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I. Introduction

The human genome is peppered with regions of repetitive DNA—hypervariable regions consisting of a short DNA sequence repeated in tandem. These regions are polymorphic in that the sequence varies in the number of copies of the repeat unit. The number of repeat units is indicated by the allele designation. For example, 14 copies of the repeat unit would be referred to as allele 14. In the early 1980s, these regions were investigated as informative markers to map the human genome (Wyman and White, 1980; Jeffreys *et al.*, 1985a), but one of the founding fathers of DNA typing, Alec Jeffreys, quickly realized that these markers could be employed for DNA-based human identification (Jeffreys *et al.*, 1985b).

A. Variable Number Tandem Repeats and Restriction Fragment Length Polymorphisms

In the early days of DNA-based identification, the hypervariable regions of interest were variable number tandem repeat (VNTR) loci, which had a high level of heterozygosity and were relatively large in size (300–10,000bp) (Nakamura *et al.*, 1987; Budowle *et al.*, 1991). VNTRs were analyzed using restriction fragment length polymorphism (RFLP), where high-molecular-weight target DNA is digested with a restriction enzyme that has recognition sites at both ends of the hypervariable region. The size of the DNA fragment resulting from the restriction enzyme digestion is dictated by the number of repeat elements. These fragments are separated by size using agarose or polyacrylamide gel electrophoresis and detected using a labeled VNTR probe. Analysis of multiple VNTR loci results in a unique pattern of DNA fragments on the gel. The patterns generated from a DNA sample of unknown origin and DNA of known origin are compared. Matching patterns indicate that the sources of the unknown and known DNA samples are likely the same. RFLP analysis of VNTR loci works well to resolve immigration and paternity disputes and for other applications where large amounts of intact DNA can be collected. However, RFLP is not ideally suited to forensic investigations because microgram amounts of high-molecular-weight DNA are required.Thus, VNTR analysis is limited to investigations where large amounts of DNA are recovered.

B. Short Tandem Repeats

Many samples recovered from crime scenes yield only nanogram or picogram amounts of DNA that is sometimes degraded, and thus not suitable for VNTR analysis. To overcome this limitation, scientists harnessed the polymerase chain reaction (PCR; Saiki *et al.*, 1985) to amplify shorter hypervariable regions known as short tandem repeat (STR) loci. STR loci consist of short, repetitive sequence elements 3–7 base pairs in length (Edwards *et al.*, 1991a; Edwards *et al.*, 1991b; Edwards *et al.*, 1992; Warne *et al.*, 1991). By using PCR, minute amounts of DNA can be amplified and analyzed and, because STR loci are much shorter than VNTR loci, degraded DNA can be successfully analyzed. This amplified fragment length polymorphism (AmpFLP) technique allows generation of full DNA profiles from trace biological samples, such as blood spatter, fingerprints, saliva, semen and other body fluids, bone, tissue and hair collected during forensic casework and missing persons investigations. In addition, PCR-based STR analysis is higher-throughput so that more samples can be analyzed in less time. For these reasons, STR analysis has replaced VNTR analysis for human identification purposes.

The human genome is organized as 23 pairs of chromosomes: the X and Y sex chromosomes and the remaining autosomal chromosome pairs, numbered 1 through 22. STR loci exist on both autosomes and sex chromosomes. Ideally STR loci for forensic use are physically separated enough so that they are inherited independently of each other (i.e., not genetically linked). Sequences on different autosome pairs are not usually linked due to independent assortment of chromosomes during meiosis. Females possess two X chromosomes, which can recombine during meiosis. However, X-STR loci can show linkage disequilibrium (Tillmar, *et al.*, 2008) and tend to fall within four linkage groups. In males, neither X-STR nor Y-STR loci are linked because these chromosomes do not pair during meiosis and cannot undergo recombination, except for a small pseudoautosomal region of the X-Y pair (Gusmão and Carracedo, 2003). This difference in genetic inheritance becomes important during data analysis. Data generated using autosomal STR loci and X- and Y-STR loci cannot be used in the same way (see the Data Analysis section). A more detailed discussion of STR analysis is provided below.

C. Single Nucleotide Polymorphisms

A single nucleotide polymorphism (SNP) is a variation in DNA sequence at a single base. SNPs can be used for human identification, although studies suggest that 50–80 loci are required to achieve the same discrimination level of 16 STR loci [Gill, 2001]. There are nearly 6 million SNPs in numerous online databases, but only a fraction of these are useful for human identification because some are less polymorphic than others or difficult to amplify in multiplex PCR. The requirement for small amplicon sizes (60–120 bp) and robust multiplex PCR with minimal nonspecific amplification further reduces the number of SNPs suitable for forensic applications. Appropriate criteria are being identified to select the most useful and robust loci and develop a core panel of SNPs for optimum discrimination in forensic applications (Phillips, 2003).

Analysis of large numbers of SNPs by direct sequencing is expensive and time-consuming, so in addition to multiplex PCR, SNPs are typed by minisequencing, which is simple, non-radioactive and easily multiplexed. Minisequencing involves amplification of an unlabeled PCR product that spans the SNP, then interrogation using a probe that anneals immediately adjacent to the SNP. This probe is allowed to hybridize to the PCR product in the presence of labeled dideoxynucleotides (ddNTPs) and DNA polymerase. The probe is extended at the 3′-end through addition of the ddNTP that is complementary to the single

nucleotide polymorphism. The extended probe is separated by electrophoresis, and the incorporated ddNTP is detected. Because each ddNTP is labeled with a different fluor, the color of the peak denotes the SNP allele. Multiplex SNP analysis is made possible by adding a defined number of nucleotides to the 5′-end of each probe so that the SNP probes can be resolved electrophoretically.

D. Mitochondrial DNA Analysis

While analysis of nuclear DNA provides full STR profiles in many situations, ancient or degraded samples often yield partial profiles or no profile. In these cases, analysis of mitochondrial DNA (mtDNA) may provide information where nuclear DNA analysis cannot. Mitochondria are cellular organelles that provide most of the energy required for various cellular functions. The number of mitochondria per cell varies with the cell type, ranging from hundreds to thousands of mitochondria per cell, and each mitochondrion contains many copies of its own DNA. Thus, mtDNA has a much higher copy number per cell than nuclear DNA. In addition, human mtDNA is a circular molecule of 16,569bp (Anderson *et al.*, 1981), and this circular nature makes mtDNA more resistant to exonucleases. For these reasons, there is often enough mtDNA, even in degraded samples, for analysis.

Samples well suited to mtDNA analysis include bones, teeth, hair and old or degraded samples, which often have little high-molecular-weight nuclear DNA. Hair is a common sample type discovered at crime scenes, but hair presents a problem to forensic examiners because, as hair is shed, genomic DNA in the root cells undergoes programmed degradation (Linch, 1998). Also, cells within the hair shaft lose their nuclei, but not mitochondria, during development. As a result, nuclear DNA analysis of hair is frequently unsuccessful. Sequence analysis of hypervariable regions within mtDNA from shed hair shafts offer an alternative, but less discriminating, approach.

mtDNA contains two hypervariable regions that are used for human identification purposes: the 342bp HVI region and 268bp HVII region. HVI and HVII polymorphisms arise through random mutation and are inherited through the maternal lineage. Thus, mtDNA analysis cannot distinguish between people of the same maternal lineage. mtDNA sequence variations, or haplotypes, are identified by sequencing the HVI and HVII regions and comparing these sequences to a reference sequence. Any nucleotides that differ from this standard are noted. However, mtDNA analysis is complicated by the fact that not all mitochondria within an organism or even a single cell have exactly the same mtDNA sequence. This heterogeneity, known as heteroplasmy, may be present as single nucleotide substitutions or variations in the length of the hypervariable region.

II. DNA Purification

Any biological material is a potential source of DNA for analysis. However, the success of DNA typing often depends on the quality and nature of the sample. In the past, DNA-typing efforts focused on samples that had a high probability of providing relatively large amounts of intact DNA and yielding a full DNA profile. However, trace evidence samples, which have limited amounts of biological material, are increasingly common in forensic laboratories due to the sensitive nature of STR typing. DNA profiles can be successfully generated from trace samples such as fingerprints, saliva and sweat stains. While success rates for analysis of these trace samples are increasing, some samples still do not yield adequate DNA amounts for analysis, and even if DNA yields appear high, the DNA may be degraded to the point where amplification is impossible. This is often the case when samples are exposed to the environment for long periods of time. Environmental exposure is not kind to DNA. Many biological samples are ideal substrates for the growth of bacteria and other microorganisms, which can degrade DNA. Exposure to ultraviolet light in the form of sunlight can induce pyrimidine dimers, which can inhibit PCR. Other PCR inhibitors can be introduced by the environment (e.g., humic acid in soil), by the substrate on which the sample is deposited (e.g., indigo dye from denim) or by the sample itself (e.g., hematin from blood samples). The ability to purify DNA free of these inhibitors can be critical to STR analysis success.

Samples collected during an investigation are often collected on a cotton swab or other solid support. The first step to purify DNA from these samples involves removing the biological material from the solid support, typically by soaking the material in an aqueous buffer. Some samples, such as blood and semen, require a proteinase K digestion at this step for maximum DNA yield and quality. The solid support is removed by placing the entire sample, including the solid support, into a spin basket assembly and centrifuging so that the liquid flows through the spin basket into a collection tube and the solid support remains in the spin basket. The solid support is discarded, and the aqueous DNA-containing fraction undergoes subsequent purification steps to remove PCR inhibitors and other components that may interfere with DNA quantitation and amplification.

Sample transfer and assembly and disassembly of spin baskets can be tedious and prone to error when large numbers of samples are processed and individual spin baskets are used. The Slicprep™ 96 Device (Cat.# V1391) offers a higher-throughput option that allows simultaneous centrifugation of 96 samples. The device is designed so that both the digestion or cell lysis step and centrifugation are performed in the same device. The Slicprep™ 96 Device consists of 3 components: a 2.2ml 96 Deep Well Plate, a 96 Spin Basket and a U-Shaped Collar. In the digestion position, the 96 Spin Basket is fully inserted into the 96 Deep Well Plate, allowing space for approximately 165µl of solution below the basket in each well. After the incubation, the baskets are raised approximately 1cm (the spin position) by inserting the U-Shaped Collar to create space for an additional 500µl of solution (Figure 14.1). The device is centrifuged, and the collar and 96 Spin Basket are discarded, leaving the DNA-containing solution in the 96

Deep Well plate. The 96 Deep Well Plate then can be used for manual DNA purification or transferred to an automated workstation for DNA purification.

More information on the Slicprep™ 96 Device....

Common DNA purification methods include organic extraction using phenol:chloroform, Chelex® extraction and the use of a silica magnetic resin. Phenol:chloroform extraction is considered by many DNA analysts to be the "gold standard" of DNA isolation methods. However, this method uses hazardous organic chemicals, is time-consuming, requires multiple centrifugations, may result in significant loss of material, is not amenable to automation and can introduce amplification inhibitors. DNA extraction using Chelex® resin is rapid but does a poor job of removing organic inhibitors. In contrast, magnetic resin-based DNA purification systems are effective at removing PCR inhibitors, do not require organic solvents and can be automated to increase throughput.

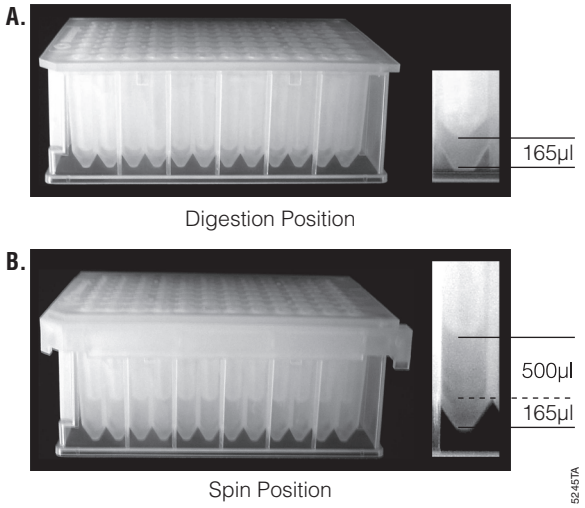

Figure 14.1. Operational modes of the Slicprep™ 96 Device. Panel A. The digestion position. The 96 Spin Basket is fully inserted into the 96 Deep Well Plate to allow incubation of solid supports with digestion or lysis buffer. **Panel B.** The spin position. The U-Shaped Collar is inserted between the 96 Deep Well Plate and 96 Spin Basket. This raises the baskets and allows an additional 500µl below the baskets.

A. DNA IQ™ System—An Example of a Magnetic Resin

The DNA IQ™ System uses a silica-based paramagnetic resin to isolate DNA from liquid samples and samples on solid supports. The DNA IQ™ System will isolate all DNA types present in a sample, including nuclear and mitochondrial DNA. Two protocols exist for the manual format, one for database samples and another for forensic casework samples. For samples with abundant DNA, such as buccal swabs collected as reference samples, the amount of DNA IQ™ Resin used is limiting so that the resin becomes saturated and any excess DNA is not bound, leading to the isolation of a consistent amount of DNA from all samples. For casework samples with limited DNA amounts, the resin is not saturated, and the amount of DNA isolated is limited only by the amount of starting material.

The DNA IQ™ System allows DNA isolation from a wide variety of sample types, including blood and blood stains, semen and semen stains, chewing gum, hair, bone, urine, cigarette butts, buccal swabs and blood card punches.

View a more comprehensive list of sample types processed using the DNA IQ™ System...

Hair, bone, sperm and tissue masses, including formalin-fixed, paraffin-embedded tissue, require proteinase K digestion to obtain reliable amounts of DNA. The Tissue and Hair Extraction Kit (for use with DNA IQ™) (Cat.# DC6740) includes proteinase K and DTT, which aid in the break up of tissue, hair and bone samples prior to DNA purification using the DNA IQ™ System.

The paramagnetic DNA IQ™ Resin is amenable to automation, and the DNA IQ™ System has been automated on a number of robotic platforms. More information about automated DNA purification can be found below

Additional Resources for DNA IQ™ System

Technical Bulletins and Manuals

TB296	*DNA IQ™ System—Small Sample Casework Protocol*
TB297	*DNA IQ™ System—Database Protocol*
TB307	*Tissue and Hair Extraction Kit (for use with DNA IQ™) Technical Bulletin*

Promega Publications

The DNA IQ™ System on the Tecan Freedom EVO® 100

Forensic extraction and isolation of DNA from hair, tissue and bone

DNA IQ™ System "frequently asked questions"

DNA IQ™: The intelligent way to purify DNA

Genomic DNA purification from cigarette butts and buccal swabs using the DNA IQ™ System

III. Differential Extraction

Sexual assault swabs make up a large percentage of samples submitted for DNA analysis. Processing of these samples involves separating sperm cells, which contain male DNA, and epithelial cells, which are mostly female-derived so that female DNA will not obscure the profile of the male DNA contributor. In 1985, Gill *et al.* (Gill *et al.*, 1985) developed a method to selectively enrich for sperm cells in the presence of an excess of epithelial cells. In the absence of dithiothreitol (DTT), detergent/proteinase K treatment preferentially lyses epithelial cells, converting the large excess of epithelial cells into soluble DNA. Sperm are resistant to lysis under these conditions. Proteinase K digestion also loosens the attachment of sperm to solid supports and reduces the degree of sperm and epithelial cell clumping, increasing yields and allowing better separation (Tereba *et al.*, 2004). The proteinase K-digested sample is centrifuged to efficiently pull sperm out of the

cotton fibers, extract the entire volume of epithelial DNA-containing buffer from the matrix and pellet intact sperm at the bottom of the tube. The aqueous phase is removed as the epithelial fraction, and the sperm pellet is washed and recentrifuged to remove any residual epithelial DNA-containing buffer prior to sperm lysis. Multiple rounds of washes and centrifugations may be required to obtain a sperm pellet relatively free of epithelial DNA.

One limitation of this traditional method is the inability to efficiently separate soluble DNA from the cell pellet. In addition, the high number of washes and centrifugations is laborious and time-consuming and necessitates a delicate balance between clean separation and loss of sperm. In spite of these issues, this method remains the preferred method in many forensic laboratories. However, the amount of time required to process sexual assault samples using this method has resulted in a steady increase in sample backlogs. Unfortunately, traditional differential extraction methods to process sexual assault samples are not amenable to automation.

To increase the throughput of differential extraction, Promega has developed the Differex™ System (Cat.# DC6800). Whereas traditional differential extraction methods use serial washes and centrifugations to separate sperm and epithelial cells, the Differex™ System uses a combination of phase separation and differential centrifugation. This protocol uses only one centrifugation instead of four or more for traditional methods, and because fewer sperm cells are lost, DNA yields are higher.

The Differex™ protocol starts by placing the entire proteinase K-digested sample in a spin basket seated in a tube containing a nonaqueous Separation Solution. Alternatively, for large numbers of samples, the Slicprep™ 96 Device can be used instead of individual spin baskets. The Separation Solution is not miscible with water and is more dense than water but less dense than sperm. During centrifugation, the sperm are pulled from the solid matrix and form a tight pellet at the bottom of the tube under the Separation Solution. The soluble DNA remains in the aqueous buffer, which forms a layer on top of the Separation Solution. The aqueous buffer containing the epithelial DNA is removed, and the DNA purified using the DNA IQ™ chemistry. The Separation Solution is washed to eliminate the thin film of epithelial DNA-containing buffer at the interface between the two layers and any droplets on the side of the tube. Up to half of the Separation Solution can be removed to eliminate any cell debris that is present at the interface between the two solutions. After washing, the tube contains the sperm pellet and remaining Separation Solution. Two or more volumes of DNA IQ™ Lysis Buffer containing DTT are added to lyse the sperm and solubilize the Separation Solution. The DNA IQ™ Resin is added, and the sperm DNA is purified using the DNA IQ™ chemistry. The Differex™ separation and subsequent DNA purification require as little as 2 hours. An adaptation of this process for automated differential extraction is described below.

Additional Resources for Differex™ System

Technical Bulletins and Manuals

TBD020 *Differex™ System Technical Bulletin*

TM331 *Differex™ System—For Use With the Differex™ Magnet Technical Manual*

Promega Publications

Tech Tips: The Differex™ System

A new, rapid method to separate sperm and epithelial cells

IV. DNA Quantitation

DNA purification methods cannot differentiate between human DNA and other DNA (e.g., bacterial and fungal DNA). Therefore, if the sample is not pristine and you want to determine the concentration of human genomic DNA present, you will need to use a human-specific DNA quantitation system. In forensics, the most common systems are based on real-time PCR and harness fluorescently labeled oligonucleotide probes or primers to detect and quantitate a PCR product in real time. These systems employ two different fluorescent reporters and rely on energy transfer from one reporter (the energy donor) to a second reporter (the energy acceptor) when the reporters are in close proximity. The second reporter can be a quencher or a fluor. Most commonly, the second reporter is a quencher, and the energy from the first reporter is absorbed but re-emitted as heat rather than light. The progress of the reaction can be monitored as the change in fluorescence of the energy donor. During the exponential phase of PCR, the change in fluorescence is proportional to the accumulation of PCR product. To simplify quantitation, specially designed instruments perform both the thermal cycling steps to amplify the target and fluorescence detection steps to measure the change in fluorescence in real time.

More information about quantitative real-time PCR...

One quantitative PCR (qPCR) approach employs hydrolysis probes that are complementary to the accumulating PCR product and are labeled with a fluor at the 5′-end and a quencher at the 3′-end. Because the two reporters are in close proximity, the fluorescent signal is quenched. During the annealing step, the probe hybridizes to PCR product generated in previous amplification cycles. The resulting probe:target hybrid is a substrate for the 5′→3′ exonuclease activity of the DNA polymerase, which degrades the annealed probe and liberates the fluor (Holland *et al.* 1991). The fluor is freed from the effects of the energy-absorbing quencher, and the progress of the reaction and accumulation of PCR product is monitored by the resulting increase in fluorescence.

The Plexor® HY System takes a different approach, one that does not require a hydrolysis probe, to determine the concentration of total human DNA and male human DNA simultaneously in one reaction. This qPCR system takes advantage of two novel bases, isoguanine (iso-G) and 5′-methylisocytosine (iso-C), which form a unique base pair

in double-stranded DNA. Amplification of each target uses two primers: one primer is synthesized with an iso-C residue as the 5′-terminal nucleotide and a fluorescent label at the 5′-end; the second primer is unlabeled. The reaction contains a fluorescent quencher covalently linked to iso-G. As amplification proceeds, fluorescence is reduced by site-specific incorporation of the fluorescent quencher opposite the iso-C and in close proximity to the fluorescent dye located on the end of the primer.

By measuring the reduction in signal during each PCR cycle, an amplification curve is generated. An amplification threshold is set within the exponential phase of PCR at a fluorescence level where all amplification curves exhibit the most significant signal decrease. To calculate the DNA concentration of an unknown sample, the cycle threshold (C_T), the point at which the amplification curve crosses the amplification threshold, is determined and compared to a standard curve. The standard curve is generated by determining the C_T values for a dilution series of a sample of known DNA quantity and plotting the log concentration on the X axis and cycle threshold on the Y axis. After amplification, a melt analysis can be performed to confirm that the correct product was amplified and expedite troubleshooting, if necessary. The kit contains an internal PCR control (IPC) to test for false-negative results that may occur in the presence of PCR inhibitors.

The Plexor® HY System is a sensitive multiplex kit that routinely detects approximately 6.4pg of total DNA. PCR setup is performed at room temperature and is compatible with automated platforms. The Plexor® HY System is optimized for use on the Applied Biosystems 7500 and 7500 FAST real-time PCR systems and Stratagene Mx3005P® and Mx3000P® qPCR systems. Protocols also exist for other qPCR instrumentation. These real-time PCR instruments are not designed to interpret a decrease in fluorescence, so specific software is required to make sense of raw Plexor® HY data. The Plexor® Analysis Software interprets these data and allows you to quickly and easily review data and create reports. Replicate samples are automatically averaged, template amounts are calculated and the necessary volume of DNA is displayed for your optimized STR amplification conditions in the Forensic Report. The forensic release of the Plexor® Analysis Software is available for free download.

Additional Resources for DNA Quantitation

Technical Bulletins and Manuals

TM293, TM294, TM296, TM299	*Plexor® HY System Technical Manuals*
EP037	*Automated Plexor® HY System Setup for the Biomek® 3000*

Promega Publications

The Plexor® HY System: Not solely a quantitation technique

The hidden benefits of real-time PCR: Assessing and addressing qualitative challenges

The Plexor™ Analysis Software

Development of a novel, fluorescent, two-primer approach to quantitative PCR

Developmental validation of a real-time PCR assay for the simultaneous quantification of total human and male DNA

Validation Guide for the Plexor® HY System

V. STR Analysis

A. STR Amplification and Detection

STR loci are amplified using fluorescently labeled PCR primers that flank the hypervariable regions. One of the biggest strengths of PCR-based DNA typing is the degree to which DNA can be amplified. Starting with a single DNA molecule, millions or billions of DNA molecules can be synthesized after 32 cycles of amplification. This level of sensitivity allows scientists to extract and amplify DNA from minute or damaged samples and obtain useful DNA profiles.

STR amplification systems can accommodate a range of template DNA concentrations. Most of the Promega PowerPlex® STR systems provide optimal sister allele balance and locus-to-locus balance with 0.5–1.0ng of DNA template, and studies performed at Promega show that full profiles can be observed with less than 100pg (Ensenberger and Fulmer, 2009; McLaren, 2007; Krenke *et al.* 2005; Krenke *et al.* 2002). However, amplification and detection instrumentation can vary. You may need to optimize protocols, including cycle number and detection conditions (e.g., injection time or loading volume), for each laboratory instrument. Most of the PowerPlex® systems use a thermal cycling program with 30 or 32 cycles for 0.5–1ng of purified DNA template. For larger amounts of input DNA (i.e., FTA® paper) or to decrease sensitivity, fewer cycles should be evaluated. In-house validation should be performed to determine the optimal amplification and detection conditions.

The sensitive nature of PCR works in a lab's favor, but it can cause problems if great care is not taken to avoid contaminating the reaction with exogenous DNA. Three main categories of exogenous DNA have the biggest impact on DNA-typing laboratories: 1) DNA from the analyst, 2) DNA from other samples in the lab and 3) allelic ladder fragments. DNA from nonhuman sources, such as bacteria and fungi, will not be amplified and detected because STR systems are species-specific. Extreme care must be taken to avoid cross-contamination when preparing sample DNA, handling primer pairs, assembling amplification reactions and analyzing amplification products. Reagents and materials used prior to amplification should be stored separately from those used following amplification. Amplification reactions should be assembled in a room

dedicated for reaction setup, using equipment and supplies reserved for amplification setup. We highly recommend the use of gloves and aerosol-resistant pipette tips. To detect reagent contamination, assemble a negative control reaction (i.e., no template) for every set of reactions, and scrutinize the reactions for the presence of unexpected peaks. After setting up reactions, wash all surfaces with a dilute bleach solution.

After assembly, reactions are subjected to thermal cycling, and PCR products are separated by size using specialized polyacrylamide gel electrophoresis or capillary electrophoresis (CE) instruments. During electrophoresis, DNA molecules move through a polymer matrix in response to an electric field. The rate of migration depends on fragment size, with smaller DNA fragments migrating more quickly through the porous matrix than larger fragments. The instruments use a laser near the anode of the capillary or polyacrylamide gel to excite and detect the fluorescent PCR products, which appear as peaks on the resulting electropherogram (Figure 14.2). Each fluor is detected in a separate dye channel. To reduce background, spectral calibration must be performed to correct spectral overlap of dyes.

Size separation of amplification products in parallel with separation of an allelic ladder, which consists of all major alleles at a particular locus, allows positive identification of each allele that makes up the DNA profile. An internal size standard is included in each analysis to control for run-to-run variation in migration.

More information about the background theory and principles of capillary electrophoresis...

B. Data Analysis

Specialized allele-calling software eliminates the need for visual comparisons to assign alleles, making the process faster, easier and more accurate. However, the software does not address data quality, making a review of each sample necessary to judge data quality and detect DNA mixtures or contamination. These reviews can be performed manually by DNA analysts, but this is often tedious and time-consuming. In many laboratories, data review has become the bottleneck for STR analysis now that other DNA-typing steps are being automated. This has resulted in an increased interest in expert system software. Expert systems for DNA-typing laboratories are defined as systems that increase the efficiency or effectiveness of genotyping samples by automatically interpreting STR data. An expert system can provide a second set of independent allele calls and allow the analyst to skip the review of sample data that the software has judged to be of high quality and focus on problematic samples. Expert systems can objectively evaluate DNA mixtures in seconds and present an analyst with the best-fit combinations of DNA profiles. Expert systems also can compare all alleles called in a sample to the alleles in every other sample in the batch and flag potential contamination events. Finally the software can summarize the data and generate reports that contain only the necessary information.

C. Statistical Analysis

Once a DNA profile is generated, statistical analysis is performed to determine if the profile matches, with a reasonable degree of scientific certainty, that of a known reference sample. The analyses performed depend on the type of STR locus.

Autosomal STR analysis employs the product rule, which calculates the probability of finding a specific DNA profile within a population by multiplying the frequencies of the alleles at each STR locus analyzed. This probability represents the number of randomly selected individuals that theoretically must be surveyed before finding the same DNA pattern in a population. To use the product rule, the STR loci must be unlinked.

Autosomal STR analysis has a high statistical power of discrimination. For example, when analyzing the 15 STR loci amplified by the PowerPlex® 16 System, the power of discrimination is as high as 1 in 1.42×10^{18} for some ethnic groups; this exceeds the current global human population, making it unlikely that two DNA profiles will match at random.

Statistical analysis can be used to determine if two individuals are related, as is done for paternity testing and in missing persons investigations where a reference sample from the missing person is not available but reference samples from close relatives exist. For paternity testing, the paternity index is calculated to estimate how many more times likely it is that the person being tested is the biological father, rather than a randomly selected individual. The typical paternity index is assigned to a locus rather than an individual case. The $PI_{typical}$ of several loci is the product of the individual $PI_{typical}$ values. Generally, a $PI_{typical}$ of less than one is indicative of nonrelatedness.

The $PI_{typical}$ is represented by the following equation:

$$PI_{typical} = X/Y$$

where X represents the chance that the alleged father transmitted the allele to the child and Y represents the chance that some other man transmitted the allele.

For more information about statistical analysis of DNA testing results, refer to *Forensic DNA Typing* (Butler, 2005).

Y-STR loci used in forensic and paternity applications are located in the nonrecombining region of the human Y chromosome (NRY) and are faithfully transmitted from father to son, unchanged except for rare mutations. Because Y-STR loci are not inherited independently, statistical analysis cannot proceed by the product rule. Instead, the frequency of a particular Y-STR profile (haplotype) is estimated by the counting method, which is based on the frequency of a specific haplotype in a population. To be useful, Y-STR databases must include thousands of Y-STR haplotypes, enough that a sufficient number of haplotypes is represented and a meaningful estimate of the frequency

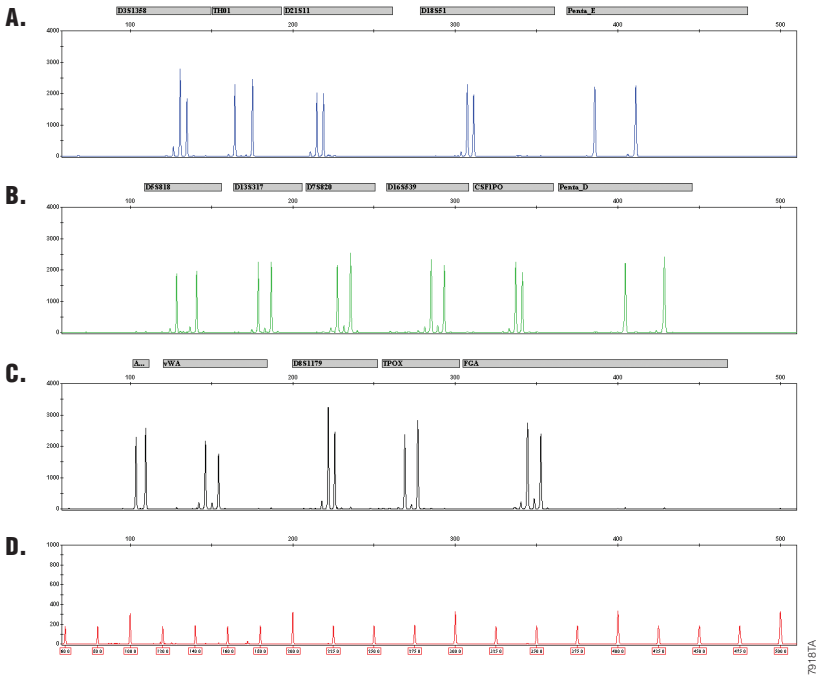

Figure 14.2. Representative PowerPlex® 16 HS System data. A single template DNA (0.5ng) was amplified using the PowerPlex® 16 HS 10X Primer Pair Mix. Amplification products were mixed with Internal Lane Standard 600 and analyzed with an Applied Biosystems 3130 Genetic Analyzer using a 3kV, 5-second injection. Results were analyzed using GeneMapper® *ID* software, version 3.2. **Panel A.** An electropherogram showing the peaks of the fluorescein-labeled loci: D3S1358, TH01, D21S11, D18S51 and Penta E. **Panel B.** An electropherogram showing the peaks of the JOE-labeled loci: D5S818, D13S317, D7S820, D16S539, CSF1PO and Penta D. **Panel C.** An electropherogram showing the peaks of the TMR-labeled loci: Amelogenin, vWA, D8S1179, TPOX and FGA. **Panel D.** An electropherogram showing the 60bp to 500bp fragments of the Internal Lane Standard 600.

of a specific haplotype can be made. Several online databases are available to assess Y-STR haplotype frequency:

U.S. Y-STR database

Y-HRD database

Additional Resources for STR Analysis

Technical Bulletins and Manuals

TMD028	*PowerPlex® ESI 17 System Technical Manual*
TMD027	*PowerPlex® ESI 16 System Technical Manual*
TMD024	*PowerPlex® ESX 17 System Technical Manual*
TMD023	*PowerPlex® ESX 16 System Technical Manual*
TMD022	*PowerPlex® 16 HS System Technical Manual*
TMD021	*PowerPlex® S5 System Technical Manual*
TMD018	*PowerPlex® Y System Technical Manual*
TMD017	*PowerPlex® ES System Technical Manual*
TMD016	*PowerPlex® 16 BIO System Technical Manual*
TMD012	*PowerPlex® 16 System Technical Manual*
TMD006	*GenePrint® Fluorescent STR Systems Technical Manual*

Promega Publications

The PowerPlex® 16 HS System.

Bringing laser microdissection and on-chip PCR together.

A solution for the split peak and n–10 artifacts at the vWA locus in PowerPlex® 16 and PowerPlex® ES Systems.

Useful resources for your laboratory.

An interview with Sir Alec Jeffreys.

An introduction to PCR inhibitors.

Debunking some urban legends surrounding validation within the forensic DNA community.

Nanotechnology and its potential in forensic DNA analysis.

Identifying and preventing DNA contamination in a DNA-typing laboratory.

Using GeneMapper® *ID* with Promega STR systems.

Comparison of multiple STR platforms and instrumentation.

The Penta BEC multiplex primers from Promega: Additional loci available for identity testing.

Tech Tips: The PowerPlex® Y System.

Validation questions and answers.

Troubleshooting capillary electrophoresis systems.

PowerPlex® 16 System validation.

VI. Automation

In the past, law enforcement officers often limited the forensic casework samples submitted for analysis to samples that had a good chance of producing a DNA profile. More recently, realizing the sensitivity of STR analysis, officers are submitting more trace samples with the hope that a DNA profile can be generated. Also, many law enforcement agencies now are collecting database samples from an increasing number of individuals such as arrestees in addition to convicted offenders. These factors increase the number of samples that require processing. To keep pace with the increased workload, many laboratories are automating key, potentially rate-limiting steps to increase throughput. Such steps include differential extraction, DNA isolation, DNA quantitation and normalization, and PCR setup.

Automation workstations vary in their capacities, from the low-throughput Biorobot EZ1™ (Qiagen), which can purify DNA from up to six samples simultaneously in approximately 20 minutes, and medium-throughput Maxwell® 16 Instrument (Cat.# AS3060), which can process up to 16 samples simultaneously in the same amount of time, to high-throughput workstations, such as the Beckman Coulter Biomek® laboratory automation workstations and Tecan Freedom EVO® liquid handlers, which can process up to 96 samples simultaneously in as little as 40 minutes.

A. Maxwell® 16 Instrument

The Maxwell® 16 Instrument is a magnetic-particle-handling device designed for low- to medium-throughput automated DNA purification from a wide range of sample types. Unlike liquid handlers, which move samples and reagents to the purification medium, magnetic-particle handlers move the purification medium through each step in the purification process. Using magnets to move the purification medium eliminates issues with clogged tips and incomplete liquid transfers. The instrument is preprogrammed with purification protocols and uses prefilled reagent cartridges, maximizing simplicity and convenience. The instrument processes up to 16 samples per instrument run. There are two configurations of the Maxwell® 16 Instrument. The SEV (standard-elution volume) system elutes DNA in 300µl of elution buffer and is suitable for DNA isolation from reference samples. The LEV (low-elution volume) system can elute DNA in 25–50µl of elution buffer and is suitable for forensic casework samples.

The DNA IQ™ Reference Sample Kit for Maxwell® 16 (Cat.# AS1040) allows DNA extraction from buccal swabs, FTA® blood card punches, liquid blood or other DNA reference samples using the Maxwell® 16 SEV Instrument. The kit contains the trusted DNA IQ™ System reagents and is optimized to yield a final DNA concentration that minimizes the need for concentration or dilution prior to amplification. Liquid samples are placed directly into the cartridges, while samples on solid supports require a preprocessing step in DNA IQ™ Lysis Buffer and removal of the solid support before the samples are placed into the cartridges. High-quality genomic DNA is obtained in approximately 20 minutes, at a concentration suitable for direct use in STR analysis.

The DNA IQ™ Casework Pro Kit for Maxwell® 16 (Cat.# AS1240) allows DNA extraction from forensic casework samples, including blood stains, semen stains, hairs, cigarette butts, tissue samples, and trace or "touch" DNA samples regularly encountered in forensic DNA analysis. The kit contains the DNA IQ™ reagents and uses the Maxwell® 16 LEV Instrument with plastic cartridges and plungers that allow DNA elution in a final volume of up to 50µl, providing a final DNA extract in a concentrated format.

B. High-Throughput Automation Workstations

There are many robotic platforms that can dispense reagents, aspirate liquids and perform manipulations of multiwell plates, including the Beckman Coulter Biomek® laboratory automation workstations and Tecan Freedom EVO® automated liquid-handlers. These high-throughput instruments are significantly larger and more expensive than low- and medium-throughput instruments. They also are complex to use and require detailed knowledge of how the instrument operates, but their increased flexibility makes them adaptable to a greater variety of tasks in the laboratory.

The Biomek® workstations use multichannel tools to pipet volumes from 1µl to 1ml with high precision. Disposable pipette tips address concerns about contamination during liquid-transfer steps. Beckman Coulter offers a range of instruments, including the Biomek® 3000, Biomek® NXP and Biomek® FXP, with a range of options, capabilities, throughput levels and price points for laboratories looking to automate their processes. With single-channel and eight-channel pipetting, plate-moving capabilities, sample-tracking functionalities and a rich programming language, the Biomek® 3000 is the lowest cost option of these three instruments. Both the Biomek® NXP and FXP workstations offer independent eight-channel pipetting and gripping capabilities as well as flexible instrument configuration. The Biomek® NXP represents an intermediate cost and throughput level; the Biomek® FXP platform adds a 96-well pipetting head to the capabilities of the Biomek® NXP. With increased size and pipetting capabilities, the Biomek® FXP offers the highest flexibility and throughput of the Beckman Coulter workstations.

The Tecan Freedom EVO® automated liquid-handler boasts independent liquid-handling and gripping arms, adjustable tip spacing, independent volume control on each tip and disposable-tip volumes of up to one milliliter. In addition, the Freedom EVO® supports multiple deck sizes and tip configurations, from a lower cost, 4-tip format to a higher throughput, 8-tip instrument.

Promega has developed methods for these instruments to automate several steps of the DNA-typing process, including differential extraction, DNA purification using the DNA IQ™ System, DNA quantitation, DNA normalization and PCR setup.

C. Automated DNA Purification

Magnetic beads, such as the silica-coated DNA IQ™ Resin, can be used to separate DNA from cellular debris and are ideally suited to automation. In addition to the standard pipetting tools, robotic workstations can be equipped with a magnet, shaking platform and thermal exchange unit to perform DNA-binding steps, washes and heated elution. DNA can be isolated from both database and casework samples without detectable cross-contamination using the DNA IQ™ System in combination with the Biomek® or Freedom EVO® workstation. Database samples that have been automated successfully include buccal swabs, blood punches (FTA® and S&S 903) and liquid blood; casework samples include sperm and epithelial fractions from differential extractions, tissue samples, hairs and blood spots on blue jeans and underwear, among others.

The Biomek® 2000 and 3000 instruments enable the user to purify DNA from 8 to 96 samples (in increments of 8) from either "aqueous" or "lysis" samples. Aqueous sample types are typically those that are preprocessed by incubating with proteinase K prior to extraction, whereas lysis samples are preprocessed by incubating in DNA IQ™ Lysis Buffer. For the Biomek® 2000, different methods were developed for different numbers of samples being processed (i.e., one method for 8 samples, one for 16, etc.) as well as for each sample type. The net result is that 24 methods exist for 8 to 96 aqueous or lysis samples. For the Biomek® 3000 this number is reduced to one method. This single method allows the user to choose between aqueous and lysis sample types and designate the number of samples being processed. Promega also has developed and optimized scripts for DNA isolation on the 4-tip and 8-tip Freedom EVO® instruments using the DNA IQ™ System. These scripts are able to process 1–96 aqueous samples and lysis samples.

Additional Resources for Automated DNA Purification

Technical Bulletins and Manuals

TB347	*DNA IQ™ Reference Sample Kit for Maxwell® 16 Technical Bulletin*
TM332	*DNA IQ™ Casework Pro Kit for Maxwell® 16 Technical Bulletin*

Promega Publications

The Maxwell® 16 Low Elution Volume System for forensic casework: Implementation and routine use in a forensic laboratory.

DNA extraction using the Tecan Freedom EVO® 200 and DNA IQ™ System.

The Maxwell® 16 applications database.

Automation in a forensic laboratory: An update.

Forensic application of the Maxwell™ 16 Instrument.

The DNA IQ™ System on the Tecan Freedom EVO® 100

Automating the DNA IQ™ System on the Biomek® 3000 laboratory automation workstation

Extraction and isolation of DNA from blood cards and buccal swabs in a 96-well format

Validation Guide for the DNA IQ™ Reference Sample Kit for Maxwell® 16.

Validation Guide for the DNA IQ™ Casework Pro Kit for Maxwell® 16.

D. Automated Differential Extraction

The Automated Differex™ System allows high-throughput, automated differential extraction of sexual assault samples and integrates with the DNA IQ™ System for subsequent DNA purification. Up to 48 samples can be processed in less than 5 hours using the automated Differex™ protocol; 40 samples can be processed if wells are reserved for DNA standards in downstream DNA quantitation. Two independent, but related, automated methods are used to process differential extractions: the automated Differex™ method and automated DNA IQ™ for Differex™ method. The automated Differex™ method involves separating samples into epithelial and sperm fractions, while the automated DNA IQ™ for Differex™ method purifies genomic DNA from each fraction. The protocol begins with a proteinase K digestion of swab samples in a Slicprep™ 96 Device, followed by centrifugation to pellet the sperm and separate digestion products from the solid support material. The plate containing the sperm pellets and supernatants is placed onto the deck of a robotic workstation, and the automated Differex™ method begins. Automated Differex™ protocols exist for the Biomek® 2000 and Biomek® 3000 workstations and Tecan Freedom EVO® 100 liquid handler.

The automated Differex™ method combines the Differex™ System reagents and DNA IQ™ Resin in a novel pellet-capping process. First, the robot dispenses DNA IQ™ Resin directly on top of each sperm pellet. Upon application of a magnetic field, the paramagnetic resin particles form a capping layer, which allows supernatant manipulation without pellet disruption (Figure 14.3). A portion of the supernatant is removed from each sample well and moved to an adjacent well for downstream processing as the epithelial fraction. The remaining supernatant is removed from the pellet and discarded or, if desired, archived. The resin-capped sperm pellets are washed four times to dilute

and remove any residual epithelial DNA-containing supernatant. During the third wash, the resin-capped sperm pellets are resuspended to release any trapped epithelial material. Resuspending the pellets requires a manual step to centrifuge the sample plate and pellet the sperm. Following this centrifugation the robot adds a second aliquot of DNA IQ™ Resin to re-establish the pellet cap. The Differex™ Separation Solution is used to float the epithelial DNA-containing wash buffer away from the capped pellet to further improve epithelial DNA removal. The fourth and final wash is performed to complete the automated Differex™ extraction. The sperm and epithelial fractions then undergo automated DNA isolation using the DNA IQ™ System. Integration of DNA IQ™ and Differex™ System components results in higher sperm fraction yields and reproducible, automated sexual assault sample processing.

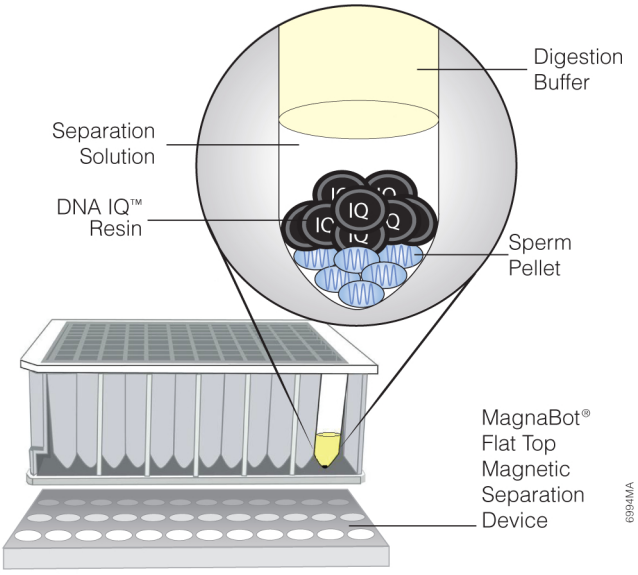

Figure 14.3. The Automated Differex™ System. The automated Differex™ method uses DNA IQ™ Resin to cap the sperm pellet after differential lysis and centrifugation. The MagnaBot® Flat Top Magnetic Separation Device immobilizes the DNA IQ™ Resin, holding the sperm pellet in place during automated wash steps. The Separation Solution helps remove any remaining digestion buffer and epithelial DNA from the sperm pellet.

Additional Resources for Automated Differex™ System

Technical Bulletins and Manuals

EP030	*Automated Differex™ System Protocol for the Tecan Freedom EVO® System*
EP031	*Automated Differex™ System Protocol for the Beckman Coulter Biomek® 2000*
EP032	*Automated Differex™ System Protocol for the Beckman Coulter Biomek® 3000*

Promega Publications

Automating the Differex™ System.

E. Automated DNA Normalization and PCR Setup

Promega has worked in conjunction with Beckman Coulter to develop a tool for DNA normalization and PCR setup on the Biomek® 2000 workstation. The Genetic Identity version of the Normalization Wizard, available from Beckman Coulter, dilutes DNA samples at different concentrations to the desired final concentration, then assembles the amplification reactions. The robot transfers PCR master mix to strip tubes or plates, then adds a fixed volume of the normalized DNA. The samples are capped manually and placed directly in a thermal cycler for amplification.

Additional Resources for DNA Normalization and PCR Setp

Promega Publications

Automated DNA normalization and STR multiplex setup methods.

VII. References

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