5-2011

Forensic DNA Analysis and the Validation of Applied Biosystems 3730 DNA Analyzer and GeneMapperID-X Software for STR Analysis

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Honors College Thesis

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May 12, 2011

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I. Introduction

With the exception of identical twins, no two individuals have identical DNA. For this reason, DNA profiling has revolutionized the field of forensic science over the past 25 years. However, any forensic technique used within a laboratory must be based on solid science and stringent procedures, to ensure obtained results will withstand scrutiny in a courtroom. Internal validations of new reagents, instrumentation, or software used within a forensic laboratory are imperative to ensure that results are accurate and reliable. The DNA Databank section of the New York State Police Forensic Investigation Center is currently in the process of validating an Applied Biosystems 3730 DNA Analyzer to be used in conjunction with GeneMapper*ID-X* software for short tandem repeat analysis.

II. History

DNA profiling was introduced in 1985 by Alec Jeffreys, an English geneticist. He and his colleagues discovered areas of high variability between individuals within the genetic code of DNA. These variable number tandem repeats (VNTRs) were applied to forensics a year later, when Jeffreys used DNA testing to exonerate a suspect and capture the perpetrator in the Pitchfork murder case (National Library of Medicine, 2006). The technique he used was restriction fragment length polymorphism (RFLP), which used a restriction enzyme to cut specific regions of DNA surrounding the VNTRs (Human Genome Project). DNA profiling techniques have since reached the forefront of forensic science, and more modern methods of identification have replaced RFLP-based analysis.

III. Background

Deoxyribonucleic acid, more commonly known as DNA, is the genetic material present in all cells of every living organism. A human contains around 100 trillion cells, all of which contain identical DNA within the nucleus. DNA contains the necessary information to replicate cells and construct proteins and determines a person's physical features (Butler, 2010). The nucleotides that make up DNA are composed of a 2'-deoxyribose sugar, a phosphate, and a nucleobase. The four nucleobases, adenine, thymine, cytosine, and guanine, are most commonly represented by A, T, C and G. Nucleic acids are linear polymers of these nucleotide units, with alternating sugar and phosphate units connected by ester links that form a structural backbone

(Hallick, 1995). *Figure 1 in the Appendix shows the structure of a nucleic acid.* The sequence of nucleobases is always listed from the phosphate 5' end to the hydroxyl 3' end, to ensure common directionality (Butler, 2010). The DNA molecule is usually found double-stranded in a helix shape, with two complementary nucleic acids linked together through hydrogen bonding between the bases. Due to steric constraints, only specific pairing of the bases occurs: adenine and thymine are connected by two hydrogen bonds, while guanine and cytosine connect via three hydrogen bonds (Hallick, 1995). This ensures that a two-ring purine base and a one-ring pyrimidine base are always across from each other, keeping the diameter of the helix consistent (Clark, 2007). *Figure 2 in the Appendix shows the structure of DNA.* Due to the restrictions of base pairing, the sequence of a DNA strand can be determined by the base sequence of its complementary strand, which forms the basis of DNA replication.

Nuclear DNA is divided into chromosomes, which are dense packets of DNA that form before cell division. The chromosomes in body cells are found in a diploid state, with one chromosome in each pair provided by each parent. The human genome contains 22 pairs of autosomal chromosomes. Although the DNA sequences in each chromosome that make up an autosomal pair are similar, they are not identical (Stanford University). Humans also contain two sex-determining chromosomes; females contain two copies of the X chromosome, while males contain one X and one Y chromosome. These chromosomes are used for gender determination in DNA analysis, and research on the Y chromosome has led to paternity testing and other applications (Butler, 2010).

The polymorphic markers that vary between individuals are found in noncoding regions of DNA that do not contain the necessary information to construct proteins. The location of a DNA marker is known as a locus, and the Human Genome Project has characterized thousands of loci by their chromosomal location. Each locus contains two alleles, which provide the variability required for DNA identification. Two identical alleles at a locus are considered homozygous, while two different alleles are considered heterozygous (Butler, 2010). DNA profiling characterizes the alleles at a particular locus to generate a genotype. The genotypes for a number of loci are then combined to form a DNA profile, which can be used to compare DNA from different sources.

IV. Short Tandem Repeats

Approximately 0.3% of the DNA base pair sequence varies between individuals, which corresponds to around 10 million nucleotides (Butler, 2010). The two most common variations found in alleles are sequence polymorphisms and length polymorphisms. Sequence polymorphisms involve the replacement of one nucleotide with another. The variability used for forensic DNA analysis, however, is based on length polymorphisms. At particular locations on the DNA molecule, a sequence of two or more nucleotides is repeated several times. The number of repeat units varies between individuals, and this variability is the foundation of DNA profiling. *Figure 3 in the Appendix shows an example of sequence and length polymorphisms.* Genotypes are developed by determining the number of repeat units at each allele of a particular locus. For example, a sample containing one allele with 11 repeat units and one allele with 14 repeat units has a genotype of (11,14) at that locus. To generate a complete DNA profile, the lengths of STRs at several loci are analyzed (Butler, 2010). STRs do not always include the entire repeated unit at the end of the sequence. Alleles that include an incomplete unit are called microvariants (Tilstone, et al.). One common example occurs at the locus known as TH01. While most common alleles contain complete units of the repeat sequence AATG, the allele 9.3 contains nine complete units and an extra three bases (AAT). Because microvariants exist, DNA analysis must provide single base pair resolution to distinguish between closely spaced alleles. Databanking laboratories utilize STR technology to develop DNA profiles from convicted offender samples, which are then searched against DNA profiles from forensic evidence in the Combined DNA Index System (CODIS). Inclusion of a DNA profile in CODIS requires analysis of 13 core loci, to ensure that submitted profiles from every lab are comparable. Commercially available STR kids allow for the amplification of target DNA at all 13 required loci (Butler, 2010). The New York State Police (NYSP) are currently using Applied Biosystems Identifiler kit, which covers 16 STR loci, further decreasing the probability of a random match.

V. Extraction and Quantitation

Before a DNA sample can undergo STR analysis, the DNA from a cheek swab or blood sample must be extracted and quantitated. Extraction removes proteins and other cellular materials that may inhibit DNA analysis, and quantitation is necessary to ensure the correct amount of DNA is present for optimal results during the analytical methods (Butler, 2010).

Saliva swabs are stored on cellulose-based FTA paper, which preserves the DNA sample at room temperature for years. The paper contains a weak base, a chelating agent, anionic detergent and uric acid, which prevent nucleic acid degradation and bacterial growth (Tilstone, et al.). The paper is punched to remove a small section of the stain and placed into a tube, where it is washed and purified. The punch can then go directly to quantitative polymerase chain reaction (qPCR), which collects real-time data during the amplification reaction to determine the amount of amplifiable DNA present (Butler, 2010).

VI. Polymerase Chain Reaction

The polymerase chain reaction was introduced by Kary Mullis in 1985, and has since dramatically revolutionized DNA analysis. PCR produces millions of copies of each DNA segment of interest, allowing for analysis of degraded or low-quality samples. The replication process is based on the complementary attribute of opposing nucleobases in DNA strands. The DNA fragments are first heated to denature the two strands of the double helix. The reaction mixture is then cooled to anneal primers, which target specific regions of the strands. Heating again causes DNA polymerase to extend the primers along the length of the target region by attaching complementary nucleotide units, forming a copy of the original double-stranded DNA. This process is completed by a thermal cycler that raises and lowers the temperature. With each temperature cycle, the DNA present in the reaction mixture doubles. After 32 cycles, the process has generated around a billion copies of the target DNA region (Butler, 2010). Multiplexing, or simultaneously copying more than one target region of DNA, can be done by adding multiple primer sets to the reaction (Tilstone, et al.). The availability of reagent kits containing the necessary reactants has simplified the process in recent years. Kits typically include primers, DNA polymerase, buffer, magnesium ion and deoxynucleotide triphosphates (Tilstone, et al.). The products of PCR are also labeled with five dyes that emit maximum fluorescence at different wavelengths, to be used during the separation process. Quantitative PCR monitors the amount of fluorescent intensity during each thermal cycle, allowing for a determination of the amount of DNA present in the sample (Tilstone, et al.).

VII. Capillary Electrophoresis

Separation of the PCR products is required to distinguish between the numerous fragments of varying lengths. Capillary electrophoresis (CE) replaced gel electrophoresis as the primary method of separation in the mid-1990s. Electrophoresis uses an electric field to separate charged molecules by forcing them through a sieving medium. In a buffer system, DNA fragments readily lose an H^+ ion from the phosphate group, forming an anion. Under an applied voltage, the negatively charged fragments will migrate toward a positive electrode (Butler, 2010). The fragments travel through a capillary filled with a polymer solution that slows down larger fragments, causing separation by size. A laser and a fluorescence detector are used to detect the fragments.

VIII. Applied Biosystems 3730 DNA Analyzer

The Applied Biosystems 3730 DNA Analyzer is a 48-capillary electrophoresis instrument. *Figure 4 in the Appendix shows a diagram of the 3730 instrument.* A polymer solution serves as the sieving medium that separates the fragments by size, and a fresh aliquot of polymer is passed through the capillaries before each run. Samples are loaded into 96-well plates and placed into the autosampler, which can hold up to 16 plates at a time. During the run, DNA molecules are forced onto the capillaries via electrokinetic injection. A voltage is applied while the capillaries are immersed in the liquid DNA samples, transferring the negatively charged fragments onto the capillary (Tilstone, et al.). The amplified PCR product solution also contains salt ions, which compete with the DNA to be loaded onto the capillary. STR kits therefore contain deionized formamide, which dilutes the solution and reduces the ionic strength, aiding in the DNA loading process. Formamide also prevents DNA strands from reannealing into a double helix by lowering the melting temperature of double-stranded DNA (Butler, 2010). Adjacent to the autosampler are a water reservoir, a waste reservoir, and the cathode buffer reservoir. Following the electrokinetic injection, buffer is pushed through the capillaries by the electric field. The DNA fragments migrate with the buffer in the direction of the positively charged anode. The polymer contained with the capillary is Applied Biosystems Performance Optimized Polymer-7 (POP-7). The polymer coats the walls of the capillaries, controlling the flow of the buffer and fragments. It was also developed to separate alleles differing in size by a single base (Tilstone, et al.). The water and waste reservoirs are used to rinse the capillaries in between each

injection. A laser at the end of the capillary excites the fluorophores, illuminating DNA fragments as they pass through the window. The fluorescence is then captured by a charge-coupled device camera, which measures the time span from sample injection to fluorescence detection. The instrument then produces an electropherogram, which plots relative fluorescence units (RFUs) as a function of time (Tilstone, et al.).

In order for genotyping software to separate the fragments into their corresponding dye colors, a spectral calibration is required to detect the dye color of each labeled fragment. The calibration is performed by testing standard fragments labeled with each individual dye to determine the wavelength of the maximum emission of each dye and produce a matrix file (Butler, 2010). *Figure 5 in the Appendix shows an example of a spectral calibration.* The software then uses the matrix file to detect the amount of overlap between the dyes, allowing for a determination of the dye color on each fragment. The use of multiple dyes allows for the simultaneous analysis of several STR loci with similar or overlapping allele sizes, as long as the alleles are labeled with a different fluorescent label (Tilstone, et al.).

The 3730 instrument is entirely automated and does not need to be attended to after the initial sample loading. However, sample preparation is essential to ensure accurate results. Samples and controls are diluted with deionized formamide and an internal size standard. The formamide helps to keep the DNA strands denatured by forming hydrogen bonds with the single strands (Tilstone, et al.). The internal size standard contains several fragments of known size, which are used by the genotyping software to determine the size of the unknown sample fragments. Each 96-well plate also contains two negative controls and two positive controls, to be used for quality assurance purposes.

IX. Genotyping Software

After the size separation of the PCR products, genotyping software is used to transfer the sizing information into the number of repeat units at each locus, developing a DNA profile. The software first uses the spectral calibration to separate each of the fragments into their corresponding dye color. Peaks are then measured in comparison to the baseline to ensure that they are over a user-defined analytical threshold. After a peak has been identified as real data, it is converted from data points in minutes to a DNA size in base pairs by comparison with the peaks in the internal size standard (Butler, 2010). *Figure 6 in the Appendix shows an example of*

the internal size standard peaks. The time-to-size transformation is often done using the Local Southern sizing method, which uses the reciprocal relationship between fragment length and mobility to determine size (Tilstone, et al.). In the five-dye kit utilized by the New York State Police, the size standard is Extended LIZ 500. The standard contains 13 DNA fragments ranging in size from 75 to 500 base pairs and is labeled in orange to be distinguished from the fragments of unknown size.

Sized peaks must then be converted into a number of repeat units, known as an allele call, by comparison with the allelic ladder of the corresponding dye. Allelic ladders are commercially available and contain the commonly observed STR alleles at each locus. *Figure 7 in the Appendix shows an example of an allelic ladder.* Each allele within the ladder has already been identified to contain a specific number of repeat units, allowing for conversion of the unknown fragments into a genotype at each locus (Butler, 2010). Multiple allelic ladders analyzed during a run can be averaged, to decrease the effects of small variations between the ladders. Samples contain either one or two peaks at each locus, depending on whether the alleles are homozygous or heterozygous. After genotypes have been determined at each of the 13 loci required for the CODIS database, the DNA profile is complete. *Figure 8 in the Appendix shows an example of a generated STR profile.*

Although allelic ladders contain commonly observed microvariants, a sample may contain a microvariant not present in the corresponding ladder. These alleles are labeled off-ladder, because they fall between two peaks present in the allelic ladder. A sample containing an off-ladder peak must be verified by rerunning the amplified product, to ensure that the peak is a microvariant and not the result of migration due to temperature fluctuations during capillary electrophoresis (Butler, 2010). Analysts must also look for extra peaks in the data known as artifacts. Spikes are narrow peaks that are often present at the same position in every dye color. They can be a result of a change in voltage or the presence of an air bubble within the capillary. Pull-up peaks occur when the software is unable to completely distinguish between the different dyes used to label the fragments. Stutter products occur due to a minor product formed during PCR amplification (Tilstone, et al.). Careful review of the generated profiles is necessary to detect these and other artifacts and ensure that the software correctly labeled the peaks.

X. Applied Biosystems GeneMapper*ID-X*

Applied Biosystems GeneMapper*ID* replaced the previously used GeneScan and Genotyper, combining the functions of each into one software program. GeneMapper*ID-X* is a recently released software upgrade that offers enhanced functionality to improve the quality of review of STR data. The GeneMapper software has several important features that were not present in earlier software programs, such as the ability to export information in a format compatible with CODIS and automated determination of the quality of a generated profile. GeneMapper*ID-X* can also be used as an expert system, which does not require analyst intervention to generate STR profiles (Butler, 2010).

XI. Internal Validation

A validation demonstrates that an instrument or technique is working properly, so a laboratory can be confident in the accuracy of results. Any new method must be proven to be robust, reliable, and reproducible in order to be introduced into a forensic laboratory (Butler, 2010). Although manufacturers perform developmental validations before releasing new products onto the market, internal validations must be completed by laboratories to ensure that the products will still work effectively within the lab. Internal validations not only verify established procedures, but also adjust the methods and parameters to fit the specific needs of the laboratory. After a procedure or instrument has been implemented into a laboratory, continuous proficiency tests must be performed to demonstrate the successful application of the technique by the forensic analysts (Butler, 2007).

The DNA Databank is performing the internal validations for the 3730 DNA Analyzer and GeneMapper*ID-X* software in accordance with the FBI Quality Assurance Standards for DNA Databanking laboratories. The standards require that extensive testing be performed and include studies for reproducibility, sensitivity, stochastic effects, precision, and contamination. The standards also require that technical and quality assurance procedures be developed and established by the end of the validation. The initial step of the validation process was to develop a method for the instrument and an analysis protocol for the software that would allow a majority of plates to be analyzed efficiently. The 3730 instrument had previously undergone a developmental validation by Applied Biosystems, which provided suggested analysis methods for both the instrument and the software. Several samples underwent analysis by the instrument

and the software using these suggested methods, and the parameters were adjusted to optimize the data.

XII. Data Optimization

The first samples analyzed had several issues that needed to be addressed, including very high allele peaks that caused pull-up, several off-ladder peaks in the first injection of each plate, data peaks overlapping with the primer peak, and a much higher baseline than seen in samples analyzed on the 3130xl instrument. The allele peaks were lowered to a more reasonable height by decreasing the sample injection time, which is how long the capillaries undergo electrokinetic injection to load the samples. The oven temperature of the instrument was also lowered. This slowed down the samples moving through the instrument, pushing the sample peaks further away from the primer peak in the data. Sample data was further improved by adjusting the 3730 instrumental method to more closely mirror the protocol used on the already validated 3130xl instruments. A new spectral calibration was completed, which helped to eliminate many of the pull-up and off-ladder peaks that were a result of overlapping fluorescent dyes. However, a resolution to all issues was not immediately apparent. Several off-ladder peaks remained in samples injected during the first injection of the day. These peaks are likely due to migration. Temperature fluctuations within the environment may affect the speed at which samples migrate through the capillary, which can cause differences in the allelic ladders and produce such peaks. However, migration was not an issue within a single injection, as temperatures remained relatively constant throughout the injection.

The analysis method used in GeneMapper*ID-X* also required several adjustments from the default method provided by Applied Biosystems. The main issue was to determine start and end points of data analysis that would include all allele peaks but remove the primer peak. As the instrumental method was adjusted, data would move through the capillaries at varying speeds, and the software method would have to be adjusted accordingly. The user-defined broad peak threshold had to be raised, to prevent every LIZ size standard peak from being flagged for having too large of a width. Although the flags only alert an analyst to a possible issue and do not necessarily result in a failed sample, an overabundance of flags can be distracting when trying to look through sample data. Another problem was the separation of allele peaks 9.3 and 10 at locus TH01 in the allelic ladders. If the two alleles are not correctly identified by the software, none of

the alleles in the entire locus will be labeled with an allele call, and the ladder will not be used for comparisons with sample data. This issue was resolved by changing the polynomial degree to 3 and the peak window size to 11, as suggested in Applied Biosystems developmental validation procedure (Applied Biosystems, 2007). Both of these settings affect the sensitivity of peak detection and have to be correctly adjusted to ensure single base pair resolution. A higher polynomial degree value results in the generation of a curve that more closely approximates the shape of the peak, allowing for increased sensitivity. The peak window size then provides a finer adjustment to sensitivity by setting the width of the window to which the polynomial curve is fitted to the data. Sensitivity is increased by decreasing the window size, capturing more of the peak structure (Applied Biosystems, 2007). Too much sensitivity would cause single peaks to be split and detected as two separate peaks, resulting in incorrectly labeled alleles.

XIII. Precision Study

After the optimization of sample data, the first part of the validation completed was the precision study. A precise capillary electrophoresis instrument is vital to generating accurate data, as even small differences in the allelic ladders could cause drastically different allele calls. The precision of the 3730 instrument was determined by injecting several samples containing the allelic ladders and calculating the base pair size standard deviation of each allele peak. A plate was prepared that contained Hi-Di formamide, LIZ size standard and allelic ladder in the 48 wells of the odd-numbered columns. The plate was placed into the autosampler of the 3730 instrument and injected twice, resulting in a total of 96 samples. The instrumental method and analysis protocol previously determined to optimize data were used. A precision table setting in GeneMapper*ID-X* listed the data points of every peak in the 96 samples based on allelic location. This table was exported to Microsoft Access, which was used to calculate the standard deviation of each allele peak. An acceptable standard deviation would be less than 0.167 base pairs, so that three times the standard deviation would not be greater than 0.5 base pairs. When standard deviations were calculated using all 96 samples, seven of the sixteen loci contained allele peaks with standard deviations greater than 0.167 base pairs, with one standard deviation reaching as high as 0.412 base pairs. The standard deviations tended to increase as the size of the DNA fragments increased. Therefore, the two injections together could not be considered precise. However, when each injection was analyzed separately, the standard deviations at all allele peaks

were under 0.167 base pairs, with the highest standard deviation being 0.152 base pairs. Therefore, precision can be proven within a single capillary injection. As a result, all plates analyzed with the 3730 will be separated into two sample files containing only the 48 samples within a single injection. Consequently, only the allelic ladders also analyzed within the same injection will be used for sample comparisons. Although the instrument was only proven to be precise within a single injection, these results satisfy the precision requirement for the FBI Quality Assurance Standard.

XIV. Future Work

Precision is only one of several necessary studies that must be completed before the 3730 instrument can be used for databanking purposes. Reproducibility will be demonstrated by injecting samples several times over an extended period of time to ensure that an identical STR profile is generated each time. Sensitivity will be analyzed by inputting varying quantities of the sample and determining the range at which full DNA profiles are generated (New York State Police Crime Laboratory System, 2006). Standard operating procedures for the instrument and the software will also be developed, to be carefully followed by all analysts who are trained to operate the 3730 instrument and analyze data using GeneMapper*ID-X*.

XV. Impact

Successful implementation of this new and upgraded technology will increase sample throughput, reduce sample costs, and improve quality control. The New York State Police are currently using Applied Biosystems 3130xl DNA Analyzers, which are 16-capillary electrophoresis instruments. Compared to the 3130xl, the 3730 DNA Analyzer will shorten the run time of a single plate from 4.5 hours to 64 minutes. The 3730 instrument can also hold 16 plates in the stacking deck, while the 3130xl instruments only hold 4 plates. This will greatly increase the number of plates that can be set up and run overnight or over a weekend. The 3730 also uses a different polymer that provides higher quality data and reduces costs, includes an internal bar code reader for tracking plates throughout analysis, and reduces hands-on time by the forensic scientists. GeneMapper*ID-X* also offers several new features that will help to improve the quality of review of STR data. Overall, the introduction of the 3730 DNA Analyzer

into the DNA Databank is expected to decrease the overall cost and greatly increase the efficiency of DNA analysis.

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Appendixes

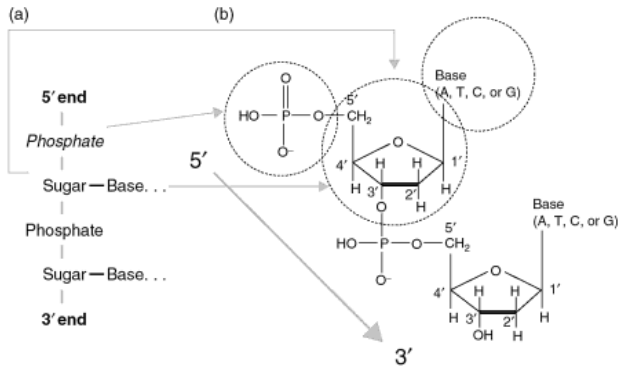

Figure 1: Structure of a Nucleic Acid

(Butler, Fundamentals of Forensic DNA Typing, 2010)

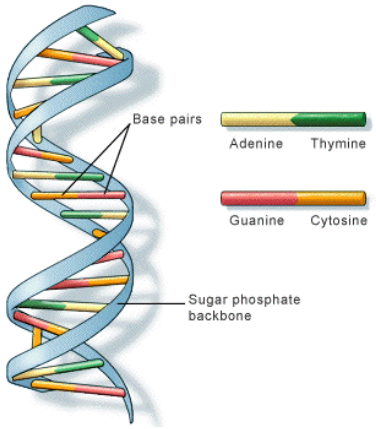

Figure 2: Structure of DNA

(Clark, 2007)

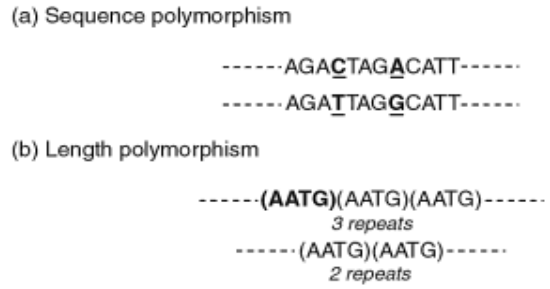

Figure 3: Sequence and Length Polymorphisms

(Butler, Fundamentals of Forensic DNA Typing, 2010)

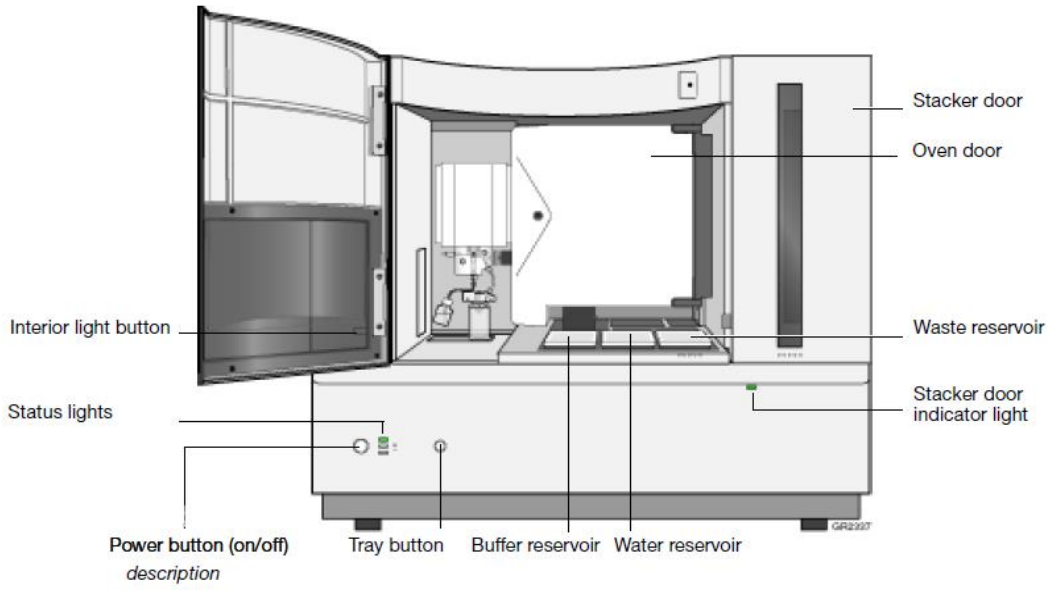

Figure 4: Applied Biosystems 3730 DNA Analyzer

(Applied Biosystems, 2010)

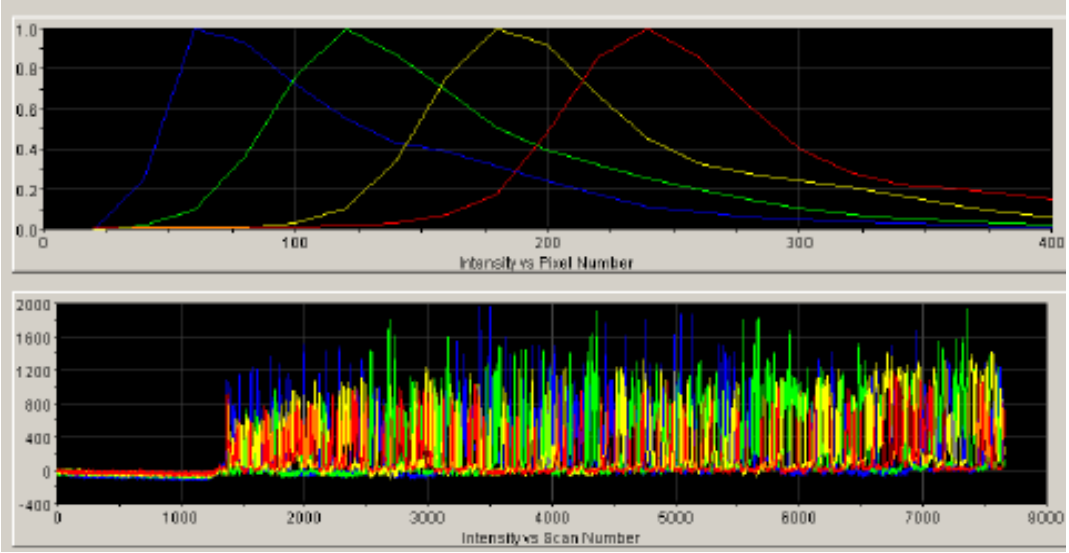

Figure 5: Fluorescence Emission Spectra from a Spectral Calibration

(Applied Biosystems, 2010)

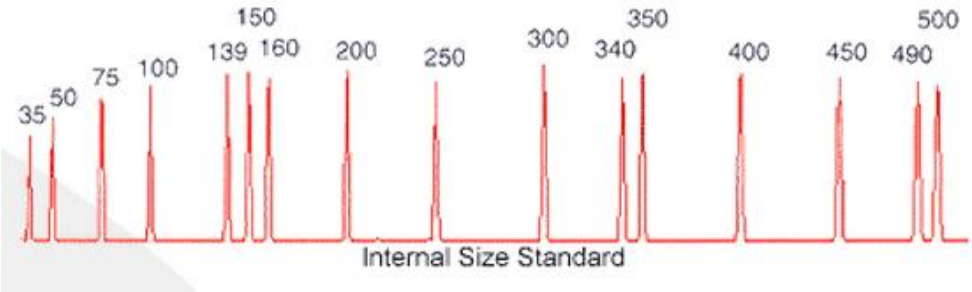

Figure 6: Internal Size Standard Peaks

(Tilstone, et al.)

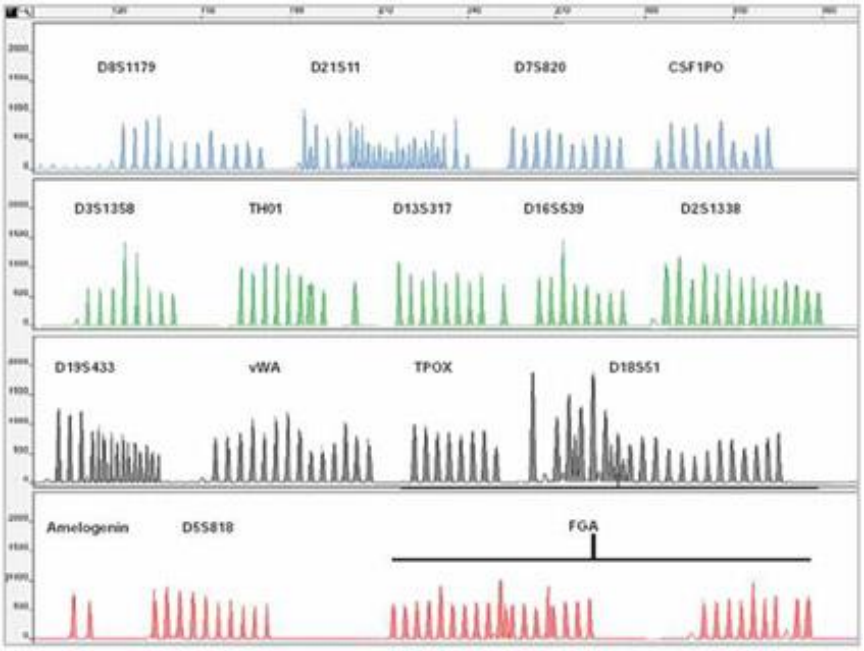

Figure 7: Allelic Ladder

(Tilstone, et al.)

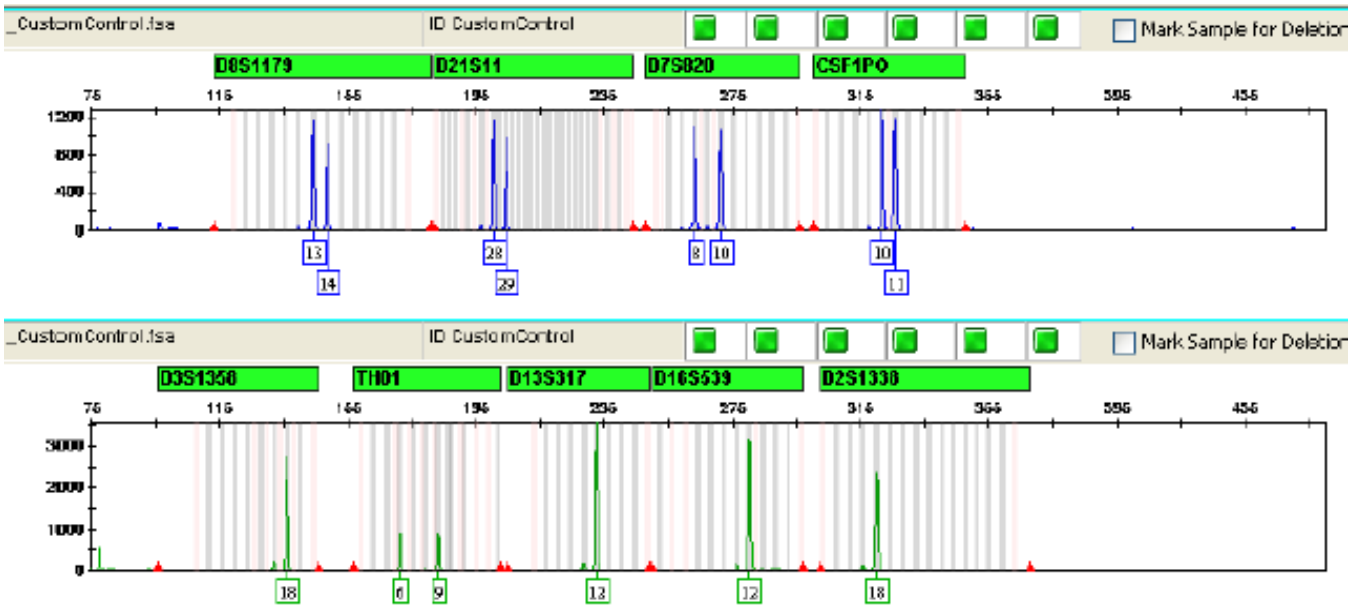

Figure 8: STR Profile (2 dyes)

(Applied Biosystems, 2007)