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Forensic DNA Typing Protocols

Edited by

Angel Carracedo

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Preface

The discovery of polymorphisms in repetitive DNA by Dr. Alec Jeffreys and coworkers in 1985 has had a tremendous impact on forensic genetics. Since then we have witnessed a revolution in the field of forensic identification, and different markers and technologies for DNA typing have moved at a breathtaking pace.

Rapid advances in technology, from serological or electrophoretic analysis of protein polymorphisms to direct investigation of the underlying DNA polymorphisms, occurred in a very short space of time in the mid-1980s. Consequently, the incorporation of modern molecular biological techniques in the forensic genetic laboratory has resulted in major benefits for justice.

DNA analysis has become the standard method applied by most forensic genetic labs, especially in criminal forensic casework (e.g., analysis of stains and hairs, identification of human remains, and paternity testing). Polymerase chain reaction (PCR)-based DNA typing systems have made it possible to analyze DNA obtained from only a few cells as well as from highly degraded human samples (recently demonstrated by the identification of relatively old human remains). The potential of DNA typing has made possible the resolution of immigration problems and complicated paternity testing cases when the father is not available. Rapid identification of individuals in mass disaster using DNA typing has also been possible. Computerized DNA databases for the identification of criminal offenders have been created in some countries.

Owing to these many impressive applications, the media have taken great interest in DNA profiling, mainly because of the value of the evidence presented through DNA profiling in certain well-known legal cases.

Initially, the use of DNA profiling was very controversial in some countries, perhaps because of a hasty introduction of this new methodology. Ironically, however, this has contributed to a much more reliable use of DNA profiling.

Two parallel upheavals concerning the introduction of DNA typing technology have been accountable for the aforementioned: the introduction of quality control and accreditation schemes and, in particular, the spreading use of the statistics in the evaluation of DNA evidence. Also, progress in standardizing the tests has proven even more important than the technical advances.

In addition to the DNA revolution, the evolution and development of DNA markers and technologies themselves have been rapid and spectacular. In only a few years we have progressed from the original multilocus DNA fingerprint

analysis of DNA minisatellites, through single locus probe analysis of specific minisatellites, to a host of systems based on the PCR technique.

Microsatellites or short-tandem repeats (STRs) have been almost completely substituted for minisatellites in forensic labs. STRs were first analyzed in manual electrophoretic systems. The introduction of fluorescent-based technology and the use of DNA sequencers have revolutionized the field, allowing the typing of large multiplexes, as well as the automation of the typing procedure. Commercially available and robust multiplexes with up to 15 STRs are routinely used by most of the forensic labs.

But new markers and methods of detection have been proposed, and the most important new advances are the introduction of the use of mtDNA and Y chromosome polymorphisms and especially the new use of single nucleotide polymorphisms (SNPs). It is now clear that SNP typing will be of prime importance in the field, owing to the potential advantages of this type of marker, especially for the analysis of degraded samples.

Because STR typing is familiar in all the forensic labs and the typing protocols are well established, we have decided to focus *Forensic DNA Typing Protocols* on the newer methods and technologies forensic scientists use to solve certain types of cases and to implement these new DNA typing methods in their laboratories. In addition, we have included a chapter on how to create large STR multiplexes, since some labs are interested in the design of STR multiplexes for specific purposes (e.g., STRs with short amplicons for degraded samples; pentanucleotide STRs for the analysis of mixtures).

Forensic DNA Typing Protocols provides protocols for the major methods of DNA analysis that have been recently introduced for identity testing, including Y chromosome, mtDNA, and SNP typing. Chapters with protocols for new applications in the forensic genetics labs—such as species identification or typing of CYP polymorphisms for the analysis of adverse reactions to drugs—have also been included. Ancient DNA is another field of forensic and anthropological interest where there is a need for well-tested protocols from laboratories with extensive experience in the field; two chapters are devoted to this topic. Finally, proper DNA quantification is a crucial requirement for the analysis of critical forensic samples, including mixtures, and new methods based on real-time PCR are now available. For this reason two chapters including protocols for DNA quantification of forensic samples and for the determination of the number of amelogenin gene copies have been added.

I am indebted to Professor John Walker, the series editor, for his helpful encouragement and patience, but I especially owe my thanks to all the contributing authors who have made this book possible.

Angel Carracedo

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Forensic DNA-Typing Technologies

A Review

Angel Carracedo and Paula Sánchez-Diz

Summary

Since the discovery of deoxyribonucleic acid (DNA) profiling in 1985, forensic genetics has experienced a continuous technical revolution, both in the type of DNA markers used and in the methodologies or its detection. Highly informative and robust DNA-typing systems have been developed that have proven to be very effective in the individualization of biological material of human origin. DNA analysis has become the standard method in forensic genetics used by laboratories for the majority of forensic genetic expertise and especially in criminal forensic casework (stain analysis and hairs) and identification.

Key Words: DNA analysis; DNA profiling; DNA typing; forensic genetics.

1. Introduction

Deoxyribonucleic acid (DNA) profiling, or typing, as it is now known, was first described in 1985 by Alec Jeffreys and co-workers ***(1)*** ,and it has had a tremendous impact in forensic genetics. Prior to that time, all forensic genetic casework (e.g., paternity testing, criminal casework, individual identification) was performed using classical serological genetic markers. Blood groups, human leukocyte antigen (HLA), and polymorphic protein and enzymes were used for solving forensic genetic casework using immunological and electrophoretic methodologies. These genetic markers were nevertheless limited when it was necessary to analyze minimal or degraded material, which is commonly involved in forensic cases. It was, in addition, difficult to analyze biological material other than blood, and therefore the information obtained from hair, saliva, and even semen in rape cases was rather limited.

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Because the polymorphic proteins and enzymes were infrequent, it was necessary to obtain as much information as possible. For this reason, sophisticated electrophoretic methods, such as isoelectric focusing, immobilines, or hybrid isoelectric focusing, were developed and applied. Despite these methods, the information that the forensic geneticists were able to report in many cases was clearly insufficient.

Since the discovery of polymorphisms in repetitive DNA by Jeffreys et al. *(1)*, highly informative and robust DNA-typing systems have been developed that are quite powerful for the individualization of biological material of human origin.

DNA typing has advantages over traditional protein, the first of which being it is more informative and can be analyzed in minute or degraded material because DNA is physically much more resistant to degradation than proteins. In addition, the same DNA genotype can be obtained from any tissue (i.e., blood, saliva, semen, hair, skin, bones), whereas the analysis of protein markers is restricted to cells where these proteins are expressed.

DNA analysis has become the standard method in forensic genetics used by laboratories for the majority of forensic genetic expertise and especially in criminal forensic casework (stain analysis and hairs) and identification.

2. DNA Polymorphisms

Hidden in the approx 3 billion base pairs of DNA of the haploid human genome are an estimated 35,000 genes *(2)*. All human genes are encoded in approx 10% of the human genome. Thus, the great majority (more than 90%) of the human genome represents "noncoding" parts of the genome because they do not contain genetic information directly relevant for protein synthesis. Genetic variation is rather limited in coding DNA with the exception of the HLA region. This is the result of the fact that expressed genes are subjected to selection pressure during evolution to maintain their specific function. In contrast, the noncoding part of the genome is not mainly controlled by selection pressure, and thus mutations in these regions are usually kept and transmitted to the offspring, leading to a tremendous increase in genetic variability. Therefore, these regions are very appropriate for forensic genetics because they are very informative and at the same time not useful for drawing conclusions about the individual other than for identification purposes.

An important percentage of the noncoding DNA (30%) consists of repetitive sequences that can be divided into two classes: tandemly repetitive sequences and interspersed elements. The majority of forensic typing systems in current use are based on genetic loci with tandem repetitive DNA sequences.

Tandemly repeated sequences can be found in satellite DNA, but from the forensic point of view, regions of repetitive DNA much shorter than satellite

DNA are much more interesting. These regions can be classified into minisatellites *(1)* and microsatellites or short tandem repeat (STRs) *(3,4)*. Minisatellites, otherwise known as variable number of tandem repeats (VNTR) loci *(5)*, are composed of sequence motifs ranging from around 15 to 50 bp in length, reiterated tandemly for a total length of 500 bp to 20 Kb. STRs are much shorter. The repeat unit ranges from 2 to 6 bp for a total length between 50 and 500 bp.

In addition, minisatellites and STRs have differences in their distribution in the human genome and probably in their biological function. Thus, minisatellites are more common in subtelomeric regions, whereas STRs are widely distributed throughout the human genome, occurring with a frequency of one locus every 6–10 kb *(6)*. The origin of the variability seems to be different as well. Although unequal crossing over and even gene conversion *(7)* are involved in the variability of minisatellites, replication slippage is mainly involved in the origin of the variability in microsatellites *(8)*.

The genetic variation between individuals in these minisatellites and STR systems is mainly based on the number of tandemly arranged core repeat elements, but it is based also on differences in the DNA sequence itself because the repeats can have slight differences in the sequence.

3. DNA-Typing Methods

Technologies used for DNA typing for forensic purposes differ in their ability to differentiate two individuals and in the speed and sensitivity with which results can be obtained. The speed of analysis has dramatically improved for forensic DNA analysis. DNA testing that previously took more than 1 wk can now be performed in a few hours.

Southern blotting with multi-locus and single-locus (SLP) DNA probes have been used for paternity testing and forensic stain typing, and they are still used (especially SLPs) by a few laboratories working in paternity testing

Briefly, the technique is as follows: First, the DNA is extracted and then cut by a specific enzyme (usually *Hinf*I in Europe and *Hae*III in the United States) into many small fragments. A tiny fraction of those fragments include the particular minisatellite to be analyzed. The fragments are then separated by electrophoresis in submarine agarose gels using TAE or TBE as buffers. After an appropriate length of time, the fragments migrate different distances in the electric field, depending mainly on their sizes, with the smaller ones migrating more rapidly. DNA fragments are then chemically treated to separate the double strands into single ones.

Because the gels are difficult to work with, the single-stranded fragments are then transferred directly to a nylon membrane, to which they adhere. This process is called Southern blotting, named after its inventor *(9)*. The next step

is to flood the membrane with a single-stranded probe, which will hybridize with the DNA fragments that contain the target minisatellite sequence and adhere to it. The membrane is then washed several times to remove any probe that does not bind to this specific DNA sequence.

Probes can be labeled using isotopic or nonisotopic methods. Nonisotopic chemiluminescent methods are more popular than isotopic ones.

Whatever the labeling method is, the nylon membrane usually is placed on an x-ray film, with the final result of an autoradiograph with the bands of the minisatellite clearly impressed in the film. The process requires several days for sufficient radioactive decay to produce a visible band on the film.

Originally, multilocus probes were proposed for forensic genetic analysis. However, this type of probe was not very successful in the forensic field because despite its informativeness, statistical problems of evaluation of the evidence in cases of match and standardization problems arose. For these reasons, this probe was substituted in the forensic field by the analysis of VNTRs using SLPs under high-stringency conditions.

Until the introduction of STR analysis by polymerase chain reaction (PCR), minisatellite analysis with single-locus probes was very popular in forensic laboratories. Nowadays, it is still used in some laboratories, particularly for paternity testing analysis.

The main advantage of SLP analysis is the enormous variability of some of the minisatellites and the adequate knowledge of the mutation rate in some of them. The main disadvantages are the time needed for the analysis and especially the need for the relatively large amount of nondegraded DNA required for SLP typing. Because DNA extracted from forensic specimens is often degraded because of environmental conditions, these techniques have often failed to produce reliable results. PCR has overcome these difficulties, and it has strongly enhanced the usefulness of DNA profiling techniques in forensic science.

PCR is a technique for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. The PCR method was devised and named by Mullis and colleagues at the Cetus Corporation ***(10)***, although the principle had been described in detail by Khorana et al. ***(11)*** more than a decade earlier. The use of PCR was limited until heat-stable DNA polymerases became available. DNA polymerases conduct the synthesis off a complementary strand of DNA in the 5' to 3' direction using a single-stranded template, but starting from a double-stranded region. The PCR uses the same principle but uses two primers, each complementary to opposite strands of the region of DNA that have been denatured by heating. The primers are arranged so that each primer extension reaction directs the synthesis of DNA toward the other. This results in the novo synthesis of the region flanked by the two primers.

Automated temperature cyclers (usually called thermocyclers) allow the exact control of successive steps of denaturation, annealing of the primers, and extension (when the DNA polymerase extends the primer by using a complementary strand as a template). All these three steps constitute a cycle, and a normal PCR reaction consists of 20–25 cycles, allowing the formation of $2^{20–25}$ molecules from a single molecule of template DNA.

Most PCR-based typing systems allow alleles to be identified as discrete entities, thus avoiding most of the statistical issues that arise in matching and binding SLPs bands and making standardization easier. Also, apart from the increased sensitivity inherent in any PCR technique, it is more likely to be successful in analyzing old or badly degraded material mainly because of the small size of some of the DNA polymorphisms (SNPs and STRs) susceptible to analysis by PCR ***(12,13)***.

Once PCR has been used to generate a large number of copies of a DNA segment of interest, different approaches may be taken to detect genetic variation within the segment amplified. Because 10^6 or more copies of the target sequence can be produced, it is possible to use nonisotopic methods of detection. A number of imaginative methods for PCR product detection have been described.

The first one was the use of sequence-specific oligonucleotide (SSO) probes ***(14)*** to detect variation in HLA–class II genes, especially in the HLA DQA1 system ***(15)***. An SSO probe is usually a short oligonucleotide (15–30 nucleotides in length) with a sequence exactly matching the sequence of the target allele. The SSO probe is mixed with dissociated strands of PCR product under very stringent hybridization conditions such that the SSO and the PCR product strand will be hybridized if there is a perfect sequence complimentary but will not be if there are different sequences. The classical format for the use of SSO probes is to spot dissociated PCR product strands onto a nitrocellulose or nylon membrane and to probe the membranes with labeled SSO; because of the fact that the samples are spotted as a "dot" on the membrane, this format is known as dot blotting. A reverse dot-blot format is much more commonly used ***(16)***. With this format (which has its antecedents in affinity chromatography), the SSO is immobilized on the membrane and is used to capture PCR products containing biotin label in the primers. Several genetic loci can be analyzed by this technology using commercially available kits. The AmpliType PM PCR amplification kit (Perkin-Elmer, Foster City, CA) was very popular in forensic laboratories some years ago. With this kit, the loci HLA DQA1, LDLR, GYPA, HBGG, D7S8, and GC are amplified in a multiplex fashion. The last five loci listed are typed simultaneously in a single reverse dot-blot strip containing ASO probes; HLA DQA1 must be typed in a separate strip. The system was validated for forensic analysis ***(17)***, and it is still used by some laboratories although most of them currently prefer to use more informative STR systems.

The efforts of forensic scientists have mainly addressed the amplification of fragment-length polymorphisms. The minisatellite D1S80 (pMCT118) was the first to be applied to the forensic routine *(18)*, but these systems have been substituted by STRs. The analysis of STRs by PCR is now the method of choice for forensic identification. Dinucleotide STRs are the most common STRs in the human genome and are the genetic markers most commonly used for linkage analysis, although they are not being used in forensic science. The reason is that analysis of these STRs has been affected by strand slippage during amplification, producing artifactual stutter bands *(19)*. Nevertheless, tetra and pentanucleotide repeats appear to be less prone to slippage and are more suitable for forensic purposes. The percent of stutters is very interesting to identify and select ideal STRs for forensic purposes because having a low percentage of stutters is critical for the analysis of mix stains. Some tetranucleotide STRs (such as TH01) are known to have a good behavior regarding these characteristics, but pentanucleotides are ideal systems for analyzing mixtures.

According to its structure, STRs range from the extremely complex STRs to the most simple *(20)*. Complex STRs have the advantages of hypervariability. Simple STRs have the advantages of easy standardization and low mutation rates. Mutation events are more frequent in the male germ line, and the rates of different loci can differ by several orders of magnitude, the structure and length being the most influential factors in the rate *(21)*. In addition to the characteristics already mentioned, the selection of ideal STRs for forensic purposes include the analysis of other artifactual bands, the robustness, and the size. In general, short sizes are desirable because the size of the amplified product is critical in degraded samples, and small fragments can be amplified when larger fragments failed *(13)*.

Another important fact is the possibility of amplifying multiple STR loci in a single multiplex reaction. This coupled with the direct detection of amplified products to polyacrylamide gels, makes STR DNA profiling amenable to automation. For this reason, the ability to be included in multiplexes is another characteristic that should be analyzed for the selection of good STRs for forensic purposes.

STRs were firstly analyzed in manual electrophoretic systems. Denaturing polyacrylamide gels are recommended for standardization purposes, given that with native gels sequence variation can also be detected making the typing prone to errors. STR electrophoretic mobility under native and denaturing conditions should also be checked since some STRs (especially AT-rich ones) have been shown to have anomalous mobility in polyacrylamide gels *(22)*.

The introduction of fluorescent-based technology and the use of DNA sequencers have revolutionized the field, allowing the typing of large multiplexes (including up to 10 systems) as well as the automation of the typing

procedure. Commercially available STR multiplexes for manual electrophoretic systems are available, but the major advantages of the use of sequencers is automation and the possibility of using intelligent systems of interpretation. The use of sequence reference allelic ladders is essential for STR typing. In general, the reference allelic ladders comprise most of the alleles of the system, but intermediate alleles are always possible even in the most simple STRs. Interpretation guidelines have been produced ***(23,24)*** to distinguish these intermediate alleles and can be easily implemented in automatic sequencers. There are many multiplexes commercially available. A very popular one is the SGM Plus (Applied Biosystems), which comprises 10 loci-HUMFIBRA/FGA, HUMVWFA, HUMTH01, D18S51, D21S11, D6S477, D8S1179, D16S539, D19S433, and amelogenine. Promega (Madison, WI) multiplexes are also very popular, especially in the laboratories using manual electrophoretic systems or monochromatic sequencing platforms. But the extremely discriminative 15-plexes are becoming more and more popular and, among these, the Poweplex16 (Promega) and the Identifiler (Applied Biosystems) are the more commonly used by forensic labs.

In general, the combined discrimination power of STRs is enormous and the probabilities of two unrelated individuals matching by chance (pM) are lower 10^{-15} for some of these large multiplexes.

4. STRs in Sexual Chromosomes and Mitochondrial (mt)DNA

Y chromosome-specific polymorphisms have proven to be especially useful in forensic work. The applications of Y polymorphisms include deficiency paternity testing, when a male offspring is in question, to different applications in criminal casework. Y polymorphisms are especially interesting in the analysis of male DNA fraction in stains involving male/female mixtures, the most common biological material available in sexual crimes. Especially important is the use of these markers in cases where preferential sperm DNA extractions fails (this is estimated to occur in 5–15% of forensic cases) and also in rapes committed by azoospermic individuals. Although the variation in the Y chromosome is low, the nonpseudoautosomal region still bears different kinds of polymorphisms, including biallelic markers, STRs, and minisatellites. SNPs and STRs are the most interesting. The most-used Y STRs are the trinucleotide repeat DYS392 and the tetranucleotide repeats DYS19, DYS385, DYS389-I, DYS389-II, DYS390, DYS391, and DYS393. This STRs comprises the so-called minimum Y STR haplotype ***(25)***, but new STRs have recently been described ***(26,27)***. Also, commercially available Y-STR plexes have been recently introduced.

As for mtDNA, statistical interpretation in cases of match is more complicated and appropriate corrections taking into account population substructure