perpetrator cut the victim all over her body. After the assailant left, the victim contacted her boyfriend and was taken to the emergency room, where a rape kit and the victim's clothes were collected. Wilton Dedge was convicted in May 1982 of this crime, despite his protestations of innocence, largely based upon eyewitness evidence. His conviction was reversed in 1983, but he was again convicted in August 1984. At trial, the prosecution relied on the victim's eyewitness identification, microscopic hair comparison, snitch testimony, and dog-sniffing evidence to secure the conviction. The prosecution's hair

expert testified that two pubic hairs were found on the victim's bed. One was similar to hairs taken from the victim. The other was from a male and, based upon a microscopical hair analysis, Dedge could not be eliminated as a possible source of that hair. The hair proved to be the only physical evidence linking Dedge to the crime. Although sperm were found on a swab in the rape kit, it did not yield the ABO blood type of the semen donor. In 1996, Dedge was one of the first Florida inmates to seek postconviction DNA testing, several years before the state passed its 2001 law providing for such testing. He won that motion in 2000, and, in June 2001, mitochondrial DNA testing proved that the pubic hair did not come from Dedge. After more legal wrangling, further testing was ordered by the Court of Appeal on the semen evidence found on the anal swab recovered from the rape kit. Initial testing had yielded only two markers because the sample was degraded. Y-chromosome STR testing was utilized in the final round of testing. The Y-STR results excluded Dedge as the contributor of the spermatozoa, conclusively proving his innocence for a second time.

100 Fifth Avenue, 3rd Floor
New York, NY 10011
info@innocenceproject.org

Low Copy Number DNA

Forensic casework items sometimes contain less than 100 picograms (pg) of template DNA, which, assuming 3.5 pg of DNA per haploid cell, is equivalent to approximately 15 diploid or 30 haploid cells. The presence of such low copy number (LCN) samples could be due to several factors including damaged or degraded DNA, oligospermic or aspermic perpetrators or from extended interval post coital samples, where sperm have been lost over time due to the effects of drainage or host cell metabolism. Trace biological evidence arising from casual handling of objects ("touch DNA") is increasingly being recovered from crime scenes. Many of these "touch DNA" samples would also be classifiable as LCN samples due to the amount of DNA recovered. Recovery of genetic profiles from LCN samples is difficult using standard STR (Short Tandem Repeats) methods and such attempts often result in total failure or recovery of partial profiles. This is an expected outcome since commercial STR kits have been optimized to produce good quality, balanced profiles with 1 ng of DNA with 28-30 PCR cycles. Hence special LCN methods, based upon increasing the PCR (Polymerase Chain Reaction) cycle number (ICN) to increase allelic signal intensity, have been developed to permit profile recovery from limited quantity samples.17 Interpretation of the data obtained from these LCN analyzed samples also requires novel considerations.17

LCN Methodologies

Various strategies have been developed in the scientific community to deal with LCN samples including increased PCR (Polymerase Chain Reaction) cycle number (ICN), nested PCR and whole genome analysis.17,18 Since the forensic community has adopted ICN as the standard LCN (Low Copy Number) DNA approach, the other methodologies will not be considered here.

A standard PCR uses 28-30 cycles. In order to improve sensitivity for LCN analysis, an increase in PCR cycle number to 34 is now standard. This six-fold increase in cycle number would be expected to produce a theoretical 2^6 fold increase in sensitivity (i.e., 64) but inefficiencies with the PCR process at later cycles reduces this significantly. Nevertheless ICN will result in increased sensitivity with the concomitant production of stochastic sampling artifacts and, in some cases, the detection of otherwise-undetectable, extraneous DNA contamination.

Stochastic Effects of LCN DNA Analysis

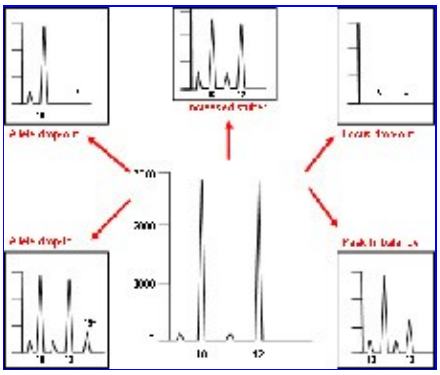
Due to the kinetics of the PCR (Polymerase Chain Reaction) process, a small number of starting genomic DNA templates will result in the production of stochastic sampling artifacts.17 Thus the bimolecular reaction

comprising a primer binding to its cognate template will be a random and rare event in the LCN (Low Copy Number) milieu, in which only a few target templates are available. Specifically, the molecular collisions required may or may not occur for each allele during each of the first few cycles, resulting in a significant imbalance between alleles or, in extreme cases, total loss of one or both alleles.19 Only those alleles that are amplified efficiently during the first few cycles will be able to produce sufficient product to surpass the detection threshold of the standard PCR endpoint analysis.

Depending upon the sample and the particular amplification, stochastically induced allelic imbalance and dropout of one or both alleles will be commonly observed in LCN profiles (see figure below). Stutter peak height ratios are also raised in some LCN samples. Allelic imbalance and increased stutter complicate mixture interpretation and single allelic dropout produce false homozygous profiles. Locus dropout due to loss of both alleles, while regrettable, does not create novel profile interpretation issues.

Contamination and Spurious Alleles

The increase in sensitivity of LCN analysis permits the detection of low levels of extraneous DNA contamination that, while often present, is not normally seen with standard 28 cycle STR (Short Tandem Repeasts) analysis.17 Thus alleles may show up in the profile that do not originate from the principal DNA donor(s) (see figure) and, in control experiments, have been shown to occur with known single donor samples. Such allelic drop-in is more-often-than-not of unknown origin but could be due to DNA from a variety of intralaboratory sources, including consumable items and personnel. As a result, LCN analysis should only be conducted in sterile laboratory facilities that have in place suitable engineering controls, akin to those employed for mtDNA analysis.



Read more about contamination in the DNA Amplification for Forensic Analysts PDF file.

Interpretation Guidelines

LCN (Low Copy Number) DNA analysis requires the development and implementation of special interpretation guidelines. While these may differ in detail between laboratories, a number of general principles can be enumerated:17

- Every allele in the evidence sample that is reported should be replicated by duplicate analysis
- Heterozygous peak ratios are not a robust measure of allelic association
- Negative controls that contain alleles consistent with the evidence profile require that the evidence samples be reanalyzed. Negative controls with alleles present that do not correspond with alleles in the evidence samples may be ignored, with caution.

- Allele dropout in the evidence sample has to be considered a possibility at those loci exhibiting an apparent homozygous profile.
- If there is a single allele in the evidence profile that does not match the potential donor's profile, then allelic drop-in should be considered.

LCN Case Example

Fourteen-year-old Marion Crofts was last seen cycling from her home in Fleet, Hampshire, England, to band practice when she was attacked as she passed some bushes close to a canal towpath in Aldershot in 1981. She had been raped, beaten, and strangled. Despite a major investigation by Hampshire Constabulary, who looked at 24,000 potential suspects, Marion's killer was never found.

A laboratory microscope slide - containing samples collected by the Forensic Science Service (FSS) from the teenager's body after her murder - lay deliberately untouched for 20 years. Scientists knew that they risked losing the evidence for good unless they waited until DNA profiling techniques became more advanced. In 1999, the Forensic Science Service (FSS) developed the super-sensitive DNA Low Copy Number (DNA LCN) technology. Using this technique, they were able to find a full DNA profile of Marion's suspected killer from some of her clothing. This profile was then loaded onto the UK's National DNA Database®. In August 2001, a match was found when Tony Jasinskyj was arrested for another crime and his DNA sample was routinely loaded onto the database. Scientists then returned to the archived laboratory microscope slide to see if they could obtain a DNA profile from the 20-year-old sample that might match the suspect's and further strengthen the case against him. A full profile was obtained that matched with the one from Marion's clothing and the suspect himself, providing strong evidence for the police.

At the time of the murder, Jasinskyj was a cook at the local army barracks and he, like 1,500 other soldiers, had been asked to fill out a questionnaire about Marion's death. On it he denied ever having been near the place where she was killed and claimed he was at work at the time. When he was questioned about how his bodily fluids ended up at the crime scene, he said they had been planted there. Tony Jasinskyj was found guilty of the teenager's rape and murder and jailed for life in May 2002.

http://www.forensic.gov.uk/forensic_t/index.htm

Selected pages from this site are available in Arabic, Français, Español and Deutsch.
Non-human DNA

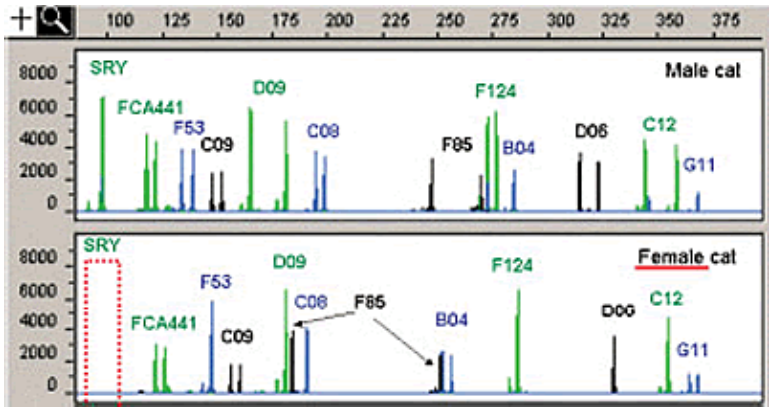
A majority of the biological evidence collected from crime scenes is material of human origin that may allow for identification of the person or persons involved in the commission of the crime. The availability of powerful and robust DNA typing strategies has made it is possible to ascertain with a high degree of certainty whether the biological evidence collected originated from a particular individual. However, according to Locard's Exchange Principal, there is a bidirectional transfer of material evidence between the crime scene and the individual participants. Some of this material evidence may be of non-human origin and include hairs, fibers, biological fluids, soils, and plant material. The collection, identification, and analysis of this evidence may associate an individual to the crime scene or a victim with a suspect. Traditionally such evidence has been examined using light microscopy and conventional serological methods. The development of molecular genetic approaches to species identification and individualization of non-human DNA (i.e., animal, plant, microbial) will increase the probative value of such evidence and is the subject of this section.

Animal DNA

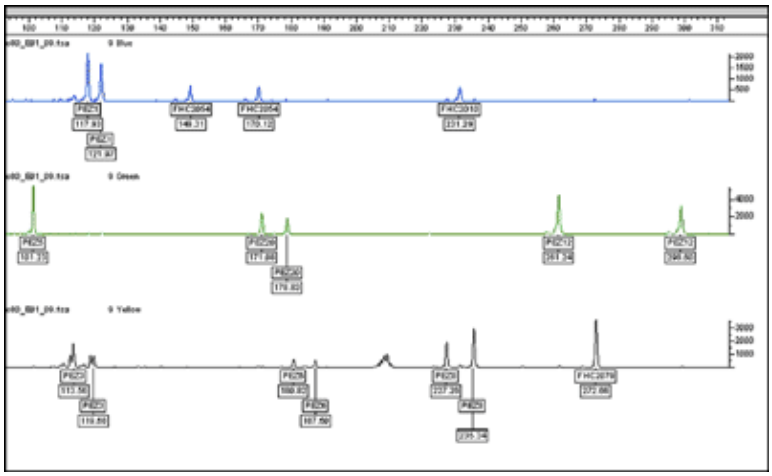
Many crimes occur in houses in which domestic pets, particularly cats and dogs, reside. For example, a survey conducted by the American Veterinary Medical Association in 2002 determined there are more than 70 million pet cats and 60 million pet dogs in the United States.20 Some studies have indicated that there is a high probability of transfer of shed pet hairs from such residences to visitors and owners alike.21 Apart from the direct transfer of dog and cat hairs from the crime scene and secondary transfer from the pet owner to another individual, there are other case circumstances which involve the domestic dog. In the case of assault on a human by a dog, there will often be a transfer of saliva from the dog to the victim. As these examples show, the ability to individualize particular dogs and cats by DNA analysis could represent an important source of probative evidence.

Extraction of DNA from the roots of cat hairs can be performed using standard methods employed by crime laboratories. A novel PCR (Polymerase Chain Reaction) based method permits quantification of the low amounts of DNA obtained from domestic cat hair samples.22 This method uses amplification of SINE (Short INterspersed Element) elements located in the Felis catus MHC and is reported to be able to detect as little as 10 femtogram (fg) of feline genomic DNA. The regions targeted by the primers in this method exhibit some degree of conservation with other species. However, if single cat hairs are used, the possibility of amplification from another species is believed by the authors to be minimal due to the washing of the root prior to DNA analysis.

Genomic sequence information is now available for the domestic cat and has resulted in the identification of numerous microsatellites.23-25 Based on the successful use of microsatellites in human individualization, attempts have been made to evaluate the use of feline microsatellites for forensic use.26 A multiplex system, MeowPlex, comprising 11 tetranucleotide STR markers and a gender determination marker, has been specifically designed to be compatible with current forensic DNA analysis instrument platforms and protocols.27 Match probabilities using this system have been reported to be in the range of 10^{-7} to 10^{-13}.27 The figure below illustrates the Meowplex STR (Short Tandem Repeats) genetic profiles obtained from a male and female cat.



Hundreds of microsatellite loci have been identified throughout the canine genome and used for genetic mapping and parentage testing.28-35 Population studies on 15 canine-specific polymorphic STR loci have been conducted to help determine their usefulness in forensic casework.26 The Stockmarks for Dogs Canine 1 amplification kit is commercially available from Applied Biosystems and comprises 10 STR loci.36 A second amplification kit, Canine 2, was discontinued since the discriminating ability of Canine 1 was sufficient for most cases.36 An example of an electropherogram from a dog using the Stockmarks for Dogs Canine 1 system is provided in the figure below.



Animal Case Example (STRs)

In the case of *State of Washington v. Kenneth Leuluaialii and George Tuilefano,* Kenneth Leuluaialii and Geroge Tuilefano were charged with the murders of Jay Johnson, Raquel Rivera, and their dog, Chief.36,37 Blood evidence that was taken from the suspects' pants and jackets was determined to be non-human, and the use of canine STR analysis allowed for identification of the blood evidence as matching that of Chief. The likelihood of the match was determined to be $1.5x10^7$ and $4.82x10^9$ for the blood taken from the pants and the jacket, respectively.36

Canine STR analysis normally requires the recovery of nuclear DNA from hair evidence taken from a crime scene. Akin to human DNA testing, the root of the hair needs to be present in order to extract sufficient quantities of nuclear DNA for STR analysis. However, mitochondrial DNA is present in higher copy number than nuclear DNA and can be recovered from the hair shaft. The sequence of the mitochondrial genome for the domestic dog (*Canis Familiaris)* is known.38 Approaches to mitochondrial DNA profiling of dog hairs have included sequence variation in the 5' end of the mtDNA hypervariable region (HVR) and also sequence and length variation within a variable and heteroplasmic tandem repeat region in the mtDNA control region (CR).39 In dogs, this region contains arrays of 10 base pair sequences that are repeated between 25 and 35 times.In addition to variability in the number of these repeat array sequences, there are two types of the 10 base pair repeats, differing in sequence by one base, known as the "informative position. "Through examination of both length and sequence variations, it was found that individual hairs from the same dog could contain different numbers of the 10-base pair repeats and therefore would not be suitable for use with single hairs. However, when sequence variation was examined between individual hairs from the same dog, they contained identical sequences and therefore this approach may be more suitable for use in forensic casework.40

Casework Example with Canine mtDNA

A dog was suspected of having caused a traffic accident, but left the scene. The driver of the car thought he knew the identity of the dog, and a lawsuit was filed against the owner's of the dog. The suspected dog had received treatment for injuries shortly after the time of the accident, which added to the appearance of the dog's guilt. However, hairs were found on the driver's car, and mitochondrial DNA testing was performed on the recovered hairs and reference hairs and saliva taken from the suspected dog. The results indicated that the recovered hairs did not originate from the suspected dog, and the lawsuit was subsequently dismissed.41

Species Identification

While domestic cat and dog evidence may be the most frequently encountered types of animal DNA found at a crime scene, there are certain instances when the identification of the species of origin and subsequent analysis of other animal DNA may be useful. Species identification could be particularly useful in cases such as poaching, illegal trade, and violations of the Endangered Species Act . Classical molecular methods for

species identification have been mainly based on immunological testing, including precipitation and agglutination reactions.42-44 However, molecular genetics-based approaches for the identification of species of origin have been developed, including the use of the mtDNA-containing cytochrome b (cyt b) gene and rRNA genes.45-52

The most common molecular genetic approaches to species identification involve analysis of the mitochondrial DNA cytochrome b gene. Amplification of the cyt b gene with primers flanking a large portion (981 bp out of 1140 bp) of the sequence and digestion of the resulting amplimer with restriction enzymes, such as ALU 1 and NCO 1, can be used to produce species specific RFLP (restriction fragment length polymorphism) fragments of various lengths.53 Alternative RFLP methods that use smaller non-contiguous amplimers from within the cyt b gene (464 bp and 358 bp) have been developed specifically for forensic use.49-51

Alternatively, direct Sanger sequencing of portions of the cyt b gene provides a more definitive, albeit more costly and time consuming, species identification strategy.46-48,50

Ribosomal RNA genes and other gene markers such as TP53 have also been reported as being useful for species identification.45,47 In a comparison study of -actin, TP53, cyt b, mtDNA D-loop, and 28S rRNA amplimers, the best species resolution was obtained using the TP53 gene.47 Sequencing of a short hypervariable 20 nucleotide fragment of the 12S rRNA gene by pyrosequencing appears to be capable of species identification.45

Plant DNA

Forensic botany, the use of plants as evidence in criminal investigations, is a well-established forensic science discipline that itself comprises a number of specialties including, *inter alia*, plant anatomy, palynology (pollen analysis), and plant systematics. During the commission of outdoor crimes, plant material may be transferred from the crime scene to the victim or perpetrators. This plant material may be probative due to the restricted geographical distribution of the plant species or individual genotype identified. Although the principal experimental tool of the forensic botanist remains the light microscope, intraspecies genetic variation is best determined by molecular genetics methods. Pre-molecular genetic forensic botany has played a significant role in a number of criminal cases. Analysis of wood evidence was utilized in the 1932 Charles Lindbergh baby kidnapping case.53 In 1994, a grave containing 32 male skeletons was discovered in Germany, and analysis of pollen spores recovered from the nasal cavities of the skulls was performed. Based on types of pollen prevalent during different seasons, identification of the type of pollen allowed for identification of the group of men and determination of when they were killed.54

Molecular genetics methods for detection of nucleotide sequence polymorphisms in plants include randomly amplified polymorphic DNA markers (RAPD), amplified fragment length polymorphisms (AFLP), and simple sequence repeats (SSR).53-56 RAPD analysis uses random primers, around 10 nucleotides in length, which hybridize to multiple sequences throughout the genome. If two primers hybridize to opposite strands within close enough proximity to one another, they will amplify a region between the two primers. Since sequence variation exists between DNA from different sources (insertions, deletions, nucleotide polymorphisms), it is possible that the primers may not hybridize in the same locations and thus result in the recovery of a different number of amplification products. AFLP utilizes restriction enzymes (RE) to detect RFLP (Restriction Fragment Length Polymorphism) variation. After RE digestion, nucleotide adaptors are ligated to the restriction fragments and amplified using appropriate adaptor-specific primers. The resulting multilocus RAPD or AFLP profiles may be sufficiently discriminatory to permit species identification or individualization. However, many of these multilocus fingerprinting methods suffer from poor reproducibility and low sensitivity. Consequently single locus microsatellite techniques, akin to human STRs (Short Tandem Repeats), are increasingly being used.

The first reported application of plant DNA evidence involved the molecular identification of seed pods from a Palo Verde tree used to link a suspect to a particular crime scene (see example below).57 RAPD analysis has been used in a lawsuit involving a suspected theft of an Italian patented variety of strawberry, "Marmolada." The RAPD amplification products of the suspected plants confirmed that the farmers had unlawfully grown the patented variety, and this evidence was accepted in court.58

Example: Seed Pods from Palo Verde Tree Help Solve Murder

In May 1992 a woman was murderd and her body dumped in an Arizona desert. The body was found close to a Palo Verde tree (*Cercidium floridum*), a bark-photosynthesizing, often leafless, plant that possesses 4-8cm long, soft seed pods and is native to the Sonoran Desert. A detective noted that one particular tree (referred to as PV-30) had a fresh abrasion in one of its branches. A suspect whose pager was found near the body possessed a truck, inside the back of which were two Palo Verde seed pods. A University of Arizona geneticist performed multiple primer RAPD analysis and was able to demonstrate interindividual variation among different Palo Verde trees. Specifically, he demonstrated that the seed pods from the truck bed matched completely PV-30 and concluded that he felt "quite confident" in concluding that the two seed pod samples most likely originated from PV-30. The suspect was convicted.

Identification of Marijuana

Identification of illegal substances is of great concern to law enforcement personnel. *Cannabis sativa*, or marijuana, is frequently encountered in criminal investigations. Current methods for identification of marijuana include microscopic examination for characteristic morphological features, chemical tests (e.g., Duquenois or Duquenois-Levine test), and cannabinoid compound identification by gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), or thin layer chromatography (TLC). A number of molecular genetic tests have been developed that also permit the identification of marijuana.59-64 These include DNA sequence analysis of the internal transcribed spacers (ITS1 and ITS2) of nuclear ribosomal DNA and the intergenic spacer region of chloroplast DNA (*trn* L-trnF IGS).

Once a substance has been identified as marijuana, it may be possible to individualize that sample to determine the originating source using DNA techniques: randomly amplified polymorphic DNA markers (RAPDs), amplified fragment length polymorphisms (AFLPs), and short tandem repeats (STRs). Such studies may facilitate the prosecution of major growers and distributors. Marijuana databases are being constructed in order to provide aid in determining the significance of a match between profiles from evidence samples.64

Microbial DNA

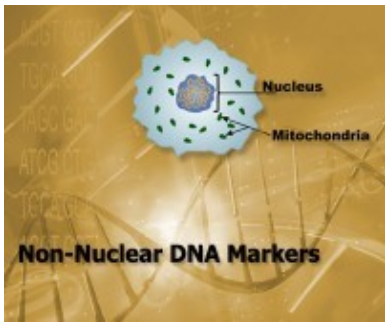
Recent terrorist attacks have raised awareness of the possible use of pathogens and toxins as bioweapons to commit bioterrorism and biocrime. Microorganisms and toxins have already been used in criminal offenses, such as the anthrax attacks of 2001.65 The field of microbial forensics has emerged in order to facilitate the identification of the infectious agent(s) used in a criminal offense, to determine the origin of such a sample, and to establish links to the perpetrators. While current DNA profiling strategies including direct DNA sequencing and the analysis of appropriate microsatellites, minisatellites and SNPs can be used to identify microorganisms. The biology of microorganisms results in additional challenges for the forensic community. Microbial source attribution is often difficult due to the clonal nature of some microorganisms, and their rapid rate of evolution (i.e., mutation).08 The former could result in false associations between the evidence and the putative source, while the latter could result in false exclusions.

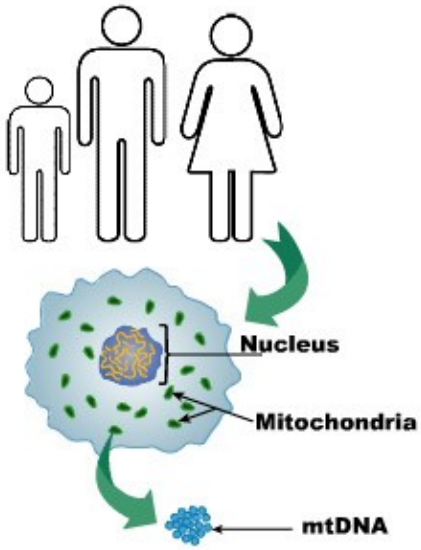
Although standard crime scene investigative processes involving the recording, collection, and preservation of evidence pertain to biocrime scene analysis, additional safety considerations apply due to the varying infectious nature of the evidence.08,09,66 Accordingly only personnel specifically trained to deal with the agents likely to be used in biocrime should process such scenes. Case-working crime laboratories are not

equipped to handle infectious agents, many of which require the use of biohazard level 3 or 4 laboratories. Quality assurance and quality control guidelines for the collection and analysis microbial DNA samples, akin to those established for human DNA profiling, have been recommended.08,66,67

Click here to read the recommendations for microbial DNA from the FBI.

Introduction





Mitochondrial DNA (mtDNA) analysis is a technique that is well-characterized, validated, and useful in the analysis of forensic evidence and identification of human remains. The technique has become more popular as a result of successes in identifying older skeletal remains where nuclear DNA (nDNA) typing was not successful. Although the maternal inheritance of mtDNA means that it is a less discriminating test than nDNA, it can produce genetic information on material where other methods will not work.

Objectives

Upon successful completion of this unit of instruction, the student shall be able to:

- Recognize the biological functions and contributions of mitochondria
- Differentiate features of nDNA (nuclear DNA) and mtDNA (mitochondrial DNA)
- Identify the different test techniques for mtDNA
- Understand the significance of mtDNA results for forensic and identity casework
- State the current and future mtDNA testing technologies

Mitochondrial DNA

Mitochondria are responsible for the bulk of adeosine triphosphate (ATP) synthesis and are often referred to as the energy powerhouse of the cell. They are the site of cellular respiration and capture energy generated by the breakdown of food during the oxidation of simple organic compounds.01

Mitochondria were first visualized as discrete cytoplasmic organelles in 1840, and in the 1960s it was determined that they contain their own DNA. Mitochondria are rod-shaped organelles that are present in all nucleated eukaryotic cells that use oxygen. They are approximately 1 to 10 micrometer (μm) in length and

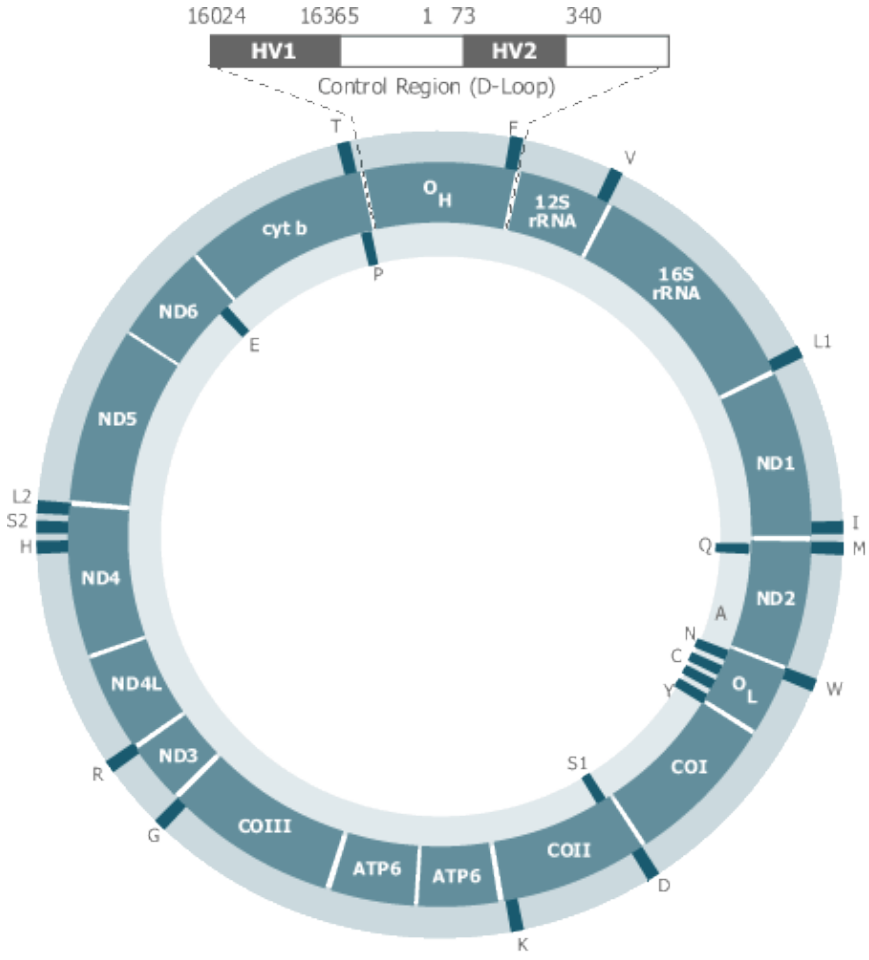
approximately 0.5 to 1.0 µm in diameter.02

A team of scientists at the Cambridge Research Institute completely sequenced the reported 16,569 bases of the mitochondrial genome (mtGenome).03 In fact, historically, this is the first component of the human genome that was completely sequenced. The DNA inside the mitochondrion is circular in structure and double-stranded. Mitochondrial DNA (mtDNA) codes for 13 polypeptides required for oxidative phosphorylation, 22 transfer RNAs, and 2 ribosomal RNAs. The heavy strand is purine rich, and the light strand is pyrimidine rich. Many scientists, especially evolutionary biologists, attribute the origin of the mitochondrion as a primitive aerobic bacterium that was once engulfed by the ancestor of present-day eukaryotic cells.04,05

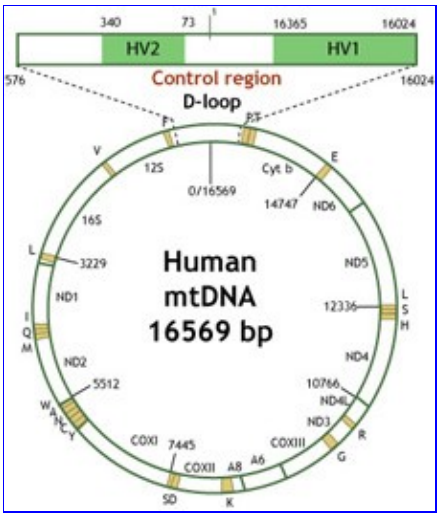
The online version of this course contains a multimedia [or downloadable] file. Visit this URL to view the file: http://beta.markers.dna.devis.com/m02/01/default_page

The mtGenome is not subjected to recombination during sexual transmission. The mtGenome is strictly maternally inherited; progeny of both males and females inherit the mtDNA from their mother (barring mutations), whereas only the daughter passes on the mtDNA to the next generation. The sperm contributes no mtDNA when fertilizing the egg. Biological maternal relatives all share their mtDNA, yet their nDNA is unique. The evolution of mtDNA has been studied in such detail that evolutionary biologist have determined that the Mother Eve of all surviving mtDNA profiles lived in Africa between 140,000 and 290,000 years ago. The low fidelity of mtDNA polymerase, lack of proof-reading, and the apparent lack of mtDNA repair mechanisms have led to a higher rate of mutation in the mtGenome as compared to the nuclear genome. Some regions of the mtGenome appear to be evolving at five to ten times the rate of single copy nuclear genes.06

The displacement loop, or D-loop, is a non-coding segment of the mtGenome that maintains elements for initiation of transcription and replication but does not code for any gene products. It is the D-loop region of the mtGenome that the forensic community routinely sequences for forensic casework. The D-loop is 1.1 Kb and is often referred to as the control region. Since the D-loop is a non-coding segment of DNA, variability within it is not lethal to the growing fetus. It is this nucleotide, or base, variability that is significant to the forensic scientist.07 Differences are observed between individuals not of the same maternal line.



Forensic Applications



Mitochondrial DNA (mtDNA) analysis is an appropriate method for:

- Charred remains
- Degraded specimens
- Old skeletal and fingernail samples
- Hair shafts

These samples either do not contain nuclear DNA or will have experienced a marked reduction in the quality and quantity of nDNA (nuclear DNA) present. In contrast, they contain much larger amounts of mtDNA and sufficient material for typing will remain even after degradation caused by environmental challenges or the passage of time.08

Additionally, mtDNA is quite useful in forensic investigations of remains recovered of a missing person or mass disaster. These remains are often highly fragmented or only very small sample sizes are recovered, such as a single tooth or a sliver of a bone.09 Biological material from known, maternal relatives, even quite distant, can be used as a reference for direct comparison to the recovered remains.

The D-loop contains two regions within its 1.1 Kb fragment that demonstrate multiple variations between individuals. The two variable regions, hypervariable region 1 (HV1) and hypervariable region 2 (HV2) are amplified, detected, and analyzed for forensic identification. Other regions within the mtGenome have been successfully analyzed but are not the typical regions tested for casework.10,11

The origin of replication is between HV1 and HV2. Counting of the bases, or the numerical address along the mtGenome, is initiated at the origin of replication on the heavy strand starting at position 1 (p1) to p16,569. As mentioned earlier, the most common practice for forensic laboratories is reporting sequence information is HV1 and HV2. The sequence information covered for HV1 is p16,024 to p16,365. Likewise, the sequence information covered for HV2 is p73 to p340.

View an animation on the D-Loop.

Unlike STR (Short Tandem Repeat) analysis where discrete alleles according to size are reported, mtDNA analysis reports the observed base sequence. The standard for the forensic community is to report the sequence information as compared to the revised Cambridge Reference Sequence's (rCRS) light strand, also referred to as the Anderson sequence.12 When the base sequence is the same, then no reference to that particular position is noted. However, if the base sequence at a position is different at one or more positions when compared to the rCRS, then the difference(s) is noted. For a common example of a transition in HV1, the rCRS is an A at p73 and the sequenced sample is a G at p73; a report is generated showing an A to G transition a p73.

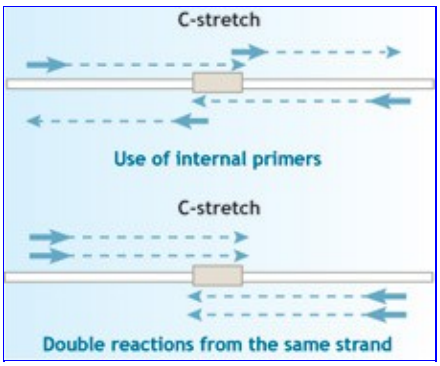
View the Cambridge Reference Sequence

In Caucasians, the most common mtDNA types from HV1 and HV2 occur in approximately 7% of the general population. On average, there are eight nucleotide differences between individuals amongst unrelated Caucasians and 15 nucleotide differences between individuals amongst unrelated Africans.13,14

Other Applications

Scientists have used mtDNA analysis for medical studies, evolutionary studies, migration studies, genealogical studies, and historical identifications.

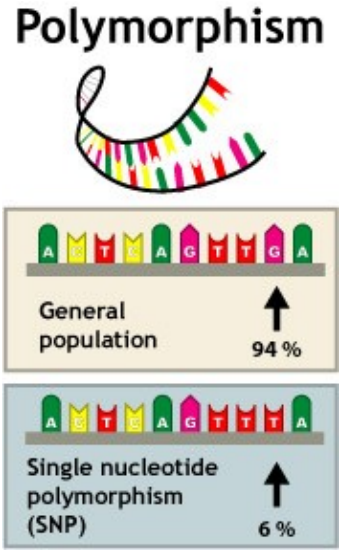
Heteroplasmy and Mutations



Heteroplasmy is when an individual exhibits more than one mtDNA (mitochondiral DNA) type. This phenomenon is usually observed as a single difference at one position exhibiting either two or more bases or a length difference often seen in a polycytosine rich region (C-stretch). Single base heteroplasmy has been documented, most notably in the identification of Tsar Nicholas II.15 Length heteroplasmy in the D-loop region has been described in both HV1 and HV2.16, 17, 18 Whereas heteroplasmy may complicate interpretation, it actually becomes a "signature" of the donor's mtDNA haplotype. Heteroplasmy can, therefore, strengthen the identification of a sample.

Point mutations or deletions in mtDNA usually increase with age. Defects in mitochondrial function produce a wide range of human diseases and can be caused by mutations within the mtDNA. The first mtDNA mutation discovered to be the cause of a disease was Leber's hereditary optic neuropathy (LHON).19 Identified in 1988, LHON is a maternally inherited form of adult-onset blindness due to death of the optic nerve. Point mutations are relevant because the person's mtDNA type can change with age.

Methodologies



In order to evaluate amplified DNA of the mtGenome D-loop, linear array assay and fluorescent sequencing technology are currently used in the forensic community. Single nucleotide polymorphisms (SNPs) and microchips are emerging technologies and are discussed in the Advanced and Emerging DNA Technologies and Techniques PDF file.

Sample Preparation

Mitochondrial DNA (mtDNA) methods are useful in analyzing degraded samples and those containing very low quantity of nuclear DNA. mtDNA is present in cells at a much higher copy number than nuclear DNA (as noted in the table/animation above). The increased copy number of mitochondrial DNA per cell increases the concern for contamination introduced in the laboratory. The procedures used in mtDNA laboratories are designed to limit the possibility of contamination.

These procedures include:

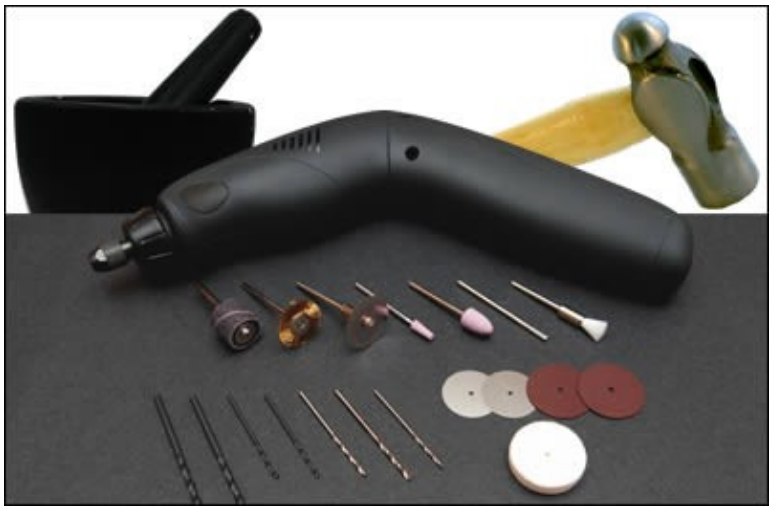
- Wearing of special clothing (e.g., face masks, sleeves, hair nets)
- Use of dedicated laboratory equipment and other supplies
- Frequent cleaning and decontamination of laboratory and equipment
- Organization of work flow
- Monitoring of extraneous DNA through controls

Several forensic-and-ancient-remains research laboratories have published cleaning and extraction procedures for recovery of DNA from bones and teeth. The specific cleaning procedures are extremely important. If these specimens are not properly cleaned, external contaminants and potential inhibitors may be extracted with the DNA.[20,21] As described in the DNA Extraction & Quantitation PDF file, inhibitors may be detrimental to the amplification process.

Published procedures include:[22-27]

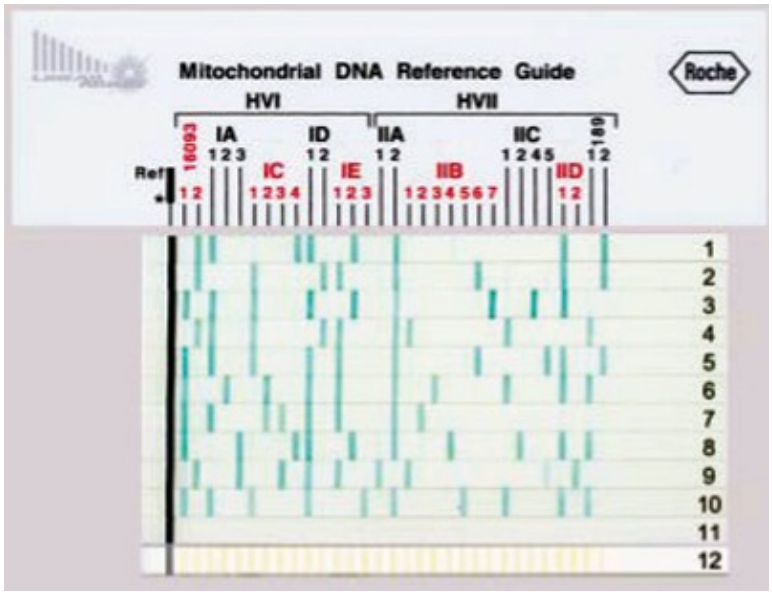
- Rinsing the specimen with double distilled deionized water
- Rinsing with a dilute hypochlorite solution
- Polishing the specimen with a grinding stone followed by water and bleach rinses
- Exposing the specimen to ultraviolet irradiation
- Vertical sectioning of the tooth's crown for removal of the dental pulp

Specialized tools are needed for many of these extraction procedures, e.g., a Dremel tool, a MicroMill grinder, a freezer mill, an aluminum oxide wheel, a grinding stone, a mortar, a pestle, a chisel, a hammer, and/or a saw with diamond blade, just to name a few. The extraction of DNA from bones and teeth is a challenging process; many of the challenges can be overcome with the appropriate procedures and equipment.



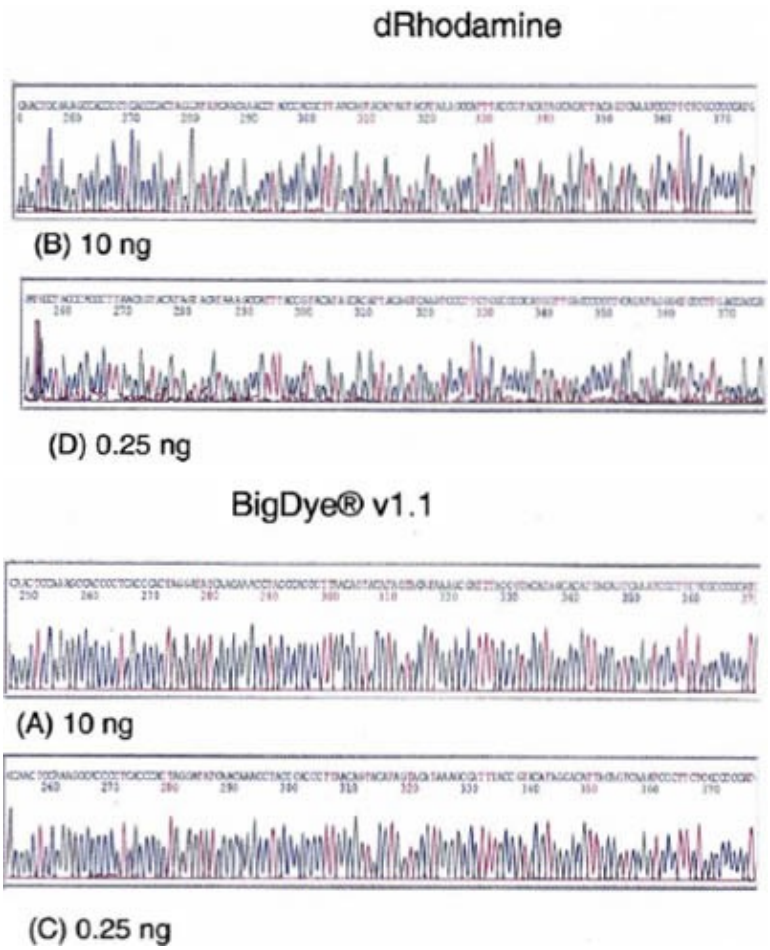
Linear Array Analysis

The Linear Array mtDNA HVI/HVII Region-Specific Typing Kit (Roche Applied Science) is a sequence-specific oligonucleotide hybridization technique.28 This technique is used as a screening assay and eliminates the need to perform more costly direct sequencing of all samples.



Example

A detective submits a single pubic hair for mtDNA analysis, the victim reference standard, and standards for ten suspects. With the linear array assay, the analyst may be able to exclude the victim and eight suspects as the donor of the pubic hair. This would leave three samples needing direct sequencing: the pubic hair sample and the samples from the two suspects not excluded.



The linear array assay has sequence-specific oligonucleotide (SSO) probes immobilized on a membrane very similar in design to the AmpliType PM + DQA1 Typing Kit.[29] Biotinylated PCR (Polymerase Chain Reaction) products hybridize to the SSO probes with the exact sequence complement. Common polymorphic sites are interrogated.[28] All biotinylated PCR products hybridized to the SSO probes are detected with streptavidine-horseradish peroxidase conjugate. A chromogenic substrate produces a blue precipitate on the membrane. It is this pattern of "thin blue lines" that detects sequence variants to the rCRS. All samples not excluded with this typing kit can be analyzed with direct sequencing methods to evaluate the full hypervariable regions, using the original amplicons. Data is reviewed to obtain additional sequence information to confirm the original match or determine it to be a non-match.

mtDNA Sequencing

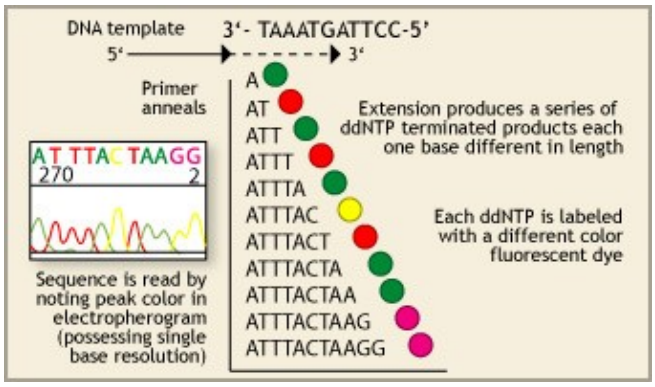
Mitochondrial DNA (mtDNA) sequencing technology is routinely used by a limited number of forensic laboratories worldwide because direct sequencing is time consuming and labor intensive.

Direct sequencing determines the order of bases along the template DNA. Automated fluorescent sequence technology has significantly increased the throughput of sequence information in the scientific communities. Often small quantity and degraded DNA are the only samples available in mtDNA forensic casework. The DNA is amplified and directly sequenced using the Sanger method.

The amplified product is:[27,30]

- Digested with SAP-EXO I (shrimp alkaline phosphatase and exonuclease I) to remove excess deoxynucleotide triphosphate (dNTPs) and PCR (Polymerase Chain Reaction) primers

- Exposed to a sequencing primer and incorporation of dideoxyribonucleotide triphosphate (ddNTPs) for the sequencing reaction
- Denatured
- Separated by electrophoresis



Each ddNTP is labeled with a different fluorescent dye. Once the ddNTP is incorporated, chain elongation is terminated. The sequencing primers direct the sequencing of the forward or reverse reaction for each of the DNA strands. Interpretation of the data can be laborious, even with sophisticated software programs.

The amplification and sequencing primer sets are not available commercially in kits similar to the STR (Short Tandem Repeat) PCR amplification kits; however, these primers are published and can be ordered through commercial vendors.[31,32] Highly degraded DNA may not amplify efficiently with the primer sets for HV1 and HV2. Miniprimer sets have been developed to amplify smaller portions of HV1 and HV2 and have been successful in overcoming failure to amplify with the larger primers.[33]

Computer software programs, such as Sequencher™ (Gene Codes), are available to aid the analyst in the tedious task of data analysis.

Sequencher™ is a sequence analysis software program that:

- Builds projects for each sample
- Removes any sequence data of the primers
- Removes poor sequence data based on signal and spacing
- Produces complementary data of the reverse sequence reactions
- Aligns forward and reverse sequencing reactions
- Compares the sequence to the rCRS

A forensic DNA analyst independently examines, interprets, and edits sequencing results prior to reporting.

Author: Jack Ballantyne

Jack Ballantyne is an Associate Professor of Chemistry at the University of Central Florida (UCF) and the Associate Director for Research at the National Center for Forensic Science in Orlando, Florida. He possesses a B.Sc. (with Honours) in Biochemistry from the University of Glasgow, Scotland, a M.Sc. in Forensic Science from the University of Strathclyde, Scotland, and a PhD in Genetics from the State University of New York at Stony Brook, NY. His current duties include teaching and conducting research in forensic molecular genetics. He teaches a variety of forensic biology courses to baccalaureate and Masters-level students in the Forensic Science Program and nucleic acid biochemistry to PhD students in the Biomolecular Sciences Program.

Prior to entering academia, he was a casework forensic scientist in Scotland, Hong Kong, and New York where he proffered expert testimony in the criminal courts of these jurisdictions. He was the full-time DNA technical leader in Suffolk County, New York, and since then has served as a part-time consultant DNA technical leader for the States of Mississippi and Delaware, the City of Dallas, and Sedgwick County, Kansas. *Inter alia*, he is the Chair of the New York State DNA Sub-committee, a regular visiting guest at the Scientific Working Group on DNA Analysis Methods (SWGDAM), a member of the DoD Quality Assurance Oversight Committee, and was a member of the World Trade Center Kinship and Data Analysis Panel (KADAP).

His research interests include Y chromosome markers, the assessment and in vitro repair of damaged DNA templates, mRNA profiling for body fluid identification, the determination of physical characteristics by molecular genetic analysis, and single cell/low copy number analysis.

Author: Erin Hanson

Erin Hanson possesses a Bachelor of Science degree in Forensic Science, Biochemistry Track, and a Master of Science degree in Forensic Science, Biochemistry Track, from the University of Central Florida. Her graduate research included development and validation of novel Y chromosome STR multiplexes for use in forensic casework. A comprehensive annotated STR physical map of the human Y chromosome was also developed through this research and made available to the forensic community. Currently, Ms. Hanson is a PhD candidate in the Biomolecular Science Doctoral Program at the University of Central Florida, and her current research includes an assessment of damage and degradation to macromolecules in dried biological stains, and the use of laser capture microdissection and whole genome amplification strategies for the analysis of single cells.

Author: Rhonda Roby

Rhonda K. Roby, MPH, has 17 years experience in the applications of DNA technology for forensic and human identification DNA testing. She is currently pursuing her doctoral degree in Forensic Genetics and Evolution at the University of Granada in Spain and is conducting research in support of the NIJ's Missing Persons Program at the University of North Texas Health Science Center. Her most recent study, Forensic DNA Databasing: Expert Systems for High-Throughput Analysis of Single Source Samples, is forthcoming from the National Institute of Justice publications department.

Author: Rhonda Roby

Rhonda K. Roby, MPH, has 17 years experience in the applications of DNA technology for forensic and human identification DNA testing. She is currently pursuing her doctoral degree in Forensic Genetics and Evolution at the University of Granada in Spain and is conducting research in support of the NIJ's Missing Persons Program at the University of North Texas Health Science Center. Her most recent study, Forensic DNA Databasing: Expert Systems for High-Throughput Analysis of Single Source Samples, is forthcoming from the National Institute of Justice publications department.

Works Cited & Online Links

1. Hinds, D. A., L. L. Stuve, G. B. Nilsen, E. Halperin, E. Eskin, D. G. Ballinger, K. A. Frazer, D. R. Cox. 2005. Whole-genome patterns of common DNA variation in three human populations. *Science* 307 (5712): 1072–9.

2. Crawford, D. C., and D. A. Nickerson. 2005. Definition and clinical importance of haplotypes. *Annu Rev Med* 56:303–20.
3. Chakraborty, R., D. N. Stivers, B. Su, Y. Zhong, and B. Budowle B. 1999. The utility of short tandem repeat loci beyond human identification: Implications for development of new DNA typing systems. *Electrophoresis* 20 (8): 1682–96.
4. Frudakis, T., K. Venkateswarlu, M. J. Thomas, Z. Gaskin, S. Ginjupalli, S. Gunturi, V. Ponnuswamy, S. Natarajan, and P. K. Nachimuthu. 2003. Aclassifier for the SNP-based inference of ancestry. *J Forensic Sci* 48 (4): 771–82.
5. Schoske, R., P. M. Vallone, M. C. Kline, J. W. Redman, and J. M. Butler. 2004. High-throughput Y-STR typing of U.S. populations with 27 regions of the Y chromosome using two multiplex PCR assays. *Forensic Sci Int* 139 (2–3): 107–21.
6. Ray, D. A., J. A. Walker, A. Hall, B. Llewellyn, J. Ballantyne, A. T. Christian, K. Turteltaub, and M. A. Batzer. 2005. Inference of human geographic origins using Alu insertion polymorphisms. *Forensic Sci Int* 153 (2–3): 117–24.
7. Frudakis, T., M. Thomas, Z. Gaskin, K. Venkateswarlu, K. S. Chandra, S. Ginjupalli, S. Gunturi, S. Natrajan, V. K. Ponnuswamy, and K. N. Ponnuswamy. 2003. Sequences associated with human iris pigmentation. *Genetics* 165 (4): 2071–83..
8. Breeze, Roger, Bruce Budowle, and Steven Schutzer. 2005. *Microbial Forensics*. Burlington, MA: Elsevier Academic Press.
9. Budowle, B. 2004. SNP typing strategies. *Forensic Sci Int* Suppl. no.146:S139–42.
10. Lahn, B. T., N. M. Pearson, and K. Jegalian. 2001. The human Y chromosome, in the light of evolution. *National Review Genetics* 2 (3): 207–16.
11. Skaletsky, H., T. Kuroda-Kawaguchi, P. J. Minx, H. S. Cordum, L. Hillier, L. G. Brown, S. Repping, et al. 2003. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* 423 (6942): 825–37.
12. Jobling, M. A., and C. Tyler-Smith. 2003 The human Y chromosome: An evolutionary marker comes of age. *National Review Genetics* 4 (8): 598–612.
13. Bandelt, H. J., P. Forster, B. C. Sykes, and M. B. Richards. 1995. Mitochondrial portraits of human populations using median networks. *Genetics* 141 (2): 743–53.
14. Roewer, L., M. Krawczak, S. Willuweit, M. Nagy, C. Alves, A. Amorim, K. Anslinger, et al. 2001. Online reference database of European Y-chromosomal short tandem repeat (STR) haplotypes. *Forensic Sci Int* 118 (2–3): 106–13.
15. Hubert, R., J. L. Weber, K. Schmitt, L. Zhang, and N. Arnheim, 1992. A new source of polymorphic DNA markers for sperm typing: Analysis of microsatellite repeats in single cells. *Am J Hum Genet* 51 (5): 985–91.
16. Kayser, M., A. Caglia, D. Corach, N. Fretwell, C. Gehrig, G. Graziosi, F. Heidom, et al. 1997. Evaluation of Y-chromosomal STRs: A multicenter study. *Int J Legal Med* 110 (3): 125–33.
17. Gill, P., J. Whitaker, C. Flaxman, N. Brown, and J. Buckleton. 2000. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Sci Int* 112 (1): 17–40.
18. Sun, G., R. Kaushal, P. Pal, M. Wolujewicz, D. Smelser, H. Cheng, M. Lu, R. Chakraborty, L. Jin, and R. Deka. 2005. Whole-genome amplification: Relative efficiencies of the current methods. *Legal Medicine* (Tokyo) 7 (5): 279–86.
19. Walsh, P. S., H. A. Erlich, and R. Higuchi. 1992. Preferential PCR amplification of alleles: Mechanisms and solutions. *PCR Methods and Applications* 1 (4): 241–50.
20. American Veterinary Medical Association and Center for Information Management Staff. 1997. *U.S. Pet Ownership & Demographics Sourcebook*. 2002. N.p.: Center for Information Management American V.
21. D'Andrea, F., F. Fridez, and R. Coquoz. 1998. Preliminary experiments on the transfer of animal hair during simulated criminal behavior. *J Forensic Sci* 43 (6): 1257–8.
22. Menotti-Raymond, M., V. David, L. Wachter, N. Yuhki, and S. J. O'Brien. 2003. Quantitative polymerase chain reaction-based assay for estimating DNA yield extracted from domestic cat

specimens. *Croatian Medical Journal* 44 (3): 327–31.
23. Menotti-Raymond, M., V. A. David, L. A. Lyons, A. A. Schaffer, J. F. Tomlin, M. K. Hutton, and S. J. O'Brien. 1999. A genetic linkage map of microsatellites in the domestic cat (Felis catus). *Genomics* 57 (1): 9–23.
24. Murphy, W. J., S. Sun, Z. Chen, N. Yuhki, D. Hirschmann, M. Menotti-Raymond, and S. J. O'Brien. 2000. A radiation hybrid map of the cat genome: Implications for comparative mapping. *Genome Res* 10 (5): 691–702.
25. O'Brien, S. J., M. Menotti-Raymond, W. J. Murphy, and N. Yuhki. 2002. The feline genome project. *Annu Rev Genet* 36: 657–86.
26. Menotti-Raymond, M., V. A. David, J. C. Stephens, L. A. Lyons, and S. J. O'Brien. 1997. Genetic individualization of domestic cats using feline STR loci for forensic applications. *J Forensic Sci* 42 (6):. 1039–51.
27. Butler, J. M., V. A. David, S. J. O'Brien, and M. Menotti-Raymond. 2002. The "MeowPlex": A new DNA test using tetranucleotide STR markers for the domestic cat. *Profiles in DNA*, Promega publication 5 (2): 7–10. http://www.promega.com/profiles/502/ProfilesinDNA_502_07.pdf.
28. Ostrander, E. A., G. F. Sprague Jr., and J. Rine. 1993. Identification and characterization of dinucleotide repeat (CA)n markers for genetic mapping in dog. *Genomics* 16 (1): 207–13.
29. Francisco, L. V., A. A. Langston, C. S. Mellersh, C. L. Neal, and E. A. Ostrander. 1996. A class of highly polymorphic tetranucleotide repeats for canine genetic mapping. *Mamm Genome* 7 (5): 359–62.
30. Zajc, I., C. S. Mellersh, and J. Sampson. 1997. Variability of canine microsatellites within and between different dog breeds. *Mamm Genome* 8 (3): 182–5.
31. Zajc, I., and J. Sampson. 1999. Utility of canine microsatellites in revealing the relationships of pure bred dogs. *J Hered* 90 (1): 104–7.
32. Schlapfer, J., G. Dolf, N. Saitbekova, and C. Schelling. 1999. Twelve novel cosmid-derived canine microsatellites. *Anim Genet* 30 (5): 387–8.
33. Jouquand, S., C. Priat, C. Hitte, P. Lachaume, C. Andre, and F. Galibert. 2000. Identification and characterization of a set of 100 tri- and dinucleotide microsatellites in the canine genome. *Anim Genet* 31 (4): 266–72.
34. Richman, M., C. S. Mellersh, C. Andre, F. Galibert, and E. A. Ostrander. 2001. Characterization of a minimal screening set of 172 microsatellite markers for genome-wide screens of the canine genome. *J Biochem Biophys Methods* 47 (1–2): 137–49.
35. Irion, D., A. L. Schaffer, T. R. Famula, M. L. Eggleston, S. S. Hughes, and N. C. Pedersen. 2003. Analysis of genetic variation in 28 dog breed populations with 100 microsatellite markers. *J Hered* 94 (1): 81–7.
36. Halverson, J., and C. Basten. 2005. A PCR multiplex and database for forensic DNA identification of dogs. *J Forensic Sci*. 50 (2): 352–63.
37. Day, A. 2001. Nonhuman DNA testing increases DNA's power to identify and convict criminals . *Silent Witness* 6 (1).
38. Kim, K. S., S. E. Lee, H. W. Jeong, and J. H. Ha. 1998. The complete nucleotide sequence of the domestic dog (Canis familiaris) mitochondrial genome. *Mol Phylogenet Evol* 10 (2): 210–20.
39. Wetton, J. H., J. E. Higgs, A. C. Spriggs, C. A. Roney, C. S. Tsang, and A. P. Foster. 2003. Mitochondrial profiling of dog hairs. *Forensic Sci Int* 133 (3): 235–41.
40. Savolainen, P., L. Arvestad, and J. Lundeberg. 2000. A novel method for forensic DNA investigations: Repeat-type sequence analysis of tandemly repeated mtDNA in domestic dogs. *J Forensic Sci*,. 45 (5): 990–9.
41. Schneider, P. M., Y. Seo, and C. Rittner. 1999. Forensic mtDNA hair analysis excludes a dog from having caused a traffic accident. *Int J Legal Med* 112 (5): 315–6.
42. Andrasko, J., and B. Rosen. 1994. Sensitive identification of hemoglobin in bloodstains from different species by high performance liquid chromatography with combined UV and fluorescence detection. *J Forensic Sci*. 39 (4): 1018–25.

43. Czesny, S., K. Dabrowski, J. E. Christensen, J. V. Eenennaam, and S. Doroshov. 2000. Discrimination of wild and domestic origin of sturgeon ova based on lipids and fatty acid analysis. *Aquaculture*. 189: 145–53.
44. Espinoza, E. O., N. C. Lindley, K. M. Gordon, J. A. Ekhoff, and M. A. Kirms. 1999. Electrospray ionization mass spectrometric analysis of blood for differentiation of species. *Anal Biochem* 268 (2): 252–61.
45. Balitzki-Korte, B., K. Anslinger, C. Bartsch, and B. Rolf. 2005. Species identification by means of pyrosequencing the mitochondrial 12S rRNA gene. *Int J Legal Med* 119 (5): 291–4.
46. Bataille, M., et al. 1999. Multiplex amplification of mitochondrial DNA for human and species identification in forensic evaluation. *Forensic Sci Int*. 99 (3): 165–70.
47. Bellis, C., K. J. Ashton, L. Freney, B. Blair, and L. R. Griffiths. 2003. A molecular genetic approach for forensic animal species identification. *Forensic Sci Int* 134 (2–3): 99–108..
48. Branicki, W., T. Kupiec, and R. Pawlowski. 2003. Validation of cytochrome b sequence analysis as a method of species identification. *J Forensic Sci*. 48 (1): 83–7.
49. Bravi, C. M., J. P. Liron, P. M. Mirol, M. V. Ripoli, P. Peral-Garcia, and G. Giovambattista. 2004. A simple method for domestic animal identification in Argentina using PCR-RFLP analysis of cytochrome b gene. *Legal Medicine* (Tokyo). 6 (4): 246–51.
50. Parson, W., K. Pegoraro, H. Niederstatter, M. Foger, and M. Steinlechner. 2000. Species identification by means of the cytochrome b gene. *Int J Legal Med* 114 (1–2): 23–8.
51. Dietmaier, W., A. Hartmann, S. Wallinger, E. Heinmoller, T. Kerner, E. Endl, K. W. Jauch, F. Hofstadter F. and J. Ruschoff. J. 1999. Multiple mutation analyses in single tumor cells with improved whole genome amplification. *Am J Pathol* 154 (1): 83–95.
52. Zehner, R., S. Zimmermann, and D. Mebs. 1998. RFLP and sequence analysis of the cytochrome b gene of selected animals and man: Methodology and forensic application. *Int J Legal Med* 111 (6): 323–7.
53. Miller Coyle, H., C. Ladd, T. Palmbach, and H. C. Lee. 2001. The Green Revolution: Botanical contributions to forensics and drug enforcement. *Croatian Medical Journal* 42 (3): 340–5.
54. Szibor, R., C. Schubert, R. Schoning, D. Krause, and U. Wendt. 1998. Pollen analysis reveals murder season. *Nature* 395 (6701): 449–50.
55. Powell, W., G. C. Machray, and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. *Trends in Plant Science* 1 (7): 215–222.
56. Tingey, S. V., and J. P. del Tufo. 1993. Genetic analysis with random amplified polymorphic DNA markers. *Plant Physiol* 101 (2): 349–52.
57. Yoon, C.K. 1993. Forensic science: Botanical witness for the prosecution. *Science* 260 (5110): 894–5.
58. Congiu, L., M. Chicca, R. Cella, R. Rossi, and G. Bernacchia. 2000. The use of random amplified polymorphic DNA (RAPD) markers to identify strawberry varieties: A forensic application. *Mol Ecol* 9 (2): 229–32.
59. Alghanim, H. J., and J. R. Almirall. 2003. Development of microsatellite markers in Cannabis sativa for DNA typing and genetic relatedness analyses. *Anal Bioanal Chem* 376 (8): 1225–33.
60. Gilmore, S., R. Peakall, and J. Robertson. 2003. Short tandem repeat (STR) DNA markers are hypervariable and informative in Cannabis sativa: Implications for forensic investigations. *Forensic Sci Int* 131 (1): 65–74.
61. Hsieh, H. M., R. J. Hou, L. C. Tsai, C. S. Wei, S. W. Liu, L. H. Huang, Y. C. Kuo, A. Linacre, and J. C. Lee. 2003. A highly polymorphic STR locus in Cannabis sativa. *Forensic Sci Int*. 131 (1): 53–8.
62. Jagadish, V., J. Robertson, and A. Gibbs. 1996. RAPD analysis distinguishes Cannabis sativa samples from different sources. *Forensic Sci Int* 79 (2): 113–21.
63. Linacre, A., and J. Thorpe. 1998. Detection and identification of cannabis by DNA. *Forensic Sci Int* 91 (1): 71–6.

64. Miller Coyle, H., T. Palmbach, N. Juliano, C. Ladd, and H. C. Lee. 2003. An overview of DNA methods for the identification and individualization of marijuana. *Croatian Medical Journal* 44 (3): 315–21.
65. Hsu, V., S. L. Lukacs, T. Handzel, J. Hayslett, S. Harper, T. Hales, V. A. Semenova, et al. 2002. Opening a bacillus anthracis-containing envelope, Capitol Hill, Washington, D.C.: The public health response. *Emerg Infect Dis* 8 (10): 1039–43.
66. Budowle, B., S. E. Schutzer, A. Einseln, L. C. Kelley, A. C. Walsh, J. A. Smith, B. L. Marrone, J. Robertson, and J. Campos. 2003. Public health: Building microbial forensics as a response to bioterrorism. *Science* 301 (5641): 1852–3.
67. Scientific Working Group on Microbial Genetics and Forensics (SWGMGF). 2003. Quality assurance guidelines for laboratories performing microbial forensic work. *Forensic Science Communications* 5 (4). http://www.fbi.gov/hq/lab/fsc/backissu/oct2003/2003_10_guide01.htm.

- Endangered Species Act Summary
  http://www.fws.gov/endangered/esasum.html
- MeowPlex
  http://www.promega.com/profiles/502/502_07.html
- Recommendations for microbial DNA
  http://www.fbi.gov/hq/lab/fsc/backissu/oct2003/2003_10_guide01.htm
- Revised Validation Guildelines by SWGDAM
  http://permanent.access.gpo.gov/websites/fbi.gov/www.fbi.gov/hq/lab/fsc/backissu/july2004/standards/2004_03_standards02.htm
- SNap shot™ assay
  https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=600762&tab=DetailInfo
- SNPstream® UHT
  http://www.orchid.com/products/lsg/products/uht.asp
- Stockmarks for Dogs Canine 1 Amplification Kit
  https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=600792&tab=Literature
- SWGDAM recommended core loci
  http://www.fbi.gov/hq/lab/fsc/backissu/july2004/standards/ 2004_03_standards03.htm
- Y-STR Haplotype Reference Database (YHRD)
  http://www.yhrd.org/index.html

1. Copeland, William C., ed. 2002. *Mitochondrial DNA: Methods and protocols.* Totowa, NJ: Humana Press.
2. Robin, E. D., and R. Wong.. 1988. Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J Cell Physiol* 136 (3): 507–13.
3. Anderson, S., A. T. Bankier, G. B. Barrell, M. H. de Bruijn, A. R. Coulson, J. Drouin, I. C. Eperon, et al. 1981. Sequence and organization of the human mitochondrial genome. *Nature* 290 (5806):457–65.

4. Gray, M. W. 1992. The endosymbiont hypothesis revisited. *Int Rev Cytol* 141:233–357.
5. Grivell, L. A. 1997. Mitochondria. In *McGraw-Hill encyclopedia of science & technology,* ed. Sybil P.Parker. 8th ed. New York: McGraw-Hill.Baechtel, F. S. 1988. The identification and individualization of semen stains. In vol. 2 of *Forensic science handbook*, ed. Richard E. Saferstein, 347–92. Englewood Cliffs, NJ: Prentice Hall.
6. Budowle, Bruce, Jenifer Smith, Tamyra Moretti, and Joseph DiZinno. 2000. *DNA typing protocols: Molecular biology and forensic analysis*. Natick, MA: Eaton Publishing.
7. Greenberg, B. D., J. E. Newbold, and A. Sugino. 1983. Intraspecific nucleotide sequence variability surrounding the origin of replication in human mitochondrial DNA. *Gene* 21 (1–2): 33–49.
8. Anderson, T. D., J. P. Ross, R. K. Roby, D. A. Lee, and M. M. Holland. 1999. A validation study for the extraction and analysis of DNA from human nail material and its application to forensic casework. *J Forensic Sci* 44 (5): 1053–6.
9. Ginther, C., L. Issel-Tarver, and M. C. King. 1992. Identifying individuals by sequencing mitochondrial DNA from teeth. *Nat Genet* 2 (2): 135–8.
10. Lutz, S., H. Wittig, H. J. Weisser, J. Heizmann, A. Junge, N. Dimo-Simonin, W. Parson, et al. 2000. Is it possible to differentiate mtDNA by means of HVIII in samples that cannot be distinguished by sequencing the HVI and HVII regions? *Forensic Sci Int* 113 (1–3): 97–101.
11. Bini, C., S. Ceccardi, D. Luiselli, G. Ferri, S. Pelotti, C. Colalongo, M. Falconi, and G. Pappalardo. 2003. Different informativeness of the three hypervariable mitochondrial DNA regions in the population of Bologna (Italy). *Forensic Sci Int* 135 (1): 48–52.
12. Andrews, R. M., I. Kubacka, P. F. Chinnery, R. N. Lightowlers, D. M. Turnbull, and N. Howell. 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nat Genet* 23 (2): 147.
13. Budowle, B., M. R. Wilson, J. A. DiZinno, C. Stauffer, M. A. Fasano, M. M. Holland, and K. L. Monson. 1999. Mitochondrial DNA regions HVI and HVII population data. *Forensic Sci Int* 103 (1): 23–35.
14. Melton, T., S. Clifford, M. Kayser, I. Nasidze, M. Batzer, and M. Stoneking. 2001. Diversity and heterogeneity in mitochondrial DNA of North American populations. *J Forensic Sci* 46 (1): 46–52.
15. Ivanov, P. L., M. J. Wadhams, R. K. Roby, M. M. Holland, V. W. Weedn, and T. J. Parsons. 1996. Mitochondrial DNA sequence heteroplasmy in the Grand Duke of Russia Georgij Romanov establishes the authenticity of the remains of Tsar Nicholas II. *Nat Gen* 12 (4): 417–20.
16. Bendall, K. E., and B. C. Sykes. 1995. Length heteroplasmy in the first hypervariable segment of the human mtDNA control region. *Am J Hum Genet* 57 (2): 248–56.
17. Marchington, D. R., G. M. Hartshorne, D. Barlow, and J. Poulton. 1997. Homopolymeric tract heteroplasmy in mtDNA from tissues and single oocytes: Support for a genetic bottleneck. *Am J Hum Genet* 60 (2): 408–16.
18. Wilson, M. R., M. W. Allard, K. Monson, K. W. P. Miller, and B. Budowle. 2002. Further discussion of the consistent treatment of length variants in the human mitochondrial DNA control region. *Forensic Science Communications* 4 (4). http://www.fbi.gov/hq/lab/fsc/backissu/oct2002/wilson.htm.
19. Wallace, D. C., G. Singh, M. T. Lott, J. A. Hodge, T. G. Schurr, A. M. Lezza, L. J. Elsas 2nd, and E. K. Nikoskelainen. 1988. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science* 242 (4884): 1427–30.
20. Baker, L. E., W. F. McCormick, and K. J. Matteson. 2001. A silica-based mitochondrial DNA extraction method applied to forensic hair shafts and teeth. *J Forensic Sci* 46 (1): 126–30.
21. Benedix, D. C., N. P. Herrmann, V. A. Haskins, and L. E. Flournoy. 1999. Analysis of dental remains from Honduran ossuary caves. *Am J Phys Anthropol* Suppl. no. 28:90.
22. Holland, M. M., D. L. Fisher, L. G. Mitchell, W. C. Rodriguez, J. J. Canik, C. R. Merril, and V. W. Weedn. 1993. Mitochondrial DNA sequence analysis of human skeletal remains: Identification of remains from the Vietnam War. *J Forensic Sci* 38 (3): 542–53.

23. Hoss, M., and S. Paabo. 1993. DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic Acids Res* 21 (16): 3913–4.
24. Potsch, L., U. Meyer, S. Rothschild, P. M. Schneider, and C. Rittner. 1992. Application of DNA techniques for identification using human dental pulp as a source of DNA. *Int J Legal Med* 105 (3):139–43.
25. Smith, B. C., D. L. Fisher, V. W. Weedn, G. R. Warnock, and M. M. Holland. 1993. A systematic approach to the sampling of dental DNA. *J Forensic Sci* 38 (5):1194–209.
26. Sweet, D. J., and C. H. Sweet. 1995. DNA analysis of dental pulp to link incinerated remains of homicide victim to crime scene. *J Forensic Sci* 40:310–4.
27. Wilson, M. R., D. Polanskey, J. Butler, J. A. DiZinno, J. Replogle, and B. Budowle. 1995. Extraction, PCR amplification and sequencing of mitochondrial DNA from human hair shafts. *Biotechniques* 18 (4): 662–9.
28. Stoneking, M., D. Hedgecock, R. G. Higuchi, L. Vigilant, and H. A. Erlich. 1991. Population variation of human mtDNA control region sequences detected by enzymatic amplification and sequence-specific oligonucleotide probes. *Am J Hum Genet* 48 (2): 370–82.
29. Fildes, N., and R. Reynolds. 1995. Consistency and reproducibility of AmpliType PM results between seven laboratories: Field trial results. *J Forensic Sci* 40 (2): 279–86.
30. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 74 (12):5463–7.
31. Wilson, M. R., J. A. DiZinno, D. Polanskey, J. Replogle, and B. Budowle. 1995. Validation of mitochondrial DNA sequencing for forensic casework analysis. *Int J Legal Med* 108 (2):68–74.
32. Fisher, D. L., M. M. Holland, L. Mitchell, P. S. Sledzik, A. W. Wilcox, M. Wadhams, and V. W. Weedn. 1993. Extraction, evaluation, and amplification of DNA from decalcified and undecalcified United States Civil War bone. *J Forensic Sci* 38 (1): 60–8.
33. Gabriel, M. N., E. F. Huffine, J. H. Ryan, M. M. Holland, and T. J. Parsons. 2001. Improved mtDNA sequence analysis of forensic remains using a "mini-primer set" amplification strategy. *J Forensic Sci* 46 (2): 247–53.

Online Links

- **Cambridge reference sequence**
  http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=337188# sequence_337188