This is the first video in a set of videos aligned to the knowledge-based portions of the ANSI/ASB Standard 115 entitled, Standard for Training in Forensic Short Tandem Repeat Typing Methods using Amplification, DNA Separation, and Allele Detection published in 2020 by the American Standards Board.

This presentation is made possible through award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST) and created by Dr. Kelly Elkins.

This standard provides the requirements of a forensic DNA laboratory's training program in forensic Short Tandem Repeat typing methods using amplification, DNA separation and allele detection.

For purposes of this document, the following definitions apply.

An allele is one of two or more versions of a genetic sequence at a particular location in the genome.

Amplification is an increase in the number of copies of a specific DNA fragment. In forensic DNA testing laboratories, this refers to the use of the PCR technique to produce many more copies of fragments at specific genetic loci from samples of known and unknown origin for the purpose of generating DNA profiles for comparison.

The analytical threshold is the minimum height requirement at and above which detected peaks on a STR DNA profile electropherogram can be reliably distinguished from background noise - peaks above this threshold are generally not considered noise and are either artifacts or true alleles – and a "Relative Fluorescence Units" (RFU) level determined to be appropriate for use in the PCR/STR DNA typing process; a minimum threshold for data comparison is identified by the specific forensic laboratory through independent validation studies.

An artifact is a non-allelic product of the amplification process, for example, stutter, non-templated nucleotide addition, or other non-specific product, an anomaly of the detection process such as pull-up or a spike, or a byproduct of primer synthesis such as a dye blob that may be observed on an electropherogram; some artifacts may complicate the interpretation of DNA profiles when they cannot be distinguished from the actual allele(s) from a particular sample.

A bin is an allele designation corresponding to the window of fragment sizes for each allele, determined by empirical testing.

Capillary electrophoresis is an electrophoretic technique for separating DNA molecules by their relative size based on migration through a narrow glass capillary tube filled with a liquid polymer.

Contamination is the unintentional introduction of exogenous DNA or other biological material in a DNA sample, PCR reaction, or item of evidence. The exogenous DNA or biological material could be present before the sample is collected or introduced during collection or testing of the sample.

Electrophoresis is a technique used in laboratories to separate macromolecules based on size and charge. Negatively charged molecules including DNA and RNA migrate towards a positively charged pole through a sieving matrix, which permits a size-dependent separation.

An inhibitor, as related to the polymerase chain reaction (PCR), is any substance that interferes with or prevents the synthesis of DNA during the amplification process.

A locus (plural loci) is a unique physical location of a gene (or specific sequence of DNA) on a chromosome.

The polymerase chain reaction (PCR) is an enzymatic process by which a specific region of DNA is replicated during repetitive cycles that consist of the following: denaturation of the template; annealing of primers to complementary sequences at an empirically determined temperature; and extension of the bound primers by a DNA polymerase. The goal of the PCR process is to generate many copies (termed products or amplicons) of a specific region of DNA for further analysis.

Spectral calibration is an examination of the contribution of overlap in the emission spectrum of fluorescent dyes used for a specific DNA test on a capillary electrophoresis instrument. It permits the color deconvolution necessary for multi-color STR typing or sequencing to be performed. A poor spectral calibration may cause artifact peaks or inaccurate peak height determinations.

Stochastic means chance, or random variation and, in DNA testing, refers to random sampling error from extracts containing low levels of DNA and/or random variation in selection of alleles amplified at a particular locus.

Virtual bins are alleles that are not present in the allelic ladder, but have been previously reported or discovered during developmental validation of a particular chemistry. Refer to the GeneMapper® ID-X Software and Bin Overlap User Bulletin.

ANSI/ASB Standard 022, Standard for Forensic DNA Analysis Training Programs shall be used in conjunction with the ANSI/ASB Standard 115 document because ANSI/ASB Standard 022 provides the foundational training program requirements upon which additional specific requirements, such as this document, will be based.

The focus of this video series is the knowledge-based training portion of ANSI/ASN Standard 115. Knowledge based training is the laboratory's training program that provides the trainee with an understanding of the fundamental principles of the theory behind PCR amplification, DNA product separation, and allele detection methods, the function of the reagents and other components used in each method, the limitations of each method, and the laboratory's own STR typing protocols. At a minimum, the knowledge-based portion of the training program requires review of the following: the laboratory's protocols for PCR amplification, DNA separation, and allele detection; the laboratory's applicable validation studies; literature used to support validation and the test methods in the laboratory; and applicable literature as assigned by the trainer. Although this series will not address laboratory specific protocols and validation studies, it will address manufacturer protocols and others from the literature used in the field.

Aligned with ANSI/ASB Standard 115, the knowledge-based portion of the training program covers the following topics: STRs in forensic DNA analysis, polymerase chain reaction, DNA separation, DNA detection, instrumentation and reagents, contamination, quality control in the amplification, DNA separation and allele detection process to include appropriate controls, storage, preservation, and retention of amplified DNA product, and troubleshooting.

The laboratory's practical training program shall provide the trainee with sufficient practical instruction for the trainee to obtain the skills for performing the STR PCR amplification, DNA separation, and allele detection protocols used by the laboratory.

At a minimum, the practical portion of the training program shall include the observation of the process at least once or until clearly understood, and exercises representative of the range, type, and complexity of routine casework or database samples processed by the laboratory. These include STR PCR amplification, DNA separation, and allele detection methods to be utilized by the trainee; the use of appropriate controls; and the proper documentation of the process.

At a minimum, the practical portion of the training program shall include exercises representative of the range, type, and complexity of routine casework or database samples processed by the laboratory. These include: STR PCR amplification, DNA separation, and allele detection methods to be utilized by the trainee; use and evaluation of controls and expected results; proper documentation of the process; and the number and quality of samples processed by the trainee shall be appropriate to demonstrate the ability to follow the laboratory's STR PCR amplification, DNA separation, and allele detection protocol(s) and to produce reliable and accurate results.

The laboratory's training program shall include knowledge-based and practical competency testing in the application of STR PCR amplification, DNA separation, and allele detection protocols used by the laboratory. The format of the test(s) shall meet section 4.3 of ANSI/ASB Standard 022, Standard for Forensic DNA Analysis Training Programs.

The trainee shall successfully complete a knowledge-based test covering the critical information obtained during the training of STR typing methods using PCR amplification, DNA separation, and allele detection methods. The test(s) shall cover, at a minimum: the theoretical and scientific basis of STR PCR amplification, DNA separation, and detection; the function of the reagents, instruments, software and other components used in each method; the proper application of each method and strategy for use; the required quality control steps pertaining to PCR amplification, DNA separation, and allele detection, including the evaluation of controls; and the laboratory's analytical procedures pertaining to PCR amplification, DNA separation, and detection.

To achieve practical competency, the trainee shall successfully complete a practical test covering each of the PCR amplification, DNA separation and detection protocol(s) for which he or she will be independently authorized to perform. Samples of known type will be used. The trainee shall be able to satisfactorily perform the following, as applicable: properly and accurately execute the analytical procedures related to PCR amplification, DNA separation and detection without contaminating the samples; apply the laboratory's analytical procedures to a variety of evidentiary casework or database type samples; operate relevant equipment, instrumentation, and software used in the laboratory; and correctly document work performed in accordance with laboratory procedures.

In order to demonstrate conformance with this standard, the laboratory shall meet Section 5 of the ANSI/ASB Standard, 022 Standard for Forensic DNA Analysis Training Programs.

The following information provides a list of the literature resources that may assist the DNA technical leader in defining the breadth and scope of the materials to be reviewed by the

trainee. This list is not meant to be all inclusive. The laboratory shall develop a list tailored to its specific needs. Updated references shall be added to the laboratory's list as new methods or technologies are incorporated into the laboratory's protocols. The documents include the FBI Quality Assurance Standards for DNA Databasing Laboratories, effective September 1, 2011, FBI Quality Assurance Standards for DNA Databasing Laboratories, effective July 1, 2020, FBI Quality Assurance Standards for Forensic DNA Testing Laboratories (QAS), effective September 1, 2011, and FBI Quality Assurance Standards for Forensic DNA Testing Laboratories, effective July 1, 2020, as well as the SWGDAM Training Guidelines and References.

This presentation is focused on the topic of STRs in Forensic DNA analysis.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3a in ANSI/ASB Standard 115.

This presentation is made possible through award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST) and created by Dr. Kelly Elkins.

The learning objectives of this video include providing trainees with an understanding of the history of STR discovery and development and use, STR structure and nomenclature, methods of analysis, STR typing systems (that is, commercially produced kits), core STR loci and the CODIS database, and limitations of the technology.

Before we get started, let us review some terms from the standard document. An allele is one of two or more versions of a genetic sequence at a particular location in the genome. A locus, or plural loci, is a unique physical location of a gene or specific sequence of DNA on a chromosome. Capillary electrophoresis is an electrophoretic technique for separating DNA molecules by their relative size based on migration through a narrow glass capillary tube filled with a liquid polymer.

Let’s start with the history of human STR discovery. Short tandem repeats, or STRs, are microsatellite DNA markers that were discovered in the 1970s. Forensic STRs were characterized by Thomas Caskey in Texas and the United Kingdom Forensic Science Service or FSS in the early 1990s. STRs are termed satellites because they were found to surround the chromosome centromere in early experimental studies. STRs are common throughout the human genome and comprise approximately 3 percent of the genome. STRs are characterized as having 2 to 6 base pair repeats.

Short tandem repeats are used for forensic DNA typing for many reasons. They are highly variable and follow Mendelian inheritance They consists of short repeats and an overall locus length of 80 to 450 base pairs which makes them suitable for PCR. Tetranucleotide repeats are easily interpreted by discrete size differences using electrophoresis. Large numbers of repeats may contain several hundred of the core repeats, but short repeats keep the size accessible for analysis. Finally, publicly available sequences are deposited in GenBank for primer design for analysis and sequence confirmation.

Let’s talk about STR structure and nomenclature. These locations were originally termed "junk DNA" as they are non-coding elements between genes or expressed units and had no apparent use or function in studies of gene-coding regions and protein expression. STRs are often described with the analogy of being like words in a sentence or boxcars on a train as the groups of nitrogenous bases are repeated one after another. The sequence and nomenclature are defined by the top coding or sense strand unless historically defined on the bottom strand in the literature. An allele is reported as the number of repeats at the locus. STRs have been found on the autosomes from chromosomes 1 to 22 and the X and Y sex-determining chromosomes and several have been selected for use in human identification for forensic applications. On this slide, the NCBI GenBank Accession G07925 locus D16S539 with tetranucleotide GATA repeats is shown with 11 repeats.

This slide schematic shows the chromosomal structure and nomenclature of STR repeats for an autosomal locus on a pair of homologous chromosomes. A homozygote has repeats of the same length as shown here with the pair of alleles at a locus with 6 repeats each while a heterozygote has repeats at the locus of different lengths as shown here at the same locus with the alleles 6 and 7.

The STR nomenclature uses either historical assigned names published in the literature or standardized names based upon the chromosome and location. An example of systematic naming is shown for a locus site on chromosome 5 in the figure on the right-hand side of the slide. The two arms of the chromosome are termed p and q and extend on either side of the centromere. The short arm is denoted p while the long arm is denoted q. The nomenclature for sub band 2 in band 1 in region 3 on the q arm of chromosome 5 for a location is 5q31.2 where the chromosome is named first by number followed by the arm then the numbered region, band and sub band. For example, some loci carry the gene name, if it was first named due to its adjacency to a gene or protein product. For example TH01 or HUMTH01 is an STR locus located on chromosome 11 at p15.5 located in human tyrosine hydroxylase gene intron 1. Its name derives from the gene and intron number. TPOX is an STR located on chromosome 2 at p 25.3 located in human thyroid peroxidase gene intron 10. Its name derives from the gene name. The STR vWA, or VWF as it is sometimes referred by, is located on chromosome 12 at p 13.31 and is located in the von Willebrand Factor gene 40th intron. Its name also derives from the gene name. In forensics, systematic naming includes the number indicating the chromosome number and the letter “s” if it is single copy. For example, STR D5S818 is located on chromosome 5 at q23.2. STR D7S820 is located on chromosome 7 at q arm position 21.11. STR D18S51 is located on chromosome 18 in the intron between exons 2 and 3 of the B cell lymphoma 2 or BCL2 gene at q 21.33.

STR structure and nomenclature varies among the STRs. STRs may be simple sequence or true repeats, simple sequence repeats with non-consensus alleles, compound repeats, or complex repeats. Simple sequence repeats or SSR elements are repeats of identical length. Some examples of SSR elements are loci TPOX, CSF1PO, D5S818, D13S317, and D16S539. For example, as shown on the slide, TPOX is characterized by AATG tetranucleotide repeats. There are 11 repeats in the GenBank M68651 sequence. Some simple repeat loci have non-consensus alleles such as TH01, D18S51, D7S820. Some have incomplete repeats or microvariants such as the TH01 locus 9.3 allele with one truncated base in the 7^{th} repeat causing the length to be one base shorter as shown. Compound repeat elements contain two or more SSRs in a string. These include the loci vWA, FGA, D3S1358, and D8S1179. For example, vWA repeat elements are TCTA followed by four TCTG repeats and then 13 TCTA repeats in an allele. This and other compound repeats are well studied and documented and the loci are reliable for genotyping. Complex repeat elements vary in length and sequence and include the D21S11 locus. In D21S11, there are four TCTA repeats, six TCTG repeats, three TCTA repeats, a TA, three TCTA repeats, a TCA, two TCTA repeats, a TCCATA, and eleven TCTA repeats in one allele. Any STR repeat that is inherited and stable and has sufficient alleles could be chosen for analysis.

A table of STRs and their chromosome locations, GenBank accession numbers, and the repeat sequences for each in NCBI in the deposited sequence are shown on this slide. Note that they are spread across chromosomes in the genome and a mix of repeat types. They represent commonly used STR loci.

Methods of STR analysis include polyacrylamide gel electrophoresis or PAGE, capillary electrophoresis, and DNA sequencing. DNA separation is the focus of another slide deck and video but are important to remember when learning about STRs for forensic DNA analysis.

STRs were selected for DNA typing for important reasons. First the STRs were selected on separate or distant chromosome locations to avoid inheritance linkage. The loci were selected were demonstrated to have high discriminating power, generally greater than 0.9, and high heterozygosity greater than 70%. They were selected for having low mutation rates and generating robust results when used by various labs, and they have been tested extensively and are known to yield reproducible results when the markers were multiplexed. Tetranucleotide repeats were selected specifically because they have lower stutter rates than di-and tri-nucleotide repeats which can have 30% or more stutter. Since they are still short repeats, the overall length is relatively short and amenable to PCR with a narrow size range of STR length and less prone to drop out. This makes the sites better for use with degraded DNA. There is a reduced incidence of preferential amplification of shorter alleles. Finally, they are easier to interpret than dinucleotide repeat loci. Still some non-tetrameric loci have been adopted including penta D and penta E with 5 repeats each.

The selected STRs were multiplexed to reduce sample testing time. One of the earliest methods of STR DNA typing was the use of PAGE gels and silver staining. Commercial STR DNA typing kits were first offered in 1993 by Promega Corporation. They targeted 1 to 4 loci in multiplex PCR reactions. Examples of kits developed from 1993 to 1996 shown in the table are the TH01 kit targeting 1 locus, the CTT kit targeting 3 loci, the FFv targeting 3 loci, the FFFL kit targeting 4 loci, an FSS kit targeting 4 loci, the SilverSTR III kit targeting 3 loci, and the GammaSTR multiplex targeting 4 loci.

The United States Combined DNA Index System, or CODIS loci, were selected by a team of scientists and used from 1998 through December 2016 as the original 13 core CODIS loci. These are diagrammed in the schematic on this slide. These included TPOX on chromosome 2, D3S1358 on chromosome 3, FGA chromosome 4, D5S818 on chromosome 5, CSF1PO also on chromosome 5, D7S820 on chromosome 7, D8S1179 on chromosome 8, TH01 on chromosome 11, vWA on chromosome 12, D13S317 on chromosome 13, D16S539 on chromosome 16, D18S51 on chromosome 18, D21S11 on chromosome 21 and the amelogenin sex marker locus.

This slide lists commercial STR kits first offered in 1996 through 1999. Since 1996, commercial kits were released using fluorescent dye detection systems by a variety of manufacturers. This slide tabulates key kits released in the early years for forensic DNA typing. To highlight a few, the earliest kits, such as AmpF*l*STR® Blue by Applied Biosystems, multiplexed as few as three loci and used one dye for detection. However, within a year, Applied Biosystems released a kit targeting 10 loci using 3 dye labels and Promega released PowerPlex™ 1.1 which targeted 13 loci using 3 dyes. Amelogenin was selected as a sex determining marker as its length varies by 6 base pairs between the X and Y chromosomes and profiled by many of the kits. Several of these kits were used in tandem to type the selected CODIS loci with 2 to 3 PCR reactions and CE injections; for example, AmpFlSTR COfiler (D3S1358, D7S820, D16S539, CSF1PO, TH01, TPOX, and Amelogenin) was intended for use with AmpFlSTR Profiler Plus (D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, FGA, vWA, and Amelogenin) with overlapping loci for quality control.

This slide lists commercial STR kits offered from 2000 to 2009. By 2000 and 2001, Promega and Applied Biosystems engineered new STR DNA typing systems that typed 16 loci simultaneously in a multiplex with 5 dyes. Over the years, additional products were released covering STR loci selected not only by the United States but also the UK, European Union, China, and other national systems. Other innovations included AmpF*l*STR™ MiniFiler released in 2007 that decreased the length of the targeted STR amplicons especially for typing DNA extracted from degraded and mass disaster samples following the September 11, 2001 attacks. Kits targeting 17 loci in a multiplex were released by Promega in 2009.

This slide lists commercial STR kits offered from 2010 to 2012. Applied Biosystems released a 17-plex kit in 2010 and Qiagen released multiplexes beginning in 2010 targeting 7 to 17 loci. The products frequently include quality sensor or QS elements. In 2011, Promega introduced the PowerPlex™ 18D system targeting 18 loci and the PowerPlex 21 kit in 2012.

Multiplex kits focused on determining male contributors using lineage markers passed from biological father to son have been offered since 2003 and this slide lists eight of these kits; these are termed Y-plex systems and include PowerPlex™ Y and PowerPlex™ Y23 by Promega, AmpF*l*STR™ Yfiler by Applied Biosystems, Investigator Argus Y-12 QS and Investigator Argus Y-28 QS by Qiagen.

The core CODIS loci were expanded to 20 as of January 1, 2017. They included the original 13 loci described previously plus D1S1656 on chromosome 1, D2S441 on chromosome 2, D2S1338 also on chromosome 2, D10S1248 on chromosome 10, D12S391 on chromosome 12, D19S433 on chromosome 19, and D22S1045 on chromosome 22. The locations of the 20 CODIS loci and loci selected by the UK and ESS are diagramed on this slide. Most of the selected loci are common to the sets.

This slide is a schematic with a NHGRI human male karyotype showing the 20 CODIS STR loci and amelogenin and their chromosomal positions.

Manufacturers' research and development teams continued to innovate and have continued to increase multiplex capacity of their DNA typing tools; eight products are listed on this slide. In 2012, Promega and Applied Biosystems released the 24plex kits PowerPlex® Fusion and GlobalFiler™, respectively, that would become standard in labs for years especially after the CODIS loci increased to 20 and amelogenin on January 1, 2017. Qiagen released its 24plex STR kits Investigator 24plex GO! and Investigator 24plex DS in 2017. In 2022, Promega released an 8 dye, 32plex multiplex PowerPlex® 35GY System. The newer STR multiplex kits include autosomal and Y chromosome lineage markers.

This slide shows a direct comparison of the loci, dyes, and amplicon sizes included in commercial 24plex STR kits including the Investigator 24plex, GlobalFiler Express, PowerPlex and PowerPlex 6C by listing the STR loci. The color dyes assigned to the various STR loci vary among the kits. The primer locations vary as well thus leading to amplicons for given loci of different sizes and size ranges. Loci that lead to long amplicons with some kits lead to short amplicons with other kits. There are other differences as well. Some kits include Y indel or more Y-STR loci as well. Some kits target pentanucleotide repeat loci Penta D and Penta E as well as the traditional tetranucleotide repeats. Some kits include the SE33 marker while others do not. Labs have choices when selecting products and have various reasons for selecting the products they do.

Here the Investigator 24plex STR loci are shown by size range and dye color schematically.

The amplified STR loci fragments must be interpreted following separation by CE. The techniques detect size differences of plus or minus 0.1 base pairs and routinely provide within 1 base pair resolution. The identification of peaks is typically performed using peak picking software. The dye colors are separated using a matrix file. Sizing is performed using analysis of the retention time of an internal lane size standard with fragments of known lengths. The blue peaks are correlated using the retention times and the standard curve to assign the size to the allele fragments. Peaks are identified with plus and minus 0.5 base pairs around each allele creating a bin shown in gray in the software. The sizes are correlated with an allele ladder to determine the allele call using the Local Southern method. With sufficient DNA and good amplification, two peaks of roughly even heights signal heterozygous inheritance at that locus while a single peak signifies homozygous inheritance.

To interpret the peaks in the electropherogram, a matrix file is first prepared using the set of dye colors employed in the kit for color differentiation and discrimination. This slide shows a matrix file and dye separation and detection on an Applied Biosystems 3500 CE instrument. Note that the six dyes in the J6 dye set are clearly resolved.

Internal sizing standards are added to the ladder and unknown samples run on the CE instrument for post run elution time correlation. This slide shows the separated peaks of the 25-peak internal size standard from 60 to 500 base pairs run on an Applied Biosystems 3500 CE instrument. If the software does not label the sizes correctly, the analyst can delete the labels and correctly assign them. The triplets at 80, 90 and 100 base pairs and 240, 250 and 260 base pairs are useful for making the assignments.

This slide shows a GlobalFiler ladder run and separated fragment peaks for the 24 STR loci and amelogenin labeled with five different dyes and run on an Applied Biosystems 3500 CE instrument. Note the baseline resolved allele peaks for all of the loci.

In the interpretation step, the analyst review the "called" alleles, compares repeat sequences in publications for the allele with the ladder, note artifacts such as pull-up, stutter, incomplete adenylation as so on, edit as needed in accordance with the lab SOP, report any incomplete repeat motif(s) such as an off-ladder allele within the range of the alleles represented by the ladder using the number of complete repeats with a decimal point and the number of base pairs in the incomplete repeat such as for the FGA 18.2 allele. For alleles that fall outside the range of the allele ladder, the allele should be designated as greater than or less than the respective ladder allele; it may be interpolated if within guidelines. The analyst may then compile the genotype table if that is within the guidelines. A technical review of the completed report will be performed by another qualified analyst examiner.

Interpretation includes the determination of the number of contributors such as a single source profile or a mixture of two or more contributors, assignment of the alleles at each locus for heterozygotes and homozygotes and drop out detected, using population data to compute probability or frequency statistics using an allele frequency table and computing the minimum allele frequency, if needed, and computing the random match probability and likelihood ratio. Statistics may be computed for single contributor and mixture profiles considering biological relationships, where applicable. For upload to CODIS, six fully deconvoluted loci are the minimum for state databases and eight loci for mixtures and partial profiles.

The single source STR profile for standard 007 is shown on this slide with the allele call in the box below each peak. Some stutter peaks exceeded the threshold and are called as well. The loci and alleles are frequently tabulated with the allele calls reported by repeat number as shown in the box to the right. The alleles are called, and the loci are color coded to correspond to the dye colors in the electropherogram.

An electropherogram for cell line and DNA standard 2800M is shown on this slide with the internal standard underneath. Under the loci are the allele peaks. Under the allele peaks are three numbers in the box. The top number is the allele call or number of repeats, the middle number is the fluorescence peak intensity and the bottom number is the size in base pairs.

Show on this slide is a table with the allele calls for 2800M, a donor of European ancestry, at the 20 CODIS STR loci and the allele frequencies using the NIST Caucasian allele frequency table. The RMP is computed for each locus using 2pq for heterozygote calls and p squared for homozygote calls. The table continues on the next slide.

On this slide, the frequencies at each locus are multiplied to the RMP using the product rule yielding a value of 3.91×10^{-26}.

Limitations of the technology include that the ladders generally contain alleles with tetranucleotide repeats, alleles may not be included in the ladder if they have an incomplete repeat or are off-ladder or rare, the target loci are not sequenced in routine CE analysis so variation in sequences is not captured reducing the number of alleles identified, and amplicons of the same size with the same dye label will be called as the same allele.

This slide shows the major peak at the TPOX locus called as off-ladder or OL with an arrow pointing to the OL peak.

This slide of a sequencing output shows some limitations of the CE technology. In the sequencing run, the sequence of the STR locus is determined whereas it is not by CE.

That's all for today regarding STRs in forensic DNA analysis. Keep abreast of new loci and product options through professional development and course opportunities.

Some questions to consider as you review and study include: Why are most of the STR's used for forensic DNA typing tetrameric as opposed to dimeric? Are there STR's used in the laboratory that are not tetrameric? If so, which ones? List the different classifications used to describe the complexity of the STR core repeat sequences. Give an example for each type. What is a non-consensus or microvariant allele, give three examples for different loci and explain the allele nomenclature. What are the CODIS STRs and why were they selected? Explain why the penta loci are more discriminatory. What is a possible limitation of these loci? Are the CODIS STR loci human specific? Are the STR loci currently used in the lab human specific? Explain linkage in the context of autosomal, X- and Y-STRs. Why can't vWA and D12S391 be used for kinship analysis? What is an off-ladder allele and how can it be interpreted? Explain how to report an STR profile. And, what is performed to ensure profile accuracy?

If you want to know more about the content in this video, review the ANSI/ASB Standard 115 available on the AAFS website, the ISFH DNA Commission 1994 report concerning further recommendations regarding PCR-based polymorphisms in STR systems, the NAS NRC document An Update: The Evaluation of Forensic DNA Evidence, the FBI Quality Assurance

Standards for DNA Testing Laboratories and Forensic Science Communications papers on convicted offender DNA databasing, the FBI NDIS Procedures manual, and Chapter 5 of Dr. John Butler's book, Advanced Topics in Forensic DNA Typing: Methodology.

This presentation is focused on the topic of the polymerase chain reaction.

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This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3b in ANSI/ASB Standard 115.

The learning objectives of this video include providing trainees with an understanding of PCR, including: the history of development and use; biochemical principles; hot-start PCR; multiplex PCR; function of reagents; specificity, fidelity, and optimization; limitations of the technology; PCR inhibitors; stochastic effects; amplification artifacts; and contamination and quality control.

Before we begin, let me provide some definitions for key terms. An allele is one of two or more versions of a genetic sequence at a particular location in the genome. Amplification is an increase in the number of copies of a specific DNA fragment. In forensic DNA testing laboratories, this refers to the use of the PCR technique to produce many more copies of fragments at specific genetic loci from samples of known and unknown origin for the purpose of generating DNA profiles for comparison. Electrophoresis is a technique used in laboratories to separate macromolecules based on size and charge. Negatively charged molecules including DNA and RNA migrate towards a positively charged pole through a sieving matrix, which permits a size-dependent separation.

An inhibitor, as related to the polymerase chain reaction (PCR), is any substance that interferes with or prevents the synthesis of DNA during the amplification process. The polymerase chain reaction (PCR) is an enzymatic process by which a specific region of DNA is replicated during repetitive cycles that consist of the following: denaturation of the template; annealing of primers to complementary sequences at an empirically determined temperature; and extension of the bound primers by a DNA polymerase. The goal of the PCR process is to generate many copies (termed products or amplicons) of a specific region of DNA for further analysis.

We just provided a definition of PCR but can restate that it is the process of copying or amplifying DNA target regions or loci outside the cell, or in vitro, in the lab. PCR consists of repetitive temperature cycling steps, typically 28 to 40 cycles of 2 to 3 steps per cycle. The first cycling step is denaturation of the template, typically at 90 to 95 °C. DNA as well as proteins including most DNA polymerases are denatured at this temperature. The second cycling step is annealing of primers to complementary sequences, typically 55 to 70 °C, and is computationally guided but the final setting is empirically determined. The third cycling step is extension of the bound primers by a heat stable DNA polymerase typically at 72 °C. The polymerase typically will amplify DNA at a lower rate at the annealing step so the extension step may be combined or deleted in the protocol. The higher the annealing temperature, the higher the specificity of the primer binding to the target sequence.

PCR is employed in the amplification and quantitation by amplification methods in a test tube as shown in the lab. PCR is a method used to copy DNA strands in vitro outside the cell in a test tube. Forensic evidence often has low quantities of biological material and cells and PCR can be used to amplify the extracted DNA so that it can be detected by the instrumentation.

Let’s discuss the components in a cell. A human cell is surrounded by the cell membrane. The cell contains organelles such as mitochondria (shown) and chloroplasts (not shown) as well as vesicles and small molecules. The nucleus contains the autosomal DNA. Products including proteins and enzymes such as proteases and DNases coded by the DNA perform the work in the cell such as recycling molecular building blocks. DNA also encodes gene transcripts, modulators and proteins as well as enzymes that are needed to copy DNA in the cell; similar proteins are employed to copy the DNA outside of the cell.

PCR was invented by Dr. Kary Mullis of Cetus Corporation a few decades ago in 1985. The E. coli enzyme DNA polymerase I was the first used to copy DNA in vitro and a schematic of its parts and activity is shown in the figure on the right. Its structure was solved by Dr. Thomas Steitz and his team at Yale University in 1985. DNA pol I can be cleaved by subtilisin to two fragments, Klenow fragment (the larger fragment) and a smaller fragment. The enzyme binds DNA and catalyzes chemical reactions. Klenow has 5' to 3' polymerase or synthesis activity to insert bases. 3' to 5' exonuclease or proofreading activity can remove inserted bases. Both of these activities are carried out by the large fragment. 5’ to 3’ exonuclease activity for primer removal is performed by the small fragment.

As described, the structure of a DNA polymerase I from *E. coli* was published in 1985. Shown on this slide is the X-ray crystal structure of the *E. coli* Klenow fragment of the DNA polymerase I complex with DNA that was published in 1993. It was used in early PCR. As it is *not heat stable*, in early PCR, DNA pol I needed to be added and readded each cycle. This resulted in additional steps and costs that scientists sought to resolve.

Yellowstone Hot Springs' *Thermus aquaticus* (source of *Taq* DNA polymerase) was discovered in 1969. *Taq* is *thermally stable* and most active at hot temperatures as shown by the growth rate graph from the paper. Unlike most enzymes, it is not inactivated by hot temperatures. It was the first identified enzyme suitable for *multicycle* amplification.

This slide is focused on the biochemical principles of DNA. DNA is short for deoxyribonucleic acid. Each unit of the DNA molecule is composed of deoxyribose sugar (numbered with primes), an inorganic phosphate or Pi, group attached at the 5'-end of the deoxy sugar, and a nitrogenous base as shown. A generic deoxyribonucleic acid triphosphate unincorporated unit is called a dNTP. DNA folds into a double helix via hydrogen bonding between nitrogenous bases. Each cytosine or C base forms three hydrogen bonds with guanine or G base. Each adenine or A base forms two hydrogen bonds with thymine or T base. Hydrogen bonds are a special dipole-dipole interaction from a partially positively charged hydrogen and a partially negatively charged atom such as oxygen.

This slide shows the structures of the four DNA bases, adenine, guanine, cytosine and thymine as well as the uracil base found in RNA instead of thymine. The bases are the informational and variable portion of the DNA strand. Adenine and guanine are bicyclic purine structures and uracil, cytosine and thymine are single ringed pyrimidines.

The DNA primer template directs the new strand base sequence. Following addition to the 3'-oxygen on the sugar, a new deoxynucleoside monophosphate (dNMP) is attached to grow the chain with pyrophosphate (PPi) driving the reaction in part through its degradation to 2 inorganic phosphates (Pi). The figure shows the nucleophilic attack from the 3’ hydroxyl on the alpha phosphorous atom of dGTP to add the dGMP to the growing strand in the 5’ to 3’ direction. This is the reaction catalyzed by DNA polymerase in PCR. The strands run antiparallel to each other.

One this slide, red blood cells are shown at 40x magnification. Red blood cells do not contain a nucleus or DNA. Other cells including epithelial cells, sperm cells, and white blood cells contain DNA. In a human cell, one copy of the genome totals 3.2 billion base pairs (which is, by definition, one haploid set of chromosomes) with a mass of 3.3 picograms of DNA. Thus, a sperm or egg cell each contain 3.3 picograms of DNA as they are haploid cells while a white blood cell or epithelial cell contains 6.6 picograms of DNA as they are diploid.

This slide shows a micrograph of a sperm cell which contains the 3.3 picograms of DNA in the head.

This slide shows the calculation of the quantity of DNA in the cell. The relative molecular mass of a DNA base pair is 618 grams per mol. Adenine has a molecular mass of 313 g/mol, guanine has a molecular mass of 329 g/mol, thymine has a molecular mass of 304 g/mol, and cytosine has a molecular mass of 289 g/mol. Thus, adenine-thymine base pairs have a mass of 617 g/mol while cytosine-guanine base pairs have a mass of 618 g/mol. There are 3.2 billion base pairs in a haploid cell so that is 3.2×10^9 base pairs times (618 g/mol per base pair) that equals 1.98×10^{12} g/mol. The quantity of DNA in a haploid cell can be computed using Avogadro's number: 1 mole = 6.02×10^{23} molecules. Thus, 1.98×10^{12} g/mol times 1 mole/6.02×10^{23} molecules) = 3.3×10^{-12} g/molecule or 3.3 picograms of DNA. A diploid cell would contain twice as much or 6.6 picograms of DNA. Thus, one nanogram of human DNA from a diploid cell can be computed by 1 nanogram or 1×10^3 picograms divided by 6.6 picograms/cell = 152 cells or approximately 303 copies of each locus.

DNA is found in the nucleus (nDNA), mitochondria (mtDNA) and chloroplasts in cells. DNA is supercoiled and packaged in chromosomes (red) as shown on the slide. Humans have 46 chromosomes including 22 paired autosomes and two sex-determining chromosomes, X and Y. DNA consists of complementary hydrogen-bonded strands which form helical secondary structures as shown. DNA wraps around histones (yellow) to pack to form the chromosomes.

This slide shows double stranded DNA in black and the DNA denatured so that primers (green) can bind for PCR. Topoisomerase is an enzyme that relaxes supercoiled DNA in the cell. Helicase unwinds DNA in vivo (in the cell) at the replication fork; heat is used for this purpose in vitro (outside the cell, in the lab). The unwound strands serve as templates for interpretation by their complement. Small strands of single-stranded RNA (in vivo) or DNA (primers in vitro) need to be complementarily bound to a DNA strand via H-bonds for the DNA polymerase I to bind to amplify the DNA. Replication is semi-conservative meaning one parent strand remains and one new daughter is produced for each of the template strands.

In Watson-Crick base pairing, adenine (A) is complementary to thymine (T) and forms hydrogen bonds with that base and guanine (G) is complementary to cytosine (C) and forms hydrogen bonds with that base. Thus, the sequence of one strand of the helix predicts the sequence of the complement as shown by the strands.

RNA primers serve in vivo while more stable DNA primers are used in vitro. Primers are assembled by primase. The DNA polymerase catalyzes the covalent attachment of the complementary nucleotide to the primer strand. Extension occurs along the strand in the sugar carbon hydroxyls in the 5' to 3' direction as shown. Short fragments result from the replication of the lagging strand which are termed Okazaki fragments; the fragments are connected by the enzyme DNA ligase.

This slide shows the structure showing the characteristic ‘fingers’, ‘thumb’, and ‘palm’ domain and the cleft containing the active site that binds the primer–template junction and mechanism of DNA polymerase I with an active site aspartic acid residue which binds to the magnesium cofactor. The 2 magnesium ions stabilize the negatively charged oxygen on the 3’ carbon of the sugar and the phosphate groups of the dNTP so that the oxygen can act as a nucleophile and attack the alpha phosphate of the dNTP. The bond is broken between the alpha phosphorus and the oxygen leading to the beta phosphorus.

This schematic shows DNA polymerase I with 2 magnesium ion cofactors catalyzing the condensation (or dehydration synthesis) reaction in the formation of the growing chain. Nucleophilic addition is initiated by a base that withdraws the hydrogen from the oxygen on the 3’-carbon of the sugar and the oxygen attacks the alpha phosphorus and 2 Pi leave. The chain is elongated by one nucleotide base as shown.

Proteases are enzymes that degrade other enzymes. The structure of a subtilisin-like endoprotease is shown in the figure. There are serine and cysteine proteases. The enzyme catalyzes an attack on the peptide chain and the enzyme cleaves the peptide bond. These need to be degraded or removed in DNA extraction so as not to disrupt the PCR process.

In PCR at 100% efficiency, each cycle of PCR leads to a copy of each parent strand to yield two new daughter strands. At less than 100% efficiency, a full set of daughter strands are not amplified in that cycle.

This slide shows the sequence specific primer (reading from 5’ to 3’) (GGAGGAACTGGGAACCACACAGGTTA) that is needed to copy a strand of a DNA template at the TPOX locus.

This slide shows the full contingent of PCR reagents and their functions. A DNA polymerase is needed. Typically, this is a heat stable native Taq, Pfu, Tfl, or Tth enzyme but can also be an engineered fusion or chimeric enzyme. Mg^{2+} ion is the cofactor needed for DNA polymerase function and to stabilize DNA duplexes, although too high of concentrations can produce non-specific PCR products or errors from the DNA polymerase in adding the correct dNTP. The dNTPs including dCTP, dGTP, dATP, and dTTP are the DNA nucleotide triphosphates for building new strands; one may need to increase concentration to amplify long fragments but too high of concentrations can inhibit PCR. A buffer such as Tris-HCl pH 8.4 is added to control the pH for DNA polymerase activity. BSA or bovine serum albumin is added to binds inhibitors such as colored components from fabrics, leather and soil. Potassium chloride or KCl is added to promote primer annealing. Additionally, an intercalating dye such as SYBR Green I can be added for real-time or post-PCR detection or the primers can be dye-labelled. TaqMan assays and reporter probes may also be used. Finally, the DNA template and specific primers must be added.

Primer specificity and stringency is impacted by the quality of the DNA template, concentration of the primers and magnesium ions, and complementarity of the primers depending on annealing temperature set. The figure shows each of these components. High stringency is achieved when there is high base-pairing homology of the primer or probe and the template strand.

Hot-start PCR can be used to reduce mispriming and nonspecific amplification. A modified DNA polymerase that is inactive until heat activated such as AmpliTaq Gold is used. It may be bound

with an aptamer or antibody (as shown) or have a modified lysine residue or a pyrophosphatase will degrade PPi bound Mg^{2+} to activate the enzyme. Often the enzyme is activated by an initial hold of 10 minutes at 95 °C prior to the cycling. These are not used for amplification of long (>2 kb) templates sensitive to long holds at high temperatures.

Lysine may be modified at its amino-terminus of the side chain. The amino acid is protonated at acidic and neutral pH. Modifiers can fall off at high temperatures and with pH changes.

Hot start activation can also remove primer inhibiting modifiers as shown.

The three steps in traditional PCR include denaturation, annealing and extension as shown in the figure. In denaturation the hydrogen bonds connecting the strands are broken. Denaturation is typically set at 90 to 95 °C. In the annealing step, the primer binds to the target or template strand. This is set most frequently between 55-65 °C but can range from 45-72 °C. In the extension step, the DNA polymerase binds the DNA template and the primer and the enzyme catalyzes new covalent bonds between the primer and dNTPs complementary to the target. This is typically set at 72 °C or the annealing temperature also serves as the extension step in two-step PCR. This is possible as while the DNA polymerase is most active at 72 °C, it has sufficient activity to catalyze the reaction at lower temperatures. In PCR, early errors will be exponentially amplified so touchdown may be employed. Touchdown assigns a higher annealing temperature (e.g., 65 °C in the first step and lowers by a value, for example 1 °C for a number of steps before continuing at the lower annealing temperature (e.g., 55 °C). Each strand produces a new stand so the amplification is exponential.

In quantitative PCR cycling using a real-time PCR instrument, the quantity of amplified product can be detected in real time. If no DNA is detected by the end of the planned cycles, additional cycles can be added.

PCR exponentially amplifies DNA strands so a lot of DNA is produced as shown in the figure! One, double-stranded copy (6.6 pg) at baseline will be copied a billion times and yield $7.08x10^9$ pg or $7.08x10^6$ ng after 30 cycles, if it is copied every time. The product concentration can be computed from the formula, $P=(2^n)T-2n$ where T is the template concentration and n is the number of cycler. Detection is set at the fluorescence threshold (C_T). The lower the cycle, the more DNA is present at the start. PCR continues until the reaction components are exhausted and a plateau is reached. A 10-fold increase in [DNA] occurs each 3.32 cycles with 100% efficiency.

The quantity of amplification product can plateau in PCR if all dNTPs are consumed or are thermally degraded, all primer molecules are consumed or are thermally-degraded, DNA polymerases are denatured or pH-inactivated (e.g., lysine amino acids can be deactivated at low pH due to protonation of their amino groups), amplified primer dimers or double stranded templates and copies bind the DNA polymerase and hinder amplification, or a high-fidelity DNA polymerase that accurately amplifies the target was not used.

If the target is poorly amplified, try one or more of these possible solutions. Increase the input DNA concentration, increase the primer concentration, apply an annealing temperature touchdown protocol or decrease the annealing temperature, increase the dNTP concentration to amplify long fragments, add BSA to bind inhibitors or repurify the target, add KCl to promote primer annealing, or increase the final extension time. If low stringency is detected, redesign the primers to have a longer or more tightly binding complement, increase the

annealing temperature, or demultiplex and test each primer set individually prior to remultiplexing.

Forensic DNA Typing is more efficient with multiplexes. A singleplex is when a single set of PCR primers is used in a PCR reaction (shown). In a multiplex, 2 or more sets of PCR primers are used in PCR reaction. For example, 24 sets of PCR primers copy 24 loci simultaneously in GlobalFiler, 24plex GO!, 24plex QS, and PowerPlex® Fusion multiplexes.

This slide shows the Qiagen Investigator 24plex STR loci with the 24 loci and 5 dye labels. Three to five loci are assigned each dye and the amplicon sizes are spread across approximately 500 base pairs so that a locus with the same dye does not overlap in size.

Quantitative PCR is used to perform an analysis of the input DNA quantity per volume in situ using fluorescence detection technology. Products include the Promega Plexor HY, Qiagen Investigator Quantiplex Pro RGQ kit, and the Applied Biosystems Quantifiler kit shown. The unknowns are amplified alongside standard DNA of known concentrations. It enables labs to meet the quantitation prior to STR typing standard as required by FBI Quality Assurance Standard (QAS) for evidence samples (Standard 9.4). Determining the quantity of evidence DNA is important so that the quantity inputted to the STR amplification reaction is within the kit range for STR typing. The commercial kits work optimally within a narrow range of input DNA of around a nanogram. Reference samples can be much more concentrated while evidence samples may need concentrating. Too much input DNA can lead to issues with pull up and detecting contamination while too little input DNA can lead to stochastic effects. The quantitation kits often employ multiplexes to quantitate human and male DNA and compute a degradation index and quality indicator simultaneously.

Human Quantitative PCR or qPCR kits employ primers specific to human DNA. They are very sensitive and detect nanogram to picogram concentration DNA. Multiplexes can amplify multiple targets simultaneously including short and long male DNA targets, short and long human autosomal DNA targets, and an internal positive control or IPC. In qPCR, the fluorescence can be monitored in real-time and the amplification can be detected above the baseline for duplicate input standard DNA as shown. The degradation index can be computed using the ratio of long to short amplicon amplification and detect inhibition.

Several commercial products employ TaqMan qPCR assays in their DNA quantitation products. A Taq Man minor groove binder (MGB) and a non-fluorescent quencher (NFQ) is positioned at one end of the probe and a fluorescent dye reporter is positioned at the other end of the probe as shown. The probe binds to the complementary DNA sequence and does not interfere with PCR amplification. The melt temperature can be increased thus enabling shorter probes to be used for shorter amplicon targets. Taq Man assays can employ fluorescence resonance energy transfer (FRET). As the target is amplified, the probe is chewed up by the polymerase and the reporter is released from the quencher and the fluorescence is detected.

Fluorescence resonance energy transfer (FRET) theory was proposed by Thomas Förster in the 1940s. Green fluorescent dye has a higher emission energy than red dye due to its shorter wavelength. If the red dye is in close proximity to the green dye, the energy emission of the green dye will transfer to the red dye due to spectral overlap. Energy is transferred from a higher energy level to a lower energy level "quenching" the green dye. The dyes must be in close proximity for the quenching, or emission suppression or absorption by the emission

acceptor, to occur. Taq DNA polymerase I will cleave the probe bound to the target in the middle of the amplicon. The fluorescent signal is specific to the DNA target being copied.

Two Applied Biosystems Taq Man qPCR assays include Quantifiler (shown) and Quantifiler Y. Quantifiler amplifies a 62 base pair short autosomal target detected via FAM, a blue, dye tethered to a major groove binder non-fluorescent quencher. A synthetic sequence is used as an IPC and is detected via VIC, a green dye. The quantitation range using the manufacturer protocol standard curve is 50 ng/µL to 0.023 ng/µL. The Quantifiler Y kit amplifies a 64 base pair short autosomal target detected via FAM (blue) dye tethered to a MGB non-fluorescent quencher. A synthetic sequence is used as an IPC and detected via VIC (green) dye. The quantitation range is 50 ng/µL to 0.023 ng/µL.

Another Applied Biosystems Taq Man qPCR assay is the Quantifiler Trio assay. Quantifiler Trio amplifies an 80 base pair short autosomal target detected via VIC (green) dye tethered to a MGB non-fluorescent quencher, a 214 bp long autosomal target detected via ABY (yellow) dye tethered to a QSY non-fluorescent quencher, a 75 bp Y-chromosome target detect via FAM (blue) dye tethered to a MGB quencher, and a 130 bp synthetic sequence used as IPC detected via JUN (red) dye tethered to a QSR quencher. The quantitation range for the standard curve is 100 ng/µL to 0.005 ng/µL.

The Plexor HY qPCR TaqMan assay shown amplifies a 99 bp short autosomal target detected via fluorescein (green) dye, 133 bp Y-chromosome target detect via CAL Fluor® Orange 560 dye, and a 150 bp IPC novel DNA sequence detected via CAL Fluor® Red 610 dye. The quantitation range is 50 ng/µL to 0.0032 ng/µL.

The Quantiplex Pro RGQ qPCR assay amplifies a 91 bp short autosomal target detected via FAMTM (blue) dye, 353 bp long autosomal target detected via TAMRA (orange-red) dye, 81 bp Y-chromosome target detect via Cy®5 (far red) dye, and 434 bp IPC target detected via JOE (green) dye. The recommended standard curve is 200 ng/µL to 0.005 ng/µL and sets the quantitation range. The Qiagen Rotor-Gene Q dye channel selection chart is shown with the excitation wavelengths of the source and emission wavelengths of the detector for the dye labels.

A qPCR standard curve is shown. DNA concentration is plotted versus cycle threshold (C_T). The R squared value is a measure of how well the data fits the standard curve. A perfect fit is a 1 or 100% with the passing threshold at greater than or equal to 98%. The slope measures the reaction efficiency; -3.32 is 100% efficient meaning that all of the DNA strands were amplified (doubled) each cycle during the PCR with passing -3.0 to -3.6. The slope and y-intercept (b) are used to compute the concentrations of the unknowns in absolute quantification. For the Quant Trio kit, the long amplicon y-intercept passing is 24.3 - 29.5 while for the short amplicon, the threshold is 24.5 - 29.5.

Shown on this slide is the RFU versus cycle detection of the standards in a Quantiplex Pro RGQ qPCR run and the standard curve. When pipetting is precise, the R squared will be higher and the other values will be in range for quality kits.

Shown on this slide is the relative fluorescence versus cycle for a few qPCR samples. The green line traces a sample that did not amplify.

Primer design is challenging. Optimal values for PCR primers include a relatively short length of 18-30 bases, relatively high annealing temperature of 55 to 60 degrees Celsius or up to 72 degrees, 40 to 60% GC content, no hairpin formation at 40 degrees Celsius or above, and less than 5 consecutive bases interacting via primer dimers as shown in the figure. Researchers and manufacturers of STR amplification kits need to consider primer dimers when amplifying 24 separate STRs and aim for a higher annealing temperature to reduce nonspecific priming, design longer primers to improve specificity, evaluate and avoid primer dimers, and use NCBI Blast to evaluate the target genome and non-target genome primer binding, if any, and select a single copy primer binding location.

There are limitations to PCR technology. The technique is limited to short amplicons of less than 500 bp with the best results for damaged, degraded, and ancient DNA being with much shorter fragment amplicons. The DNA polymerase may commit errors in processing. Primers may exhibit nonspecific annealing to the target template. If annealing/extension times are too short, incomplete amplification products can result as shown and those peaks will show up in the electropherogram. Primer dimers will lead to extended primer amplicons rather than target amplicons. PCR is also sensitive to contamination (especially with more cycles) and can amplify even one target above the threshold after dozens of successive cycles.

Other limitations are PCR inhibitors that may be coextracted from the evidence samples. Inhibitors prevent PCR amplification, often by binding the requisite Mg^{2+} cofactor at hydroxyl, carbonyl, and carboxylic acid groups, but also by digesting the DNA polymerase or interfering with detection. PCR inhibitors can include humic acids and other natural organic matter (NOM) such as fulvic acid found in soil. Fluorescence experiments have demonstrated evidence of magnesium binding to acidic NOM including by the fluorescence enhancement shown in the figure. Other PCR inhibitors include heme from hemoglobin and myoglobin, indigo dye from blue jeans, proteases from soil, melanin from hair and skin, and tannins from leather. Developmental validation of new PCR reagents includes tests with these known inhibitors. The structures of these molecules are shown on subsequent slides.

Shown on this slide are the structures of natural organic matter metal chelators such as humic and fulvic acid, the most acidic of the complex humic group.

Shown on this slide are the structures of metal chelators such as melanin and heme.

Shown on this slide are the structures of metal chelators such as tannic acid and indigo.

In PCR reactions, PCR inhibition is mitigated by several approaches. These include diluting the genomic DNA template, which will in turn also dilute coextracted inhibitors and reamplifying if low yields were observed. Analysts also may add more DNA polymerase to compensate for PCR inhibitors present or add bovine serum albumin (BSA) which binds the inhibitors in the sample thus mitigating inhibition. The BSA protein is shown colored by charges and, from the figure, can be seen to bind charged and nonpolar substances. NaOH can be added to neutralize inhibitors of Taq DNA polymerase. Additional purification/washes can be performed during DNA extraction.

Shown on this slide is a figure with the stochastic threshold marked. Another term for stochastic is random. For chemical reactions to occur, the components must come together. In a tube with all of the PCR reagents and many copies of the full DNA genome, there are many chances for the DNA template of interest to bond with its primer and for the complex to become bound by

DNA polymerase and for it to find the dNTPs it needs to grow the chain, and so on. However, in the case of only one or a few copies of the DNA genome, there will be limited chances for the primer to find the template in the mixture. With each PCR cycle and new copy produced, the number of incorrect binding molecules increases so the chance decreases. This can lead to some targets not being extended or insufficiently extended so they are not detected. This is called allelic dropout. Preferential amplification is a phenomenon when some targets are copied well while others are not. The observed result is peak imbalance as shown with the peaks. Once bound, sometimes the template strand slips in the DNA polymerase when copying repeat regions causing a repeat (or more) not to be amplified known as stutter.

Issues that lead to poor amplification include inputting low quantity of template DNA. This may lead to stochastic effects such as allele dropout, extreme drop out or undetectable amplification below the thresholds as shown.

Issues that lead to poor amplification include preferential amplification of primer dimers, as opposed to target DNA and non-specific amplification in which the primer binds weakly at a location other than the desired location on the template and is extended at that location by DNA polymerase rather than the target. The figure shows how hot start PCR can mitigate this issue and how no antibody/non hot start polymerase can lead to less target produced as shown by the weaker bands and non-specific addition as shown by the smaller bands produced outside of the target length.

Amplification artifacts are issues with PCR amplification. These include primer dimer formation which can be mitigated by decreasing the length of self-complementary stretches in design. Other issues include the formation of heteroduplexes which can be mitigated by decreasing the number of PCR cycles, stochastic effects to due amplification bias which can be mitigated by increasing the input DNA, chimeras for which the reaction can be rerun to verify, and amplicon accuracy for which the amplicon can be sequenced and the sequence can be checked for specificity in NCBI Blast. Other issues include deletions which can be avoided by avoiding amplifying homostretches of a base, insertions which can be avoided by avoiding amplifying homostretches of a base, base substitution which can be avoided by decreasing the dNTP concentration, poly A addition which can be reduced if the primer ends with an adenine base, and stutter (shown) which can be reduced by reducing the template concentration.

An approach to reducing terminal addition artifacts include inducing poly-A addition to encourage it as shown by allowing sufficient annealing/extension time to extend the target strand to add adenine to all strands and not get the split peak shown or by creating a primer ending in an adenine base one base longer than planned so that adenine is added or by PIGtailing in which GTTTCTT is added to the 5'-end of the reverse primer.

PCR is very sensitive, and quality control is essential. Trace and touch DNA can serve as templates for amplification and compete with evidence target template. Quality control samples include positive controls such as a known standard (that is 9948 (shown), K562, 2800M, NIST SRM 2391d, among others), negative control samples such as a no template control and substrate/reagent blank control, and elimination samples for comparison such as analyst and CSI DNA samples.

To avoid contamination and implement quality control, collect elimination samples from CSI, lab personnel and visitors, clean surfaces regularly, wear PPE including a lab coat, hair covering, gloves, and a mask as shown, follow clean technique, implement positive ventilation,

as indicated, process samples in time and space from evidence to sampling, extraction, amplification and analysis but not back again, and store post-amplification samples separately from pre-amplification.

That’s all for today regarding PCR. Keep abreast of new technology and options through professional development and course opportunities.

Some questions to consider as you review and study include: Define PCR. What is the purpose of PCR? What are the advantages of PCR? What are the limitations of PCR? Explain the functions and reactions of DNA polymerases. List and explain the functions of the PCR reagents. Explain what nucleases are and why it is important to remove them during extraction. List examples of PCR inhibitors. Explain how the probe works in a TaqMan assay. Explain how multiplexing is employed in qPCR assays. Explain the meaning of R squared, slope, y-intercept, IPC, inhibition index, and degradation index in qPCR assays. What are "passing" values? What is a standard curve? How is it generated? How do we use it to determine quantitation values of unknown samples? What does it mean when a value for IPC is not obtained for a sample?

If you want to know more about the content in this video, review the ANSI/ASB Standard 115 available on the AAFS website and Chapter 3 Quantitation in John Butler’s book Advanced Topics in Forensic DNA Typing: Methodology as well as the papers: “Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder,” “A Duplex Real-Time qPCR Assay for the Quantification of Human Nuclear and Mitochondrial DNA in Forensic Samples: Implications for Quantifying DNA in Degraded Samples,” “Sensitive and specific quantitation of human genomic DNA in forensic specimen specimens: casework examples,” “A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts,” and “A rapid chemiluminescent method for quantitation of Human DNA.”

This presentation is focused on the topic of DNA Separation.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3c in ANSI/ASB Standard 115.

This presentation is made possible through award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST) and created by Dr. Kelly Elkins.

The learning objectives of this video include providing trainees with an understanding of theory of electrophoresis; capillary electrophoresis, including advantages and disadvantages; function of reagents; electrokinetic injection; DNA sieving; sample preparation; electrophoresis artifacts; contamination and quality control; and limitations of the technology. Shown here is a photo of an agarose gel.

Before we begin, let me provide some definitions for key terms. An allele is defined as one of two or more versions of a genetic sequence at a particular location in the genome. Amplification is defined as an increase in the number of copies of a specific DNA fragment. In forensic DNA testing laboratories, this refers to the use of the PCR technique to produce many more copies of fragments at specific genetic loci from samples of known and unknown origin for the purpose of generating DNA profiles for comparison. Electrophoresis is a technique used in laboratories to separate macromolecules based on size and charge. Negatively charged molecules (e.g. DNA and RNA) migrate towards a positively charged pole through a sieving matrix, which permits a size-dependent separation.

In electrophoresis, an electric field is applied to separate charged molecules, such as DNA or proteins in a liquid or gel medium. The prefix electro (from the word elektro) refers to electron flow or electricity and the suffix phoresis means travel or movement. Separation in electrophoresis is influenced by several factors including the length of the molecule, charge and relative charge of the molecule, fold or shape of the molecule and if the molecule is complexed with another molecule or molecules, cofactor, or group. As shown in the figure and the bands from the top to the bottom of the gel, intact genomic DNA migrates the least followed by circular but nicked DNA, linear but double-stranded DNA, supercoiled but intact circular DNA, circular single-stranded DNA, double-stranded PCR products and degraded DNA which travels the furthest, on average and depending on the degree of degradation.

In DNA electrophoresis, the DNA fragment size can be determined using a gel. The smaller fragments travel further in a given time while the larger fragments interact with the gel and are decelerated or retarded. Molecule size in base pairs or Daltons can be determined using a ladder of known sizes that is run on the same gel in a lane parallel to the sample(s).

Separation of DNA molecules is dependent on several factors including the viscosity and composition of the polymer used for sieving, the capillary or gel length, the buffer composition and pH, and the voltage applied or electric field strength applied. The figure on this slide shows the separation of DNA fragments using a slab gel; DNA migrates to the positively charged anode when the voltage is applied, and the DNA fragments are separated as viewed with a green dye.

During electrophoresis, the negatively charged DNA sample is introduced, or injected, in a well or capillary at the negative electrode or cathode. A buffer such as a working strength (1X) tris-

acetate-EDA, tris-borate-EDTA, or a proprietary capillary electrophoresis buffer is applied to cover the slab gel and complete the circuit at the ends of a capillary or gel at the anode and the cathode. The run voltage for a slab agarose gel is set to 120 volts and run for 30 minutes to 2 hours depending on the percent polymer makeup of the gel and the gel length. For CE, the DNA is electrokinetically injected at 15 kilovolts and run for 20 to 45 minutes or more depending on the capillary length and polymer percentage. DNA migrates to the anode. It is run out of the capillary in CE for reuse of the capillary with new polymer. Gel bands on a slab gel are visualized in situ so the sample must not be run off the gel. A photo of a slab gel set up is shown the figure; the anode, cathode and gel wells are labelled. A blue tracking dye was loaded with the DNA samples.

In DNA electrophoresis, an electric field is generated by plugging into or turning on a power source. The relative position(s) of the molecule(s) can be determined when the field is terminated on a slab gel using detection methods such as autoradiography, silver staining, or fluorescence by comparison with a ladder. Fluorescence can be viewed using a UV transilluminator.

In DNA sieving via electrophoresis, the size and shape of the sieving pores are not uniform. There are two major sieving models: Ogston sieving and reptation. In the Ogsten sieving model, the larger spherical particles are described as sterically hindered from entering the nanopores and coiled molecules move more freely through the gel matrix. In reptation, complex DNA strands are described as snaking or crawling through the gel's network of polymers as shown.

Shown here is a schematic of gel electrophoresis used to separate DNA fragments. The longer molecules do not migrate as far as the smaller molecules and, for each locus represented by the green, orange, and blue bands, a heterozygous individual will have two alleles. The banding pattern of the crime scene sample can be compared to that of the suspects. Suspect 2 shares the banding pattern of the crime scene sample in this example.

In DNA electrophoresis, the migration of DNA molecules decreases exponentially as the pore size decreases and the migration distance increases as the gel concentration is decreased. The gel and graph show the distance migrated is highest with the shortest fragment or strand lengths and shortest for the longest strands in base pairs. The gel is a 1.5% agarose gel run with 1x TAE with the TrackIt 100 bp ladder detected by exciting SYBR Green and detecting its fluorescence on a UV transilluminator.

Capillary electrophoresis using performance optimized polymer or POP is routinely used to separate, size and call amplified and fluorescently-tagged STR fragments. The sample is drawn into the capillary by electrokinetic injection at the cathode from the plate sample well. The capillary is made of glass and the ends are inserted into cathode and anode buffer, respectively. The capillary must remain immersed in the buffers to complete the circuit. DNA migrates to the anode due to its negative charge when the power is turned on and the electric field is applied. The DNA molecules are detected using fluorescence when they pass the detection window in the capillary; small fragments such as the 90 base pair fragment shown reach the detector first. The eluted DNA molecules are discarded at the anode outlet. The 130, 250 and 270 bp fragments migrate and are detected by increasing size.

The anode and cathode buffers maintain a constant pH and conduct electricity as they consist of proton donor acids. The prepackaged and barcode labelled anode and cathode buffers are shown in the images. Buffers include 1X Tris-acetate-EDTA, or TAE, buffer made with 40 mM

Tris, 20 mM glacial acetic acid, and 1 mM EDTA which is best when working with large DNA fragments or genes and 1X Tris-borate-EDTA, or TBE, buffer made with 89 mM tris-borate, 89 mM boric acid and 2 mM EDTA adjusted to pH 8.3. It has a higher buffering capacity but inhibits many enzymes. It also has a higher resolution with small DNA fragments (<2 kb) and can be used for longer run times as it is less prone to overheating. The EDTA in the buffer chelates metal ions. pH is often 8.0, with a range 7.6-8.2. The conductivity resistance is 1500 - 2000 µSiemens/cm.

Electrokinetic injection is the method used for introducing charged samples and controls to the capillary. Samples are dissolved in Hi Di formamide which is highly polar. Samples are heat denatured to generate single strands and flash cooled. A voltage of 5-20 kilovolts is the range with frequently 15 kilovolts used as the setting. The maximum recommended time is 30 seconds, but shorter times are frequently used. Negatively charged molecules will be drawn into the capillary by attraction to the opposite charge. Shown here is the structure for formamide, HCONH2, and the voltage that draws the ions in the fixed layer, diffuse layer and bulk solution and the movement of the ions influences how the charged sample is introduced to the capillary.

Samples are prepared in 12 µL Hi-Di (or Seq-Di) formamide for CE. The Hi-Di is highly deionized which helps in denaturation of double-stranded DNA and for optimal salt levels for electrokinetic injection. A GeneScan 500 LIZ size standard is shown in the photo. 0.5 µL of internal size standard such as BTO, 500 LIZ, 600 LIZ, or 500 WEN is added as well as 1 µL of amplified DNA sample. The samples are heated to denature for 3 to 5 minutes at 95 degrees Celsius and snap cooled for 2 to 3 minutes on ice or at 4 degrees Celsius. The plate is then loaded to the CE instrument.

This slide shows the run parameters with the Promega PowerPlex Fusion 6C kit on a ABI 3500 Genetic Analyzer. These include a capillary length of 36 cm, POP-4 polymer, the Promega J6 dye set, the HID36_POP4 (xl) run module, 15 seconds of injection time, 1.2 kilovolts injection voltage, 13 kilovolts run voltage, and WEN 500 internal size standard with a 25 minute run time.

This slide shows the run parameters with the Applied Biosystems GlobalFiler kit on a ABI 3500 Genetic Analyzer. These include a capillary length of 36 cm, POP-4 polymer, the J6 dye set, the HID36_POP4 run module, 15 seconds of injection time, 1.2 kilovolts injection voltage, 13 kilovolts run voltage, and LIZ 600 internal size standard with a 25.8-minute run time.

There are several advantages of capillary electrophoresis over slab gels. It requires only a small amount of sample, DNA cleanup not required, samples can be multiplexed, the process is automated, there are fast run times, a discrete sizing resolution, high sensitivity and it is cost-effective for large numbers of samples, does not require prior knowledge of the fragment size or sequence, high throughput with multi-capillary arrays, and the data analysis is standardized and (relatively) easy. Shown here is a photo of the instrument with the capillary array, electrodes, and detection window labelled.

There are some disadvantages of capillary electrophoresis as well. These include that it is difficult to discretely separate large molecules, the separation is sensitive to fluctuations in temperature and buffer composition, conductivity failures can occur when bubbles or dust are introduced to the system, it is only cost-effective when all capillaries in the array are utilized, there is poor concentration sensitivity due to nanoliter injection volume small loading capacity, salts such as buffer ions and chloride from PCR in samples will lead to poor sample loading, it is

prone to artifacts, a poor matrix can lead to a raised baseline and extra peaks called, and that the loci are not sequenced in fragment analysis.

Shown on this slide is an output electropherogram from a CE run plotted as relative fluorescence units of RFUs versus the retention time converted to size using the internal size standard or ISS and allele number of STR repeats by correlation with a ladder as shown underneath the peaks.

Electrophoresis artifacts are shown on this slide. These include reverse stutter, dye blobs, poly A addition, spikes and pullup as shown in the blue channel. Spikes are detected in all color channels while pull up is an artifact of insufficient matrix color differentiation as shown here in the green channel. Dye blobs are dye labels that become detached from primers. PCR artifacts include poly A addition in which a longer extension time leads to a final adenine base added and stutter is an artifact of the fragment folding on itself in PCR and the polymerase amplifying a shorter fragment such as the one shown of four bases shorter.

On this slide, a CE electropherogram is shown for standard 007 and locus in which the sample is homozygous at the locus is circled. Four color channels are shown. The bars on the top of each color channel are labels for the loci while the numbers in the boxes below the peaks are allele call labels. At each autosomal locus, two even height peaks characterize heterozygosity at those loci and one peak indicates homozygosity for a single source sample. The single homozygous peak at a locus is typically twice the size of a heterozygous locus if there is no degradation as the fluorescence emission of the two copies is additive. The extra labeled small peaks that are one repeat less in TH01, D19S433 and FGA are stutter peaks.

On this slide, the same electropherogram is shown and a locus in which the sample is heterozygous at the locus is circled.

On this slide, an electropherogram is shown for a reagent blank and no peaks are detected above the interpretation threshold and called.

Here the CE separated internal sizing standard is shown with relative fluorescence units versus size and the lengths in base pairs are labelled above the peaks.

Shown here is a CE separated size standard best fit line plotted as relative fluorescence units (RFU) versus Retention time of dye-labelled known size fragments internal size standard, or ISS, and sizing using the local Southern method.

For comparison, on this slide, an internal sizing standard is shown with low fluorescence intensity and few peaks detected and called.

In CE separation, there can be sizing issues as previously described. Here, an example of an off-ladder peak is shown. The label under the peak is OL instead of a allele call with a number for the repeats detected. An off-ladder falls outside of the bin for the allele call and does not correspond to allele sizes in the allele ladder. The red color of the locus box is an issue indicator flag.

On this slide, dye blob electrophoresis artifacts are shown by the circled region. Dye blobs are dye labels disassociated from PCR primer oligos that elute early and are detected by the detector and may be detected in positive and negative control samples as well as unknowns.

On this next slide, unextended primer primer front electrophoresis artifacts are shown by the circled region. Unextended primers are oligos with dyes attached that elute after dye blobs and are detected by the detector. As shown, these can be tall peaks.

On this slide, a spike electrophoresis artifact is shown by the circled region. Spikes are intense peaks that appear in all color channels although only green is shown here and are due to voltage fluctuations or air bubbles in the capillary.

On this slide, a pull-up or bleed through electrophoresis artifact is shown by the red arrow. Pull-up or bleed-through occurs when dyes of similar emission wavelengths are co-detected under the sample peaks due to a failure in the software matrix file to discriminate the colors; they distort the peak height and also can be detected in other color channels.

On this slide, a pull-down electrophoresis artifact is shown by the yellow arrow. Pull down (or negative pull-up) artifacts occur when dyes of similar emission wavelengths are over subtracted; they distort the peak height RFU as well.

On this slide, a shadow peak electrophoresis artifact is shown by the red arrow. Shadow peak artifacts are unexpected peaks that arise before the allele peak due to contaminated formamide, rehybridized ssDNA fragments, incompletely denatured dsDNA samples, or interactions with the capillary.

On this slide, stutter peak electrophoresis n-1 and n+1 artifacts are shown by the yellow arrows. Stutter is a PCR artifact that is generated with a length of one of more bases more or less than the parent allele targeted in the assay when the DNA template ribbon folds up and two or more bases are skipped or four bases are added by the DNA polymerase when the target folds. Stutter can include n+1, n-1, n-2, half-back stuffer, and off-ladder stutter peaks.

On this slide, forward or n+1 and n+2 stutter peak electrophoresis artifacts are shown. These types of stutter have extra repeats due to the new strand folding while the polymerase amplifies the strand. N-1 stutter results when the template folds and the amplicon is copied with one less repeat than it should after copying.

On this slide, forward or n+1 and n+2 stutter peak electrophoresis artifacts are shown. These types of stutter have extra repeats due to the new strand folding while the polymerase amplifies the strand. N-1 stutter results when the template strand folds and the amplicon is copied with one less repeat than it should after copying.

The figure shows forward or n+1 stutter peak electrophoresis artifacts are shown by the yellow arrows.

On this slide, reverse or n-1 stutter peak electrophoresis artifacts are shown by the several black arrows. In this type of stutter, the amplicon has one less repeat unit than the actual STR allele. It is the most common stutter pattern.

On this slide, reverse n-2 stutter peak electrophoresis artifacts are shown by the yellow arrows. The amplicon has two less tetranucleotide repeat units than the actual STR allele.

On this slide, a half back stutter peak electrophoresis artifact is shown by the black arrow. The amplicon has two less base pairs than a tetranucleotide STR allele.

On this slide, off ladder stutter is shown. In this case the stutter is in an off-ladder position outside of a bin.

On this slide, shoulder peak electrophoresis artifacts are shown by the black arrows. Shoulder peaks are one base pair shorter or longer than the true allele due to issues such as non-template extension or incomplete adenylation.

On this slide, split peak due to N bands such as +A/-A adenylation electrophoresis artifacts are shown by the black arrows. Split peaks are allele peaks that are separated into two due to incomplete adenylation at the terminus of an amplicon. They can also arise due to matrix over subtraction.

On this slide, the amelogenin peak is split as shown by the black arrows. Matrix oversubtraction from deconvolution software in which overlapping signals are reduced too much can lead to a split peak as shown here.

On this slide, an elevated baseline is shown by the yellow arrow. An elevated baseline can occur due to several reasons, such as contamination of reagents and fluorescence detection issues.

Avoiding contamination is very important in the DNA typing process. Shown is a labelled tube. There is a low risk of contamination post-PCR with low level exogenous DNA as it is not fluorescently-tagged. Post-amp samples should be carefully handled to avoid contaminating any sample. Waste DNA should be carefully handled to avoid contamination and transfer.

Let’s talk about quality control and the steps and processes that should be performed. Control samples should be run to verify instrument performance. Sample locations must be carefully tracked. The analyst must check that sufficient polymer remains for the number of samples to be run and the polymer, buffer, capillary and other products must be checked and verified that they are not expired and are performing as expected. An internal standard must be included in controls and samples to correlate fragment size with retention time. A spectral file is needed to quantify dye overlap. An allele ladder must be run to call the alleles based on size. Constant room temperature, such as 25 °C, must be maintained and a heated plate is used for to maintain the temperature of the capillary and reduce secondary structures from forming during the run. Buffers can become depleted and need refreshed and must be monitored. Capillaries can become clogged and fail. New or cleaned capillaries are required circa every 100 runs. Fresh polymer should be used for best results: Polymer polymerizes due to reactions with air over time.

Limitations of the technology include variations due to temperature fluctuations and polymer crosslinking. Lower concentration polymers cause fragments to elute faster and may lead to co-elution of fragments of different lengths and higher temperatures decrease elution times and may lead to co-elution of fragments of different lengths. Other limitations include poor performance with large DNA fragments and that co-eluting fragments of different sequences will not be differentiated; that is STRs of the same length - but different sequences - will not be differentiated.

That’s all for today regarding DNA separation. Keep abreast of new technology and options through professional development and course opportunities.

Some questions to consider as you review and study include: Explain how electrophoresis works. How does DNA move through a gel? What is the purpose of an allelic ladder? What is the purpose of the internal size standard? Explain how electrokinetic injection works. What can be varied or changed to inject more or less sample? Why do the capillaries need to stay immersed in liquid before, during, and after electrophoresis? List each of the reagents used to set up CE and describe their function. Explain and sketch a spike, primer peak, pull-up, split peak, reverse stutter, forward stutter, and dye blob.

Some more study questions include: Describe how DNA is separated in capillary electrophoresis. Describe how the GeneMapper software analyzes the STR raw data and assigns allele calls to a sample.

If you want to know more about the content in this video, review the ANSI/ASB Standard 115 available on the AAFS website and Chapter 6 in John Butler's book Advanced Topics in Forensic DNA Typing: Methodology.

This presentation is focused on the topic of DNA detection.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3d in ANSI/ASB Standard 115.

This presentation is made possible through award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST) and created by Dr. Kelly Elkins.

The learning objectives of this video include providing trainees with an understanding of the history of DNA detection methods, fluorescent dye detection including excitation and emission, dye-labeling of PCR primers, computer software programs for DNA detection, multicomponent analysis/spectral calibration/spatial calibration, analytical threshold, fragment sizing and allele calling, bins (including virtual bins), and limitations of the technology.

Before we get started, let us review some terms from the standard document. The analytical threshold is the minimum height requirement at and above which detected peaks on a STR DNA profile electropherogram can be reliably distinguished from background noise - peaks above this threshold are generally not considered noise and are either artifacts or true alleles – and a "Relative Fluorescence Units" (RFU) level determined to be appropriate for use in the PCR/STR DNA typing process; a minimum threshold for data comparison is identified by the specific forensic laboratory through independent validation studies. An artifact is a non-allelic product of the amplification process, for example, stutter, non-templated nucleotide addition, or other non-specific product, an anomaly of the detection process such as pull-up or a spike, or a byproduct of primer synthesis such as a "dye blob" that may be observed on an electropherogram; some artifacts may complicate the interpretation of DNA profiles when they cannot be distinguished from the actual allele or alleles from a particular sample.

A bin is an allele designation corresponding to the window of fragment sizes for each allele, determined by empirical testing. Spectral calibration is an examination of the contribution of overlap in the emission spectrum of fluorescent dyes used for a specific DNA test on a capillary electrophoresis instrument and permits the color deconvolution necessary for multi-color STR typing or sequencing to be performed. A poor spectral calibration may cause artifact peaks or inaccurate peak height determinations. Stochastic means chance, or random variation and, in DNA testing, refers to random sampling error from extracts containing low levels of DNA and/or random variation in selection of alleles amplified at a particular locus.

Let's start with the history of detection in human DNA typing. From the mid-1980s to the early 1990s, restriction fragment length polymorphism or RFLP of variable number tandem repeat or VNTR minisatellites were probed using single and multi-locus probes. From the early to mid-1990s, reverse dot blot DNA Hybridization Polymarker such as AmpliType HLA DQα1 were used. From the mid1990s to present, short tandem repeat or STR microsatellite loci have been probed and separated with capillary electrophoresis and from the 1990s to present, single nucleotide polymorphisms or SNP loci have been probed and sequenced with massively parallel sequencing or MPS. Sample data from each of the techniques are shown on the slide. Drawbacks of RFLP and VNTRs was poor sensitivity (pre-PCR), many alleles and few loci. PCR improved sensitivity but SNPs offer few loci and alleles. STR typing is highly sensitive and STRs offer many alleles and loci. PCR with MPS offers more loci and higher sensitivity. Examples of data from each assay are shown on the slide with references.

The first human DNA typing kit was introduced by Promega in 1993 and targeted the locus TH01. It used gel separation and silver stain detection. The CTT STR DNA typing kit, also from Promega, was introduced in 1994. Its targets were four loci: CSF1PO, TH01, TPOX, and Amelogenin. In silver stain detection, silver binds to negatively charged DNA strands. Silver ions (Ag+) are from silver nitrate are reduced to metallic silver (Ag^0) by alkaline formaldehyde. The method is sensitive and has a limit of detection (LOD) of 2.5 nanograms, or approximately 5 times that of ethidium bromide stains. The linear range is 5 to 30 nanograms. A silver-stained glass slide from a case with a suspect (s) and ladder (l) band profiles is shown at the right as well as profiles from MF, L, FF, V1 and L using the CTT kit.

Some more on the history of forensic DNA detection. The Applied Biosystems 310 Capillary electrophoresis instrument was introduced in 1995. STR detection using fluorescent dyes was introduced in 1996. The GenePrint PowerPlex™ 1.1 System by Promega included 8 loci and used 3 dyes, fluorescein (green), TMR (red), and CXR (blue) to label them and the internal standard. Another kit, the AmpF*l*STR® Blue kit by Applied Biosystems analyzed 3 loci using 1 dye. Shown is an FBIO image collected by scanning sequentially the fluorescence emission of the GenePrint PowerPlex 1.1 three dyes at 505 nm, 585 nm, and 605 nm for samples amplified with an eight-locus multiplex. Emission at 505 nanometers, 585, and 650 nanometers revealed the D16S539, D7S820, D13S317, D5S818, CSF1PO, TH01, TPOX and vWA STR loci sample and ladder bands. The sample bands were assigned by the linear migration relationship of the ladder bands when log of the base pair fragment size is plotted against the distance migrated from the well when the samples were introduced to the gel.

Before we proceed, let's go over the theory of fluorescent dye detection using a Jablonski diagram. In excitation, an electron in the fluorophore is boosted by a laser or LED energy from the ground state to an excited state. The excited state energy depends on the absorption (10^{-15} seconds) wavelength and energy of the radiation used in excitation. Emission (10^{-8} seconds) is the energy emitted by fluorophore as electron relaxes to ground state following nonradiative transitions resulting in a longer wavelength than excitation. The colors of the arrows correspond to increasing energy in the visible spectrum from red to purple with red being the lowest and purple being the highest and the corresponding excited states.

Fluorescence detection is a sensitive technique with a nanomolar detection limit. Many molecules absorb UV-Vis radiation but only some molecules fluoresce which makes fluorescence much more specific than UV-Vis spectroscopy detection. Fluorescent molecules such as dyes are used to detect DNA in PCR reactions and capillary electrophoresis. Absorptions occur in the 200 to 700 nanometer range and emissions occur at longer wavelengths than the excitation in long wave UV or visible range. Dyes with absorptions in the 480 to 650 nanometer range are used. Peaks do shift with pH changes as well as changes in environment. In fluorescence, light is emitted by the fluorophore and is detected by a fluorimeter or fluorescence spectrometer charge coupled device, or CCD, detector. The dots show the fluorescence of a green dye such as SYBR green.

On this slide, four fluorescent dyes, HEX, Texas Red Rhodamine Red and TET are shown. The dyes are attached to the oligo at the succinymidyl ester or NHS ester (circled in red). Note that they contain highly conjugated molecules with numerous substituent groups modulating electron effects.

There are several fluorescent dyes that have been synthesized and characterized; they have absorbance and emission maxima throughout the visible spectrum. A schematic with the visible spectrum is diagrammed with the emission maxima of various dyes including 6-FAM, TET, 6-JOE, TAMRA, ROX, CXR, and CY5. Absorption and emission maxima can be shifted to the red by additional pi bonds and conjugation and substituent groups.

On this slide is a table showing the excitation and emission wavelengths for numerous fluorescent dyes used in biology and forensic biology including DAPI, SYBR Green, 6-FAM (6-carboxyfluorescein), 6-JOE, TET, HEX, BODIPY TMR, TAMRA, ROX, CXR, Radiant Red, and Cy5. Some details for proprietary dyes such as AQA, BTG, BTR2, BTR, BTY, CCO, TOM, and WEN were unable to be included. The band gaps between some excitation and emission maxima are larger than others.

In fluorescent dye detection, molar absorptivity (ϵ) is the measure of how strongly the fluorophore absorbs light. Quantum Yield, or phi, is the ratio of photons emitted to absorbed or the measure of the efficiency of converting absorbed photons to light emission. A higher quantum yield leads to higher signal intensity. The range is 0 to 1.0 or zero to 100%. The highest sensitivity is at the absorption and emission maxima. The Stokes shift is the difference in wavelengths between the excitation and emission maxima. As is shown, some energy is lost to non-radiative transitions between the excitation and emission so the emission peak height is lower in intensity. The technique is sensitive; the limit of detection is 125 picograms of DNA.

Fluorescent dye detection is complicated by overlap between and among dyes. Shown is the overlap for the blue, green and yellow dyes FAM, JOE, and TAMRA upon excitation at 532 nanometers. Software for deconvolution is often set to detect outside of the emission maxima to reduce the detection overlap. The same laser may be used to excite some or all of the dyes. An narrow emission filter as shown with the rectangles can be used to filter the wavelengths of interest.

PCR primers can be labeled by fluorescent dyes at the 3' and 5' ends of the oligos but most commonly the 5’-end to enable chain extension. Modern kits have up to 8 dye detection. Amplification via PCR generates labeled amplicons as the PCR primers are extended. Shown is a PCR oligo with the TET dye attached on the base.

The Applied Biosystems 310 capillary electrophoresis instrument was introduced in 1995. It was a single capillary, four-dye detection instrument with laser induced fluorescence, or LIF, detection. It consisted of a laser, filters, lens, sample window, lens, filters, and detector such as a photomultiplier tube (PMT) or charge coupled device (CCD).

Fluorescently labeled fragments generated by PCR are detected in CE using fluorescence detection. Here we show how the fluorescence spectrometry system is incorporated into the CE instrument. The parts include a excitation laser, filter, lens, excitation monochromator, capillary window for sample detection, diffraction grating/emission monochromator, slit, CCD, software, and an electropherogram output. The laser is directed at a window in the capillary glass and, as the labelled amplicons pass through, they are excited. A detector offset by 45 to 90 degrees collects the fluorescent signal which is plotted on the y-axis of the electropherogram for each sample.

Computer Software Programs for DNA Detection include the Applied Biosystems Data Collection and secondary analysis software GeneMapper ID and GeneMapper ID-X, the

Promega Spectrum Software, Open Source and Independent Review Interpretation System or OSIRIS, TrueAllele by Cybergenetics, and GenoProof 2 and GenoProof Mixture.

Shown on this slide is the number of dyes detected in various CE instruments. A number of instruments have been manufactured and employed by forensic labs over the years. The Applied Biosystems 310 instrument detected four dyes, the 3130 detected 5 dyes, and the Applied Biosystems 3500 instrument detects six dyes while the Promega Spectrum CE detects eight dyes. The table lists several CE instruments, the number of dyes they detect, capillaries they are equipped with and the manufacturer.

Multicomponent analysis includes both spectral calibration for color deconvolution and spatial calibration of the physical space of the capillary. To perform spatial calibration, the relationship between the elution of the dye from the capillary and position of the signal as detected by the CCD camera must be determined. The figure shows spatial calibration discrete peak results on an Applied Biosystems 3500 CE instrument.

On this slide, dye spectral calibration with six dyes is shown. To perform the spectral calibration, the dyes are injected into the capillary or each capillary in an array. Separate emission files are created for each capillary in an array. The spectral calibration must be performed for each dye set. Shown here is the J6 dye set containing orange, red, yellow, green, blue, and purple run on an Applied Biosystems 3500 CE instrument.

The image here shows the spectral calibration of eight dyes using the Promega Spectrum Compact CE system from the manufacturer. This is the most dyes currently used for STR DNA typing using CE.

To perform fragment sizing using an internal size standard, the internal size standard is added to the sample postamplification and prior to CE separation. The internal size standard is composed of DNA fragments of known size labeled with a unique fluorescent dye from the STRs. From their elution time and known sizes, a sizing curve is constructed by plotting the DNA size versus the migration time for the standard fragments. A regression line is added and the slope is used to compute the size of the sample fragments of unknown size using their retention times. Shown here is the elution profile for the GS600_LIZ 60-460 base pair size standard and one channel for 2800M with its allele calls (top number in the box below the peaks).

As previously stated, the internal size standard is added to the sample prior to separation. It contains DNA fragments of known size. CE separates the amplified fragments in the sample. A sizing curve is constructed and a plot of DNA size versus migration time from the standard fragments is constructed, a regression line is constructed, and the fit is determined.

In the Global Southern Method, the slope is used to compute the size of the sample fragments using their migration times. Forensic STR size calculations use Local Southern sizing in which the peaks in the size standard above and below the peak of interest are used to compute the size of the sample peak.

The allele ladder is a mixture of DNA fragments of known size and sequence. Shown here are the dye-labeled GlobalFiler ladder fragments in the blue, green, yellow, red, and purple channels and the internal size standard in the orange panel. It includes the known and most common alleles for each locus. The internal size standard is included in the ladder and the

sample to correlate the migration time of the ladder peaks to the sample peaks. The fragments elute from smallest to largest.

Let's talk about thresholds. Peaks above the interpretation threshold are reproducible and reliable and are called. The analytical threshold is the limit of detection, or LOD, and can be used for exclusion. The analytical threshold is 50 relative fluorescence units (RFU) and the interpretation threshold is 200 RFU in the figure shown. A peak or baseline noise below the analytical threshold is stochastic and noise and not reliable for interpretation.

There are several types of bins that are used to assign the alleles. A bin or allele bin as previously discussed is a region that defines an allele within a locus. The bins are separated to 1 base pair sensitivity and can be separated by 0.1 nucleotides. A physical bin is a region physically defined by the allele ladder for the kit used. A virtual bin are alleles not present in the ladder but that have been reported in the literature by users. The bin offset is the size difference between the physical bin and allele ladder fragments. If bin overlap occurs, rerun the ladder or replace with one that did not produce overlap. The bins shown here in the figure are shown in the shaded areas and are fully resolved.

Limitations of the technology include limits of the range of visible spectrum wavelengths available for a range of colors and the spectral overlap not being fully removed by matrix deconvolution. Spectral overlap can lead to pull-up in other dye channels. The size - but not the sequence - is determined in STR forensic DNA typing kits for fragment analysis. The figure peaks are labelled with examples of these limitations with the red line and arrow.

That's all for today regarding DNA detection. Keep abreast of new technology and options through professional development and course opportunities.

Some questions to consider as you review and study include: Define fluorescence. What methods have been used to detect DNA? How can DNA be detected using fluorescence? What is a spectral calibration and why is it performed? Define analytical threshold. Define interpretation threshold. How are alleles detected and called? Define a bin and virtual bin. Using fluorescent STR allele detection technology, how many reaction primers are labeled and what are the dye labels and colors? And, what are limitations of using fluorescence to detect DNA?

Additional questions include the following. Why are allelic ladders kit specific? What can cause a sample not to analyze properly? You are troubleshooting a CE run. What are possible explanations for the following observations? No PCR product is present in all lanes, but the size standards are visible. Most samples look OK, but one sample has neither standard nor alleles. The smaller fragments peaks are sharp but later peaks get progressively lower and wider. All of the peaks are present in all of the color channels.There are spikes in almost every lane.

If you want to know more about the content in this video, review the ANSI/ASB Standard 115 available on the AAFS website and the FBI Quality Assurance Standards for DNA Testing Laboratories and read Dr. John Butler's book, Advanced Topics in Forensic DNA Typing: Methodology.

This presentation is focused on the topic of Instrumentation and Reagents.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3e in ANSI/ASB Standard 115.

This presentation is made possible through award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST) and created by Dr. Kelly Elkins.

The learning objectives of this video include providing trainees with an understanding of thermal cycling instruments and parameters, DNA separation and detection instruments and parameters, software parameters associated with instruments, maintenance and calibration, and storage of STR typing kit and DNA separation reagents.

Shown on this slide is the DNA typing process and instrumentation. The process includes sampling (the first step, circled), DNA extraction, DNA quantitation, DNA amplification, DNA separation, allele call interpretation, and statistics. The instrumentation and tools for each step are shown under the step in the process and includes the use of an alternate light source or ALS, DNA extraction robot or manual method, thermal cycling instrument, capillary electrophoresis instrument, GeneMapper or other allele calling software, and statistical software tools.

Prior to DNA extraction being performed, the stain is located visually or using the ALS and sampled using a swab as shown here or by cutting with scissors, picking with tweezers, scraping with a tool such as a razor, or pulling with a tool such as a vacuum filter.

The second step to be performed is the DNA extraction process using an robotic or manual method. There are several options on the market available via commercial suppliers and lab grown methods.

A cell is shown on this slide. DNA extraction is defined as isolating DNA from the cellular nucleus, mitochondria and/or chloroplast.

DNA extraction may be performed manually or robotically using an extraction instrument. Manual DNA extraction methods include the organic / phenol-chloroform isoamyl alcohol or PCIA method, DNA IQ method, DNA investigator kit, PrepFiler kit, microGEM reagent kit, Zygem kit, to name a few. The pros of manual DNA extraction methods include that they are typically cheaper or lower in cost, are more flexible for incorporating user-defined protocols, as desired, and they use standard laboratory equipment. However, they are often more time-consuming than robotic methods, and leave more opportunity for tube switching, mislabeling, protocol error, and contamination events due to the number of manual handling steps.

Robotic DNA extraction instruments include the EZ1, QIACube, and Maxwell robots, to name a few. These instruments minimize sample handling and the risk of sample switching or cross contamination, are more efficient and minimize DNA extraction time, are higher throughput but do have a higher upfront cost as a drawback. Additionally, training is needed to use the specialized instruments.

DNA extraction instruments including three models of the Qiagen EZ1 and QIACube DNA extraction instruments are shown on this slide.

Here is an EZ1 extraction DNA Investigator consumable and reagent kit. It is packaged in a box which is marked with the date received. The quality seal should be intact when received. Unpack the kit wearing gloves to avoid contamination of the plastics with human DNA that could later be transferred to the tubes, tips, or reagent cartridges is advised. The kit includes specialized pipette tips, tip holders, reagent cartridges, sample tubes, and elution tubes, as well as proprietary G2 buffer and room temperature stable proteinase K, and carrier RNA as marked.

DNA extraction pre-extraction steps include incubation in a thermal mixer, centrifuge, substrate removal using a spin basket and tweezers, and mixing with a vortex machine as shown. Thermomixers are used for incubation and mixing. Centrifuges are used for separation, wash and pelleting steps.

The EZ1 robot is used by many labs. We will focus on the use of an EZ1 extraction robot as an example and go over run steps and parameters. The DNA extraction process includes incubation to lyse cells, loading the pre-incubated sample with or without the cutting to the sample tube and placing it uncapped in the instrument, loading the instrument with consumables and reagents, running the program to bind the DNA, washing the other molecules away, and eluting the DNA by raising the pH. The process steps lyse the cells through the heat incubation with the G2 buffer and proteinase K and rupture epithelial cells to release DNA but not sperm cells. The proteinase K breaks down proteins such as DNases that degrade DNA but not the DNA molecules themselves. The DNA binding and washing steps are facilitated first by loading the consumables and samples onto the EZ1 instrument. The DNA is bound, purified and washed using paramagnetic resin beads. The DNA strands bind to the rough beads (like hair on a tennis ball) in presence of chaotropic salts contained in the reagent cartridge. If added, carrier RNA binds the DNA and increases DNA binding to the beads. At low pH, ethyl alcohol washes are used to remove the clinging cellular material and salts. The final step in the program is elution in which the bound DNA binding is released from the beads and dissolved in TE buffer or water as selected as the elution buffer. As the pH is increased, the DNA releases from the beads.

The first step in the use of the EZ1 robot is to turn on the power at the back of the instrument. The panel will show the instrument is starting up as shown in the pictures of these two older and newer models.

On the touchpad of the EZ1, the user will be prompted to select from tools or quality assurance test protocols by selecting the appropriate button, 1 or 2, respectively.

Select and apply a manufacturer's or validated protocol. Selections include Trace, Trace Tip Dance (TD) protocol for when the sample cutting remains in the tube, normalization which eliminates the need to quantify post-extraction, and large volume which is a protocol for larger input buffer and reagent volumes for samples including bone.

The next step is to select an elution solvent, either water or TE buffer. Water is a pure substance that contains no EDTA to chelate magnesium which stabilizes DNA duplexes; magnesium is needed stabilize DNA duplexes and as a cofactor for DNA polymerase activity in PCR and this can improve PCR results. TE consists of Tris buffer and EDTA. Tris buffers pH and EDTA chelates divalent cations like magnesium and calcium. Calcium and magnesium are cofactors for DNase activity which breaks down DNA. The EDTA is present at a low molarity and the buffer stabilizes the DNA.

Next, users select the elution volume: 50, 100, or 200 microliters by selecting 1, 2, or 3 as shown on the screen. Selecting a lower volume such as 50 microliters will result in more concentrated DNA. Eluting in a higher volume will yield more but more dilute DNA. The EZ1 robot can be used to concentrate DNA from a larger volume to a smaller volume.

Next, the EZ1 instrument prompts the user to load the consumables to the instrument. Load the number of cartridges as samples into the metal cartridge holder.

Next, load the 1.5 milliliter elution tubes with the caps removed in the front row nearest the user. Pushing any key will advance the tutorial or escape to get out.

In the second row, load the tips into the tip holders and load these into the EZ1 and push any key to continue.

In the fourth row, load 200 microliters of sample in 2 milliliter tubes without the caps as shown and push any key to continue. The loaded instrument with the tubes, tips and cartridges is shown in the photo.

Push start as shown on the screen to run the program with the input parameters and loaded samples. Each cartridge foil covering is pierced by air when the instrument starts. The program performs all binding, washing, and elution steps. The entire process takes approximately 20 minutes and yields a high-quality double-stranded DNA product.

The EZ1 screen shows the step it is on such as binding in this photo.

The DNA extraction process includes lysis, binding, washing and elution steps as shown by the figure. In the lysis step, amphipathic phospholipid bilayer cellular and nuclear membranes are disrupted and the chromosomes are released. In the binding step, DNA binds to magnetic silica resin particles (or a membrane in the Qiagen Investigator manual method) at low pH and high chaotropic salt and this isolates the DNA from proteins (including DNases), lipids, carbohydrates, and organelles. In the wash step, the DNA remains bound to the particles or membrane and the protein, sugars, lipids, other macromolecules and salts are washed away and the DNA is rinsed off. DNA is eluted from the beads with an alkaline or basic buffer and low salt. The process yields high quality DNA concentrated for downstream analysis.

What are the functions of the DNA extraction reagents. The process is akin to a washing machine.

Specifically, the lysis reagents include chemical detergents and agents, enzymes, and RNA molecules. The G2 buffer is made with 800 mM guanidine hydrochloride; 30 mM Tris•Cl, pH 8.0; 30 mM EDTA, pH 8.0; 5% Tween 20; and 0.5% Triton X-100. Proteinase K is a non-specific serine protease enzyme active from pH 4 to 12.5, at high temperatures, and in chemical conditions. It digests proteins including histones and enzymes including DNases that are a threat to DNA integrity. Its activity is enhanced by Tween 20, a non-ionic surfactant that solubilizes proteins. Carrier RNA improves DNA yield especially for short fragments by increasing the surface area for binding to the particles or membrane.

Other chemical agents used in DNA extraction include sodium dodecyl sulfate, or SDS, guanidinium hydrochloride, guanidinium thiocyanate, and sodium hydroxide. SDS is an anionic amphipathic detergent that disrupts hydrogen bonding and solubilizes the phospholipids and proteins. Guanidinium hydrochloride or GdHCl, is a chaotropic agent that denatures proteins

and disrupts hydrogen bonding networks among water molecules and in proteins. Guanidynium thiocyanate or GdSCN is a chaotropic agent, meaning that it disrupts the structure of water and macromolecules. Sodium hydroxide, or NaOH, is a strong base that denatures DNA and proteins by disrupting H-bonding networks.

Other DNA lysis chemical agents include Triton X-100, a mild non-denaturing non-ionic surfactant and emulsifier detergent. The structure is shown.

Shown here is the chemical lysis agent Tween-20, a non-ionic detergent for emulsifying and stabilizing protein. A reagent bottle and structure are shown.

Shown here is the chemical lysis agent dithiothreitol or DTT. It reduces disulfide bonds in proteins including sperm nuclear membrane proteins. The photo shows the reagent.

Shown here is the chemical lysis agent ethylenediaminetetraacetic acid or EDTA. It can be used to demineralize bone and teeth. It binds divalent metal ions including calcium and magnesium at its carboxylates. The photo shows the reagent and the structure is shown.

Shown here is the chemical agent ethanol or EtOH. Cold ethanol can be used to precipitate DNA. The photo shows the reagent bottle, and the compound is shown.

Other lysis agents include instruments for heat denaturing and physically agitating the sample. The thermomixer shown at 56 °C and 900 rpm can perform these tasks. The cell membrane fluidifies at 56 °C lysing the cell contents and proteins are denatured with heat rendering them inactive. Physical agitation aids in upsetting the membrane.

In the EZ1, the reagents for lysis include the general lysis buffer (G2) and proteinase K for forensic samples including semen, blood, saliva, trace cellular material and DTT is used for sperm to reduce disulfide bonds. G2 and 0.5 M EDTA followed by MTL are used for the extraction of DNA from bone and teeth. Animal tissue lysis buffer (ATL) and proteinase K is used for extracting DNA from epithelial cells. In the binding step, the positively-charged magnetic silica resin particles bind the DNA and a magnet binds the particles; the DNA is bound in the presence of a chaotropic salt. Wash buffer is used to wash away cellular debris and degraded protein. DNA is eluted in low salt conditions with TE buffer at pH 8.0 or nuclease-free water.

The EZ1 manual DNA extraction incubation volumes are shown on this slide. For blood and saliva up to 50 µL, use 140-190 µL of G2, 10 µL of ProK and the Trace program on the instrument. For FTA 4x3 mm punches, add 290 µL of G2, 10 µL of ProK, and use the Trace or Tip Dance program. For a surface swab, use a swab or cutting, 290 µL of G2, 10 µL of ProK, and the Trace or Tip Dance program. For up to 0.5 g of soil, use 100 µL G2 and the Trace or Tip Dance program. For a blood or saliva stain for 0.5 centimeters squared, add 290 µL of G2, 10 µL of ProK, and use the Trace program. For hair of 0.5 to 1 cm in length, add 160 µL of G2, 20 µL of ProK, and 20 µL of DTT with the Trace program. For a nail clipping, add 160 µL of G2, 20 µL of ProK, and 20 µL of DTT with the Trace program. For semen of a stain greater than 0.5 centimeters squared, add 455 µL of G2, 25 µL of ProK, 20 µL of DTT, and the Large Volume method. For tissues of up to 10 mg, add 190 µL of G2, 10 µL of ProK, and the Trace program. For chewing gum, up to 40 mg, add 190 µL of G2, 10 µL of ProK, and the Trace program. For bone of up to 150 mg, add 225 µL of G2 buffer, 25 µL of ProK, 250 µL of 0.5 M EDTA pH 8.0, after incubation add 400 µL of MTL buffer, 50 µL of 3 M sodium acetate at pH of 5.2, and 1 µL

carrier RNA, and use the Large Volume program. For cigarette butts of 1 cm^2, add 190 µL of G2, 10 µL of ProK and use the Trace or Tip Dance programs.

The process for the Qiagen supplementary large volume protocol DNA extraction from bone or teeth is diagrammed here showing the addition of the G2 buffer, Proteinase K, 0.5 M EDTA pH 8.0, before incubation on the thermomixer for 24 hours at 56 °C at 750 rpm followed by centrifugation. After incubation, the MTL buffer, 3 M sodium acetate at pH of 5.2, and carrier RNA are added, and the sample is loaded to the EZ1 instrument.

For comparison, the Promega DNA IQ manual DNA extraction kit method steps are shown here. To the sample, add 386 µL Casework Extraction Buffer, 10 µL ProK (18 mg/mL), and 4 µL of 1-Thioglycerol. Vortex for 5 seconds and incubate at 56 °C for 30 minutes. Remove the substrate with tweezers, if applicable, and separate with a spin basket. Centrifuge for 2 minutes, add 40 µL of lysis buffer to the eluent, and vortex 5-10 seconds. Vortex the DNA IQ™ Resin for 10 seconds and add 7 µL to the sample. Vortex the sample/buffer/resin mixture for 3 seconds. Incubate at room temperature for 5 minutes. Vortex for 3 seconds once every minute and 2 seconds at the end. Place the tubes on the magnetic stand. Remove the liquid carefully and do not remove any beads. Add 100 µL of lysis buffer and remove the tubes from the magnetic stand and vortex the samples for 2 seconds. Place the tubes back on the magnetic stand and remove/discard the lysis buffer. Add 100 µL of 1X wash buffer, remove from the magnetic stand, and vortex for 2 seconds. Place the samples on the magnetic stand and remove/discard wash buffer. Repeat twice more for 3 washes. Remove all wash buffer, open the tubes and air-dry the resin for 5 minutes. Add 25-100 µL of elution buffer, as desired, vortex for 2 seconds, and incubate at 65 °C for 5 minutes. Vortex for 2 seconds and place the tubes on the magnetic stand and obtain the DNA-containing solution by pipetting it to new tube. Store frozen at –20 °C.

A differential extraction procedure for DNA extraction is shown on this slide. First the analyst cuts out a 2 cm^2 stain or cut swab tip and adds it to a spin basket in a sterile tube, then adds 15 µL of 20 mg/mL Proteinase K and 285 µL of Gill Extraction buffer to each sample. The sample is incubated at 60 °C for 1 hour, centrifuged for 5 min. The substrate is removed from the tube as well as the spin basket, taking care to not disturb the pellet. The supernatant is moved to a new non-sperm fraction tube. The tube is retained and labeled as containing the pellet as sperm fraction. Next, wash the sperm pellet with 300 µL of Gill Extraction buffer. Centrifuge for 3 min. Remove the supernatant and discard; if no pellet is visible, retain 15-20 µL of solution. Add 10 µL of 20 mg/mL ProK, 250 µL of Gill buffer, and 20 µL 0.8 M DTT to each sperm pellet tube. Vortex for 20 seconds and incubate at 60 °C for 2 hours. In a fume hood, add 300 µL buffer saturated phenol/chloroform/isoamyl alcohol (PCIA) (25:24:1 ratio) at pH 8.0 to each of the sperm and non-sperm fractions. Centrifuge 5 minutes at top speed and remove the top aqueous layer to a new tube. Re-extract the sample with additional PCIA reagent and combine aqueous layers in a new tube. Add 100 µL of TE buffer to a Microcon 100/Microcon concentrator/filter assembly. Centrifuge for 5 minutes. Transfer up to 400 µL of the aqueous phase to the Microcon filter and centrifuge. Discard the waste effluent. Add 100 µL of TE buffer to the Microcon to wash and centrifuge. Invert the Microcon filter upside down inside a new Microcon tube. Elute DNA with 50 µL of TE buffer or water. Store the extracted DNA solution at –20 °C.

For DNA extraction using a QIAcube robot shown, apply a validated or manufacturer's protocol. There are several to select from depending upon sample type. There are slots for tubes and reagents and a centrifuge in the instrument. The robot can perform differential and non-

differential extractions, fraction separation, and can wash and lyse the sperm pellet. As a pre-run reminder, make sure the tubes click in or they will dislodge.

For DNA extraction using a Maxwell instrument robot, apply a validated or manufacturer's protocol. There are slots for tubes and reagent kit. As a pre-run reminder, open the reagent kit and tubes before running.

For DNA extraction with the Zygem one-step sperm lysis one-step single-tube purification kit, using acrosolv mesophilic and forensicGEM thermophilic proteases, there are 52, 75, and 95 °C incubation steps. This kit is used for sexual assault (SA) cases involving a biological male assailant and a biological female victim. Do not use on SA cases that are: 1) Biological male (s) and biological male (v) cases, 2) Biological female (s) and biological female (v) cases, or 3) Biological female (s) and biological male (v) cases.

Sonication as shown can be used to aid in DNA extraction. A digestion buffer and proteinase K is added to a sample and the sonicator is run for the prescribed time.

Following DNA extraction, the DNA can be concentrated if it is too dilute for the testing. This can be performed using ethanol precipitation, Microcon unit as shown, or by re-extraction and elution with a smaller volume on a EZ1 instrument.

The next step in the DNA typing process is DNA quantitation as circled.

Thermal cycling instruments (shown) that can be used for DNA quantitation include a standard or gradient thermal cycler followed by a quantification gel or a real-time PCR instrument such as a Qiagen Rotor-Gene Q with fluorescent detection. Other instruments include the Applied Biosystems 7500 real-time PCR instrument or a BioRad real time PCR instrument.

For using the thermal cycler, such as the Veriti shown, use thin-walled tubes and tubes or plates that fit snuggly into the well for best heat exchange, apply the locking ring to the Rotor-Gene prior to starting so that the tube caps do not detach, and the plastic does not get into the instrument, melt and get lodged in, apply mineral oil to the surface of the PCR reaction mix to reduce evaporation if the thermal cycler does not have a heated lid, and input the validated/published PCR temperature and other parameters for DNA quantitation or STR typing when entering the run program.

Thermal cycler parameters vary by kit polymerases, primer sequences, reagent concentrations, and multiplex optimization. Variables include temperature, time, number of step repeats, ramp speed, and excitation and emission wavelengths for dye detection (for real-time PCR).

PCR DNA quantitation kits for forensic applications include the Plexor HY kit from Promega and the Qiagen Investigator Quantiplex Pro RGQ kit as well as the Applied Biosystems Quantifiler, Quantifiler Y, Quantifiler DUO, and Quantifiler TRIO kits.

As shown here for the Quantifiler kit, the quantitation kits include master mix for PCR, the primer set, standard positive control DNA, and nuclease free water. The user needs only to supply the unknown samples for testing.

The Quantifiler kit thermal cycler run parameters are shown here as loaded into the Rotor-Gene Q software. The standards from 0.023 to 50 ng/mL are prepared and inputted on another screen. For each reaction, 10 µL of primer mix, 12.5 µL of PCR reaction mix, and 2 µL of sample, standard, or control are pipetted to tubes or a plate. The PCR steps are an initial hold at

50 °C for 2 minutes, a denaturation step at 95 °C for 10 minutes, forty cycles of a two-step denaturation at 95 °C for 15 seconds followed by 60 °C for 60 seconds of annealing/extending. The amplification can be detected in real time.

The Plexor HY quantification kit thermal cycler run parameters are shown here. The standards from 0.0032 to 50 ng/mL are prepared and inputted. For each reaction, 1 µL of primer/IPC mix, 10 µL of PCR master mix, 7 µL of amplification grade water, and 2 µL of sample, standard, or control are pipetted to tubes or a plate. The PCR steps are a denaturation step at 95 °C for 2 minutes, 38 cycles of a two-step denaturation at 95 °C for 5 seconds followed by 60 °C for 35 seconds of annealing/extending. The amplification can be detected in real time.

The Quantiplex Pro RGQ quantification kit can be programmed and analyzed in the Q-Rex software. The standards from 0.0025 to 50 ng/mL are prepared and inputted. For each reaction, 9 µL of primer mix, 9 µL of PCR master mix, and 2 µL of sample, standard, or control are pipetted to tubes or a plate. The PCR steps are a denaturation step at 95 °C for 3 minutes, 40 cycles of a two-step denaturation at 95 °C for 5 seconds followed by 60 °C for 10 seconds of annealing/extending. The amplification can be detected in real time data using the green, yellow, orange, red and crimson channels with auto-gain optimization.

This slide shows the screen in the Qiagen Q-Rex software following login where a template can be used or new experiment can be created for the run or recent run data can be viewed or printed.

This slide shows the screen in the Qiagen Q-Rex software where the user can create a new experiment by inputting parameters.

This slide shows the Quantiplex Pro RGQ run parameters in the Q-Rex software. The PCR steps are a denaturation step at 95 °C for 3 minutes, 40 cycles of a two-step denaturation at 95 °C for 5 seconds followed by 60 °C for 10 seconds of annealing/extending. The amplification is set to be detected in real time using the green, yellow, orange, red and crimson channels.

This slide shows a Quantiplex Pro RGQ run standard input in the Q-Rex software with the concentrations inputted and the sample type standard selected.

The next step in the DNA typing process is DNA amplification as circled.

The Applied Biosystems GlobalFiler STR PCR amplification kit components are shown with the component master mix, primer set, control standard DNA, and nuclease-free water.

The GlobalFiler thermal cycler parameters are shown inputted in the Applied Biosystems Veriti instrument. The PCR steps are a denaturation step at 95 °C for 1 minute, 30 cycles of a two-step denaturation at 94 °C for 10 seconds followed by 59 °C for 90 seconds of annealing/extending and a final extension at 60 °C for 10 minutes followed by incubation at 4 °C.

The Qiagen Investigator 24plex QS kit thermal cycler parameters are shown inputted in the Applied Biosystems Veriti instrument. The PCR steps are a denaturation step at 98 °C for 30 seconds, annealing at 64 °C for 55 seconds, extension at 72 °C for 5 seconds, 27 cycles of a three-step denaturation at 96 °C for 10 seconds followed by 61 °C for 55 seconds of annealing, and 72 °C extension for 30 seconds and final extensions at 68 °C for 5 minutes and 60 °C for 5 minutes followed by incubation at 10 °C.

The Promega PowerPlex Fusion 5C thermal cycler parameters are shown inputted in the Applied Biosystems Veriti instrument. The PCR steps are a denaturation step at 96 °C for 1 minute, 30 cycles of a three-step denaturation at 94 °C for 10 seconds followed by 59 °C for 60 seconds of annealing and 72 °C for 30 seconds of extension and a final extension at 60 °C for 10 minutes followed by incubation at 4 °C.

The Promega PowerPlex Fusion 6C thermal cycler parameters are shown inputted in the Applied Biosystems Veriti instrument. The PCR steps are a denaturation step at 96 °C for 1 minute, 30 cycles of a three-step denaturation at 96 °C for 5 seconds followed by 60 °C for 60 seconds of annealing and 72 °C for 30 seconds of extension and a final extension at 60 °C for 10 minutes followed by incubation at 4 °C.

The Promega PowerPlex 35GY thermal cycler parameters are shown inputted in the Applied Biosystems Veriti instrument for a 25 µL reaction volume. The PCR steps are a denaturation step at 96 °C for 1 minute, 25 cycles at a 6 °C / second ramp speed of a three-step denaturation at 98 °C for 5 seconds followed by 60 °C for 60 seconds of annealing and 72 °C for 15 seconds of extension and a final extension at 60 °C for 10 minutes followed by incubation at 4 °C.

The Veriti thermal cycler tools menu is shown in the photo. Routine maintenance and calibration should be performed according to the lab's requirements for accreditation and lab SOP. Examples include temperature verification and run cycle performance.

The next step in the DNA typing process is DNA separation, as circled.

DNA separation and detection instruments include the Applied Biosystems 3130 capillary electrophoresis instrument for CE with 5-dye detection and 4 to 16 capillaries, the Applied Biosystems 3500 CE instrument shown with 6-dye detection and 8 capillaries, the Promega Spectrum CE instrument with 8-dye detection and 8 capillaries, and the Promega Spectrum Compact CE instrument with 8-dye detection and 4 capillaries.

In preparing to run the DNA separation and detection instruments, login, open the software, and input the run parameters in the software.

The screen with the Applied Biosystems 3500 CE software is shown on this slide.

The Life Technologies Applied Biosystems 3500 CE software version 3.1 is shown on this slide and upcoming slides and several troubleshooting topics are addressed.

The Applied Biosystems 3500 Dashboard is shown on this slide with the instrument not connected so the instrument parameters and consumables are showing unknown in red.

The Applied Biosystems 3500 Dashboard is shown on this slide with the instrument connected so the instrument parameters and consumables are showing with samples remaining or not remaining. You can see that the anode and cathode buffers need changed.

To replace the anode and cathode buffers, retrieve new barcoded buffers from the fridge and remove their plastic seals on top. Face the barcode to the reader in the back for the anode buffer and add the rubber septa to the cathode buffer. Replace as shown.

Now the 3500 CE dashboard shows the buffers have been replaced with the dials reading at a full quantity of injections remaining.

The Applied Biosystems 3500 CE Maintenance Wizard options are shown on this slide. The wizard guides the user through the actions in a step-by-step fashion. Options include installing a capillary array, removing bubbles from the polymer pump, washing the pump chamber and channels, filling the array with fresh polymer, replenishing the polymer, changing the polymer type, instrument reactivation and instrument shutdown.

This slide shows the Applied Biosystems expiration warning for polymer installed on the 3500 CE. To replace the polymer, fill the water block and overflow using a 50 mL syringe, flush polymer, wait 2 min, then select finish or one can override the expiration date warning, if desired. Once the polymer is replaced, the array can be installed and filled with polymer.

This slide shows the AB 3500 CE wizard to replace the capillary array. The wizard leads the user through the steps when they select next.

This slide shows the AB 3500 CE wizard to replace the capillary array. Step 1 is shown; a new capillary array is needed.

This slide shows the AB 3500 CE wizard to fill the array and remove bubbles.

This slide shows the AB 3500 CE wizard to fill the array and remove bubbles. Step 1 is shown; the user is prompted to select where the bubbles are found (before or after the array) or if they are gone.

This slide is step 2 of the wizard to remove bubbles from the array in which the array may be filled with polymer.

This slide shows an AB 3500 CE spatial calibration output.

This slide shows an AB 3500 CE spectral calibration output with the J6 dye set. Note the clear detected orange, red, yellow, green, blue and purple dyes.

This slide shows an AB 3500 CE spectral calibration output with the BT6 dye set. Again, note the clear detected orange, red, yellow, green, blue and purple dyes.

Once the CE is configured, samples are prepared to run. The steps are dilute, denature and load. To a 96-well skirted plate, combine 0.5 µL size standard (vortexed well) and 8.5-12 µL Hi-Di formamide per sample as the protocol indicates. Create master mix for the total number of samples to reduce pipetting. To the mixture, add 1 µL of amplified sample or allele ladder (before and after samples). Add 10-12 µL total per well to plate, pipetting down the side to avoid bubbles. Lay a clean septum on the plate and press in. Denature the plate for 5 minutes at 95 °C and snap cool on a cold block or ice. Load all 8 wells in a column per injection (fill with formamide if you have an empty lane). Discard any used formamide.

This slide shows the new plate setup tool in the AB 3500 CE. Copy in or type in the name and select number of wells, plate type, capillary length, and polymer type from the pull down menus.

Next assign the plate contents in the plate table and the assay, file name convention and results groups.

This slide shows a screenshot with the file name convention options listed to be added to the plate file name including sample name and date.

Here a user is loading a plate to the AB 3500 CE instrument. Select the tray button on the front of the instrument to home the tray. Insert the plate in a white and blue cartridge. To run in A, put your plate in the A spot. To load the plate, pull side tab to put in and close.

The photo shows the loaded plates and buffers. Close door and push the button to home the tray and start the run program as on the next slide.

To start the CE run, in tray view, select sample type: sample, positive or negative control, or allele ladder. In plate view, highlight all samples to run. Link the plate to run, the default is plate A. When asked to save the changes select yes. Start the run.

This slide shows the software while a plate is running on the CE.

The next step in the DNA typing process is allele call interpretation, as circled.

The software GeneMapper can be used to analyze raw .hid files from the Applied Biosystems 3500 CE. You can create a new file and add new samples to the project.

As shown here, the STR kit panel and ISS used must be indicated for interpretation in GeneMapper.

The analysis method and thresholds must also be defined.

The analysis method for each sample is set based upon the lab's validated thresholds. Interpretation thresholds are commonly set at 200 or 300 RFU and a common analytical threshold is 50 RFU.

The next step in the DNA typing process is statistics, as circled.

Instruments require care and maintenance. Warnings will be given in the software when maintenance is due or consumables need replaced as shown in the picture. Best practices include replacing rubber septa on plate and buffers when they are changed, replacing the anode buffer every 14 days, replacing the cathode buffer every 14 days, replacing the capillary array every 160 injections, refilling the polymer every 384 injections, and replacing the fittings and O-rings as needed.

DNA separation reagents including POP-4 (shown), anode buffer and cathode buffer should be stored in the refrigerator (2-8 °C).

DNA separation reagents including Hi-Di formamide, internal size standards, and allele ladder should be stored in the freezer (-15-25 °C). Hi-Di formamide is a highly deionized formamide prepared by formamide passing over ion exchange resin. Allele ladders are specific to each kit with DNA fragments of the allele sizes.

That's all for today regarding instrumentation and reagents. Keep abreast of new technology and options through professional development and course opportunities.

Some questions to consider as you review and study include: What component of blood, semen and saliva contains the nuclear DNA? What agents are used for cell lysis and how does each work? What pH is used for DNA binding and elution in DNA extraction? What type of DNA is the final EZ1 eluted product? How is DNA separated in extraction instruments? What steps are performed by robotic DNA extraction instruments? What is three-step PCR and what happens at each step? What functions are performed by CE instruments? What calibration steps must be

performed prior to running CE instruments? What maintenance steps must be performed prior to running the CE?

If you want to know more about the content in this video, review the Andersen and Bramble's paper "The effects of fingermark enhancement light sources on subsequent PCR-STR analysis of fresh bloodstains," "PCR analysis from cigarette butts, postage stamps, envelope sealing flaps, and other saliva stained material" by Hochmeister et al., "Application of deoxyribonucleic acid (DNA) polymorphisms to the analysis of DNA recovered from sperm" by Giusti et al., "Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material" by Walsh, and chapters 2 (extraction methods), 6 (capillary electrophoresis), and 11 (low-level DNA testing) from the book Advanced Topics in Forensic DNA Typing: Methodology by Dr. John Butler.

This presentation is focused on the topic of Contamination.

This presentation is made possible through award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST) and created by Dr. Kelly Elkins.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3f in ANSI/ASB Standard 115.

The learning objectives of this video include providing trainees with an understanding of sources environmental, procedural and other contamination, sample handling strategies and preventative methods, decontamination procedures, detection limitations, root cause analysis including corrective action when contamination occurs.

Before we begin, let me provide some definitions for key terms. Contamination is the unintentional introduction of exogenous DNA or other biological material in a DNA sample, PCR reaction, or item of evidence; the exogenous DNA or biological material could be present before the sample is collected or introduced during collection or testing of the sample. It is not native to the evidence. Contamination may be accidental or unintentional. The image shows William Dickson's 1894 5-second video, "Fred Ott's Sneeze" which depicts a source of contamination.

Sources of contamination include exogenous DNA. Exogenous DNA is DNA contamination from outside of the organism or case of study. Exogenous DNA includes environmental and procedural contamination. Environmental contamination includes genomic DNA from the air, surfaces, soil, and water rather than the human - or wildlife – suspect or victim sample. Procedural DNA includes DNA contamination from lab analysts such as sneezing as shown on the previous slide. It can also include contamination from reusing dirty tips or forgetting to change tips or not changing gloves frequently enough or mixing or commingling samples on plates or in runs. Removing seals from 96-well plates can lead to contamination. Using filter tips (shown) with pipettes can reduce contamination from aerosols.

Sources of contamination include first responders, laboratory personnel, crime scene technicians, law enforcement, medical personnel, and objects or surface transfer, to name a few.

It can never be known with certainty that a casework or database sample is contamination-free.

The sensitivity of testing instrumentation and methods in human forensic DNA laboratories has steadily increased and has resulted in a greater chance of detecting low-level contamination events.

Labs should have documented procedures designed to minimize contamination and loss.

The DNA typing process is a multi-step process. It includes evidence collection at the crime scene, sampling the evidence, extraction of DNA from the sample or samples, and amplification of DNA for quantification and STR typing. The process concludes with interpretation, analysis, and reporting.

Contamination can occur at any of the crime scene and lab handling stages including sample collection, sampling, DNA extraction, PCR and CE. Being aware of steps in which contamination may occur, and potential sources of contamination can reduce contamination.

In the sample collection step, sample contamination can occur with genomic DNA, body fluids, tissue or exogenous DNA already present in the environment. Samples can be contaminated with genomic DNA traces on crime scene equipment. During sampling, sample contamination can arise from DNA present on lab surfaces, from DNA present on scissors, lab paper, swabs, and so on, and from DNA contamination between samples during preparation. DNA extraction is the most susceptible step. Samples can be contaminated from DNA present on scissors, lab paper, swabs, and so on as well as DNA contamination in extraction reagents. Thus, it is important to obtain reagents and consumables such as tubes and tips from an ISO 18385 compliant manufacturer or PCR grade products, and document lot numbers in documentation. Contamination can also occur in PCR and CE steps. Sample DNA can be contaminated with amplified DNA from a previous PCR reaction, DNA contamination in PCR reagents or consumables, and contamination with other amplified and dye labelled samples.

When evidence is collected at the crime scene, samples can be contaminated with genomic DNA, body fluids, tissue or exogenous DNA already present in the environment.

During sampling, samples can be contaminated with DNA present on lab surfaces, from DNA present on scissors, lab paper, swab (shown), and so on and DNA contamination from other samples during preparation.

Contamination can arise during DNA extraction due to DNA present on lab surfaces, from DNA present on scissors (shown), lab paper, swabs, and so on and DNA contamination in extraction reagents. Contamination can also be found on consumables such as pipette tips or tubes.

Contamination can occur in preparing and conducting PCR reactions. DNA can be contaminated with amplified DNA from a previous PCR reaction or from PCR reagents or consumables as well as dirt or other molecules as shown.

The working environment and procedures should mitigate contamination.

There are several methods for avoiding contamination including restricting lab access, use of PPE, awareness of how and where contamination can occur, use of effective decontamination procedures, and processing samples separately. Separating handling, space use, using controls and employing an elimination database can reduce and eliminate contamination.

Let’s talk some more about those preventative methods of avoiding contamination. To best avoid contamination, access should be limited to necessary personnel. PPE should be worn and changed when contamination is suspected or changing activities. Personal protective equipment (PPE) includes gowns and lab coats, booties, gloves, hair coverings, and face masks; beware of static cling when removing PPE. Awareness of how contamination is introduced can be informative in avoiding contamination. For example, avoid excessive speaking or activities that could introduce biological material over samples and bench surfaces. Also, avoid opening more than one sample at a time. Utilize tested and approved decontamination procedures frequently. Separate processing such that evidence (crime scene) samples are processed separately from reference (exemplar) samples. Separate handling of samples and open tubes with tube openers and one tube at a time. Separate spaces such that PCR amplification samples are prepared in a designated area in PCR prep hoods. Do not move post-PCR amplification samples or waste to the pre-PCR amplification areas; store them in each space in the appropriate freezer or refrigerator. Equip pre- and post-amplification areas fully so that equipment can remain in place to avoid contamination in moving between areas. Implement and use appropriate controls such

as reagent blanks, extraction controls, and amplification controls and clearly label tubes following labelling procedures or conventions as written. Establish a DNA elimination database for laboratory staff and visitors and rules for searches and notification policies for privacy purposes.

For safety, quality and avoiding contamination purposes, there are several activities that are not allowed in the lab. The items may fall into evidence, cause a chemical reaction or fire, or become contaminated by evidence or chemicals that could cause injury or harm to the analyst and the surrounding staff. The prohibited activities include smoking, eating, drinking beverages, makeup application including chapstick, and wearing hanging jewelry as it may pose a safety and quality risk.

Personal protective equipment or PPE for reducing risk and avoiding contamination include googles as shown; they reduce the risk of samples and reagents entering the eyes. Additionally, gloves and lab coats as shown reduce the risk of DNA transfer to surfaces, equipment, other evidence and reference samples and extracts as well as microorganisms from samples to staff.

Preventative methods including the use of personal protective equipment (PPE) include wearing googles, gloves, hair coverings and lab coats, as shown, as well as booties, hair coverings, heat protection gloves, Tyvek or Kleengard suits, and a respirator as needed. In the event that a foreign material enters the eye, an eyewash located in the lab should be used immediately. Analyst safety is the utmost priority and essential for quality work.

Use biohazard labels to signify biohazard waste. Dispose of contaminated gloves, masks, suits and consumables in red biohazard bags as shown.

Preventative methods for avoiding contamination include the use of a PCR preparation hood as shown on this slide. UV light is used to destroy surface DNA that could be introduced to samples. The surface is cleaned with 10% bleach followed by 70% ethanol before using the space. Paper can be used to cover the surface and changed to prevent carryover and contamination of samples. Dedicated filtered pipette tips, pipettes, and equipment in pre- and post-PCR amplification areas eliminate DNA carryover. Biosafety laminar flow hoods (not shown) should be used when working with potentially pathogenic materials as the airflow carries the airborne pathogens away from the analyst and from contaminating samples.

Decontamination procedures should be used prior to opening evidence or samples and reagents and consumables to destroy cellular and DNA material on surfaces. Solutions include 10% bleach, 70% ethanol, DNA off, soap and water, and ethylene oxide. UV light as well as steam via autoclaving are also widely used. Do not spray bleach, water, ethanol, and other decontamination and cleaning sprays directly on pipettes/instruments when cleaning.

10% bleach oxidizes DNA and cleaves the strands into smaller pieces. Wear PPE and avoid contact. It is corrosive and discolors clothing. Prepare by diluting 1 part bleach with 9 parts water. Bleach degrades over time in the presence of light and at room temperature. Prepare 10% bleach fresh daily or weekly per SOP. Apply to the surface by spraying. Apply and let stand on surfaces for at least 10 minutes or according to SOP before wiping. Bleach damages metal surfaces and sensitive equipment so an alternative method may be indicated. Wipe bleach with a paper towel or cloth and rinse the surface with water or ethanol. When decontaminating bones, soak it in at least 3% bleach for at least 15 minutes. Gloves should be worn when handling.

70% ethanol is flammable, an eye irritant and toxic. It can be diluted from 200 Proof (shown) or purchased as 70% ethanol. It evaporates quickly so 10-minute application is not achievable. Apply following bleach to wipe down surfaces and equipment. Small tools, such as tweezers, scissors or another item, may be soaked in the solution for at least 10 minutes or following the SOP.

DNA off (shown) is commercial agent that should be applied according to the manufacturer's instructions. According to the manufacturer, DNA-OFF is a proprietary "non-alkaline, non-corrosive, and non-carcinogenic cleaning solution" that contains a surfactant. Store at room temperature. Heat at 37 °C to redissolve if precipitates form. Apply directly to surfaces such as PCR prep hoods for a few minutes, wipe with a paper towel, rinse with water and wipe again with a paper towel. Wear PPE as the product "may cause eye or skin irritation; may be harmful if inhaled." Cationic surfactants such as octyl-trimethyl-ammonium bromide (OTAB), dodecyl-trimethyl-ammonium bromide (DTAB) and cetyl-trimethyl-ammonium bromide (CTAB) induce structural changes in DNA.

Soap and water is shown on this slide. Soap disrupts and dissolves cell lipid membranes. The released DNA is water soluble and dissolves and can be wiped away. Water, especially at high temperatures, hydrolyzes DNA into fragments. Wash surfaces with hot soapy water, wipe away and rinse with water. Cells are persistent and items soaked in water at neutral pH can still yield DNA profiles.

UV light is used to decontaminate lab surfaces. Short wave UV-B (290-320 nm) or UV-C (254 nm) light is typically employed. UV-B causes pyrimidine, such as thymine and cytosine, cyclobutane formation / dimerization. UV-C causes strand breaks and generates reactive oxygen species including singlet oxygen, hydrogen peroxide, and hydroxyl radicals that oxidize DNA bases. Decontamination procedures should be written in SOP and scheduled routinely. A length of time of 15 to 30 minutes depending upon distance to the surface and intensity of the source is used.

An autoclave (shown) is used to sterilize consumables or packs, lab glassware, stainless steel die presses and other items. Autoclaving destroys DNA templates using high heat, steam, and pressure. Heat induced hydrolysis of DNA strands breaks them into small fragments. Autoclave items as indicated or for 80 minutes at 121 °C to remove intact DNA that may serve as a template in amplification. Consumables such as tips and tubes (packs) are typically autoclaved at 121 °C for 20 minutes.

Ethylene oxide is a probable carcinogen and PPE should be worn. The structure is shown. Ethylene oxide is an SN2 (substitution nucleophilic biomolecular) alkylating agent in which the molecule is reacted via backside attack of the bond. It destroys DNA via hydrolytic deamination via hydroxy group on alkyl side chain.

Shown on this slide is an example of swab decontamination of a thermal cycler. Soak a cotton swab in isopropanol or 1:10 v/v dilution of 5.25% bleach or apply with an atomizer. Clean the sample wells by swabbing. Let the isopropanol evaporate. Rinse the block with deionized water following bleach treatment. Avoid excessive use of bleach as the sample blocks can corrode.

Shown here is the screen to decontaminate a EZ1 Extraction Robot using UV light for the set time.

Detected contamination should be documented. Documenting includes logging events and when they likely occurred. Access should be monitored and restricted. PPE should be worn and changed when moving to different areas of the lab. Detection and control of contamination includes evaluation of environmental DNA on surfaces and consumables, creating an elimination database for comparison, performing intra-batch comparisons and cross-contamination checks, and using probabilistic genotyping software to aid in detection.

Contamination should be monitored by staff when visitors and cleaning staff come through the lab but also in air and ventilation, surfaces, and from medical personnel, production staff, crime scene technicians, forensic staff, and interns.

DNA, cellular material, and substance contamination monitoring should occur through regular evaluation of lab surfaces, instruments, and equipment for detectable and amplifiable DNA.

The lab should have an elimination database to monitor sources of DNA contributors. Voluntary samples should be collected from lab and law enforcement staff including technicians, analysts, technical leads, interns, investigators, and administrators. The elimination database should not be associated with the crime scene samples but should include people who had access to the crime scene or samples such as people working with samples can shed DNA on the samples. We cannot detect all forms of contamination, but we can avoid contamination from happening.

There are several detection limitations that are important when detecting contamination. PCR is very sensitive, and caution must be taken to avoid contamination. We can detect amplifiable DNA that amplifies in the assay using fluorescent-tagged primers or intercalating dyes, but we need sufficient amplicons to exceed the fluorescence detection threshold. We can detect the equivalent of one cell (6 pg) if the DNA is intact and amplifiable with the primers, but to avoid stochastic effects, 100 pg to 1 ng is recommended for most reactions so very low concentrations may not be detected.

What can we do if contamination is detected? A root cause analysis can be performed. We can identify possible observations, symptoms or issues indicating contamination, such as detectable quantifiable DNA in negative amplification control or reagent blank/extraction control or obtaining an STR profile or partial profile for a negative or substrate control. The tree shows the process if an STR profile is obtained for a reagent blank. The first step is to identify the issue or problem. Next, the cause is considered and determined. This may be sample contamination with exogenous DNA at collection, contamination from a lab surface when sampling, contamination from tubes or tips used in DNA extraction, or contamination from PCR reagents used in amplification.

The root cause analysis steps include 1) define the problem, 2) Determine the "Why" or cause of the problem, 3) Collect data about the steps preceding the problem, and 4) Identify or locate the root cause of the contamination and eliminate that cause using a well-developed and effective corrective action.

The types contamination are considered in root cause analysis. These include material cases such as contaminated consumable tips, plastics and/or gloves or contaminated reagents and solutions as well as environmental causes such as a contaminated bench, surface or airflow, a machine cause such as a contaminated instrument, a method issue such as altered procedural steps, and/or a staff cause, such as skills that need remediation.

Defining the problem is the next step. "Problems" may be categorized into three main types: Type 1: Deviation, non-conformity or inconsistency where the actual and expected results differ. For example, an erroneous result obtained during proficiency testing, Type 2: An undesirable situation or event. For example, accidents such as the loss of a sample, contamination events, loss of electronic data, or Type 3: Undesirable performance. For example, failure to follow established validated and documented protocols. The level of difficulty to detect and analyze the problem is dependent upon the type/category it falls within.

Controls may be used to uncover the problem. Types of controls include the negative control such as an amplification reaction in which no DNA template is added to the primer and reaction mix to ensure the method produces no detectable DNA or a negative fluorescence response. Substrate negative control is a control to test if environmental DNA is present on the surface or swab the biological material is sampled from. A reagent blank (extraction negative) control is a control to test if the extraction reagents are contaminated. A quantitation negative control is a control to test if the quantitation reagents are DNA free. A reagent blank (amplification negative) control is a control to test if amplification reagents are contaminant free. A no template control (NTC) is an amplification reaction in which DNA molecules are not added to the reaction and primer mix.

Reference samples can be used to compare to the sample test results. Reference samples are samples of known origin to compare to evidence sample. They include buccal swabs from victims / suspects and hair samples from victims / suspects. It is best practice that they are tested after the evidence sample(s).

A contamination tolerance level is the level of detection at or below which contamination does not interfere with a confident interpretation of the data based on validation. In DNA typing the tolerance threshold is the interpretation threshold.

Shown on this slide is a figure with an analytical threshold set at 50 RFU and an interpretation threshold set at 200 RFU. Peaks below 50 RFU here are considered noise. The analytical threshold is 1) The minimum height requirement at and above which detected peaks on a STR DNA profile electropherogram can be reliably distinguished from background baseline noise; peaks above this threshold are generally not considered noise and are either artifacts or true alleles. 2) A “Relative Fluorescence Units” (RFU) level determined to be appropriate for use in the PCR/STR DNA typing process; a minimum threshold for data comparison is identified by the specific forensic laboratory through independent validation studies.

There are several ways to determine the analytical threshold and if a peak is real. The peak is reportable if it is above the stochastic or interpretation threshold setting determined in validation studies such as 150 or 200 RFU and the allele has not dropped out due to primer binding site mutation or another issue. The peak is real if it reliably amplifies in concordance studies and is above the analytical threshold, such as 30 or 50 RFU. The peak is real if the ratio of heterozygote loci peaks is less than 60%.

Failed controls can be identified if positive or negative controls produce an unexpected result.

There are several types of contamination events. These include contamination in a negative control or reagent blank, contamination in a positive control or contamination in a forensic or reference sample.

Controls can fail for many reasons. These include handling error, contamination with exogenous DNA and a faulty lot of reagents. For example, in 2007, there was contamination found in an allelic ladder in PCR reagents sold by Applied Biosystems. Once the root cause was identified, a manufacturing change was implemented to prevent this in the future.

Identifying the contamination issue is not easy. First it must be determined if the event was an isolated instance or one of multiple related events. A single contamination event may be due to a contaminated pipette tip or tube. If low level contamination is detected in all samples but has no impact on interpretation, it may be a DNA profile from a high-quality single source or two-person mixed DNA profile with a very low-level minor component consistent with the profile in the negative control and possibly other samples below interpretation threshold. Sometimes there is an isolated cross contamination, or event could not be determined. Contamination events remind us that we need to change gloves frequently.

In interpreting failed controls and contamination events, conduct a risk assessment and determine the cause and impact. If the results are not suitable for interpretation, it may be due to results that are compromised and are not suitable for retesting or a contaminated control and sample that contained a mixture of more individuals than the validated interpretation procedure permits. If the results are suitable for interpretation, it must be within constraints of the lab's internal validation studies and documented interpretation protocols. The source may be identified by name, employment position or other descriptor as permitted by law and agency policies (this may include the analyst’s name whose DNA was detected in negative control). Apply the appropriate statistical analysis to assess the similarities or differences of the two DNA profiles.

When corrective actions are needed, create an actionable strategy and confirm the solution worked. The strategy may include purchasing new kit(s) or consumables, cleaning or decontaminating instrument(s) and/or surface(s), retesting the steps prior to error or issue, and/or providing retraining or additional training. Following the solution implementation, confirm the solution worked, evaluate the technician or analyst skill with proficiency test samples, and run QC samples with new products or cleaned instruments and/or surfaces.

Retesting is sometimes, but not always, possible. Sufficient material must be available to sample or retest. When retesting, analyze the new extract or material prior to comparing to the previous results. Retest when the issue can be determined and ameliorated and the associated profiles are needed for comparisons. Retesting is not possible when the sample was consumed during the initial analysis. Do not retest when additional testing would exhaust the remaining portion of the sample or DNA extract and eliminate the possibility of future testing or when the associated profile or profiles would not be suitable for comparison if the controls produced the expected results.

Contamination events should be documented, tracked by lot numbers, case numbers, and dates, remediated and the lab process improved. Transparency is key and both original and retested data should be reported. Communication can include the lab management, staff, and legal discovery.

A case study example is provided on this slide. First, an observation is made by someone. It could be a manager, technician, scientist or even an intern. In this case, contamination by a crime scene technician was identified in multiple samples in a year using the QC elimination database and laboratory management system. Next, a corrective action is planned and

undertaken based on observation and consideration of the approach most likely to fix the problem. In this case, a technician was not changing gloves as frequently as required in the SOP. This was confirmed by observation and sample tracking data. Knowing the problem makes finding a solution possible. In this case, maintaining a quality control elimination database of employee profiles is key.

That's all for today regarding Contamination. Keep abreast of new technology and options through professional development and course opportunities.

Some questions to consider as you review and study include: Define contamination in DNA analysis. List some causes of contamination. List some processes and procedures to avoid contamination. List some decontamination methods. Explain how root cause analysis can be used to trace contamination events. Explain which failed controls can be interpreted and why.

If you want to know more about the content in this video, review the ANSI/ASB Standard 115 and 136 available on the AAFS website and the OSAC 2020-S-0004, Standard for Interpreting, Comparing and Reporting DNA Test Results Associated with Failed Controls and Contamination Events available on the OSAC website.

This presentation is focused on the topic of Quality Control.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3g in ANSI/ASB Standard 115.

This presentation is made possible through award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST) and created by Dr. Kelly Elkins.

The learning objectives of this video include providing trainees with an understanding of the history of quality control in the amplification, DNA separation and allele detection process to include appropriate controls.

Quality control is a part of a quality management system (QMS). A quality system is the organizational structure, responsibilities, procedures, processes and resources for implementing quality management and is often managed with a records or laboratory information management system, or LIMS system. It includes Quality assurance (QA), Quality control (QC), and inspections.

An overview of quality management principles is shown on this slide. The items include a process approach, evidence-based decision making, leadership, engagement of people, improvement, relationship of management and customer focus.

Features of a QMS include goals and objectives, organization and management, personnel, facilities, direction, annual review, training review and approval, proficiency testing, data quality checks, data interpretation guidelines, and mixture interpretation guidelines.

Quality control is a system of maintaining standards by testing the reagents, platforms, and products to ascertain that a specification or threshold is met. Quality control is used to detect non-conforming results and fulfills QMS requirements. Routine quality control is part of the process. Each part of the process is inspected, tested and measured. Quality control includes using premade and purchased chemicals and products including kits and reagents as well as in-house prepared reagents. QC samples are run with each set of samples, daily, or for a lot number, as per the lab's SOP.

A part of quality management and quality control is the use of standards. Standards include control DNA isolated from a known, documented, and reproducible source and may be purchased as shown in the image. Standards are consensus-based, scientifically sound and proven to work, well-characterized, and can be incorporated into accreditation processes.

Quality assurance ensures that all functions perform as intended, includes a review of education and training to meet legal and professional requirements, includes technical review of casework and testimony, has a staff person coordinate and review proficiency testing, accreditation and audits and lead remediation efforts. QA can also uncover and mitigate process issues using data-driven approach.

The use of controls, references and standards is necessary to interpret data and assess if instruments and products are working properly and avoid instrument bias.

The are quality controls in all aspects of the DNA typing process. When sampling an evidence sample, reference samples and elimination samples of other investigators or staff should also be collected if they have not been in the past. In the DNA extraction step, a positive control,

substrate control or No Template Control, and/or reagent blank, RB, should be run. In the DNA quantitation step, quantitation standards must be run and a positive control and reagent blank is also typically included. In STR fragment analysis, an allele ladder, positive control and negative control / non template control and/or reagent blank, RB, should be included. An elimination sample may need to be included. An internal lane size standard, ILSS, should be included in each ladder, sample, and control. Be sure to consult your lab's SOPs to be sure that all controls are performed.

Let's talk more about those controls used in forensic DNA analysis and why they are used. A reagent blank control is an analytical control sample that contains no template DNA and is used to monitor contamination from extraction to final fragment or sequence analysis. This control is treated the same as, and parallel to, the forensic and or casework reference samples being analyzed. No DNA template, control DNA or standard is added. A positive amplification control is an analytical control sample that is used to determine if the PCR performed properly. This control consists of the amplification reagents and a known DNA sample and is frequently a standard DNA template. A negative amplification control is used to detect DNA contamination of the amplification reagents. This control consists of only amplification reagents without the addition of template DNA. Background controls are used to test that the background is negative or as expected for the test run without a sample. These are used in fluorescence detection for example.

A negative control is an amplification reaction in which no DNA template is added to the primer and reaction mix to ensure the method produces no detectable or a negative response. An internal standard is a known component is added to the evidence or control samples. Some examples of negative controls include substrate negative controls, reagent blanks, quantitation negative controls, and no template controls. A substrate negative control is a control to test if environmental DNA is present on the surface or swab the biological material is sampled from. A reagent blank or extraction negative control is a control to test if the extraction reagents are contaminated. A quantitation negative control is a control to test if the quantitation reagents are DNA free. A reagent blank or amplification negative control is a control to test if amplification reagents are contaminant free. A no template control or NTC is an amplification reaction in which DNA molecules are not added to the reaction and primer mix.

Reference material and known samples and standards are used to check that the reagents and instrumentation are performing properly and the expected result is obtained. Reference material including certified or standard reference materials is a material for which values are certified by a technically valid procedure and accompanied by, or traceable to, a certificate or other documentation which is issued by a certifying body. Known samples are biological material whose identity or type is established. These are important samples in DNA testing.

For quality control standards, you or your lab may use standard control DNA included in the kit or purchased separately (for example 007, K562, 9948, 2800M) for quantitation and amplification controls. The standards are used to make the standard curves and generate high quality DNA quantitation and typing results. Standards have a known profile, are reproducible, and can be used for troubleshooting if a non-conforming result is detected. Process each amplification set with proper controls. Each batch run must include at least one positive control.

An example of a standard is the internal lane size standard (ILSS) or internal size standard (ISS) for electrophoresis sizing used to determine a correlation coefficient to size sample

fragments. For troubleshooting, if all of the peaks for the sizing standard are not present, it suggests a temperature, run time, or injection problem.

Some sources of controls and standards are independent institutes and government entities. These include Coriell Institute for Medical Research cell lines and extracted DNA, NIST's Standard Reference Materials or SRMs, manufacturer's included standards such as 007, 2800M, 9947A, 9948, K562, and ISS and standards from molecular biology suppliers such as OriGene.

Another practice necessary to obtain quality lab results is preparing surfaces prior to use. Surfaces can be cleaned, disinfected and sterilized in preparation for use. Cleaning and sterilization agents that are frequently used include soap and water, 70% ethanol, 10% bleach, commercial specialty products such as DNAZap, DNA OFF, DNA OUT, and CIDEX, and ethylene oxide. UV irradiation can also be used to prepare surfaces.

An approach is batch processing. In batch processing, exemplars and evidence must be handled separately (never placed on the same tray) and can run on the same instrument, but on separate plates (separate runs). Each amplification should be tested with amplification controls. Correctly document and ensure set-up is witnessed. The controls must match or exceed injection sensitivity used for samples. Stagger incubation by approximately 1 hour if running multiple batches in a day.

Regarding instrument and computer maintenance, handle the instruments gently and keep them clean. Monitor instrument performance to detect issues such as leaks, bubbles, and calibration issues. Defragment hard disks and delete non-essential files to improve system performance. Report any issues to Quality Assurance.

Safety data sheets or SDS are important documents in the lab that should be reviewed prior to working with chemicals. SDS documents describe the handling safety, storage, transport, disposal, and chemical and physical properties of a product or chemical. They can be downloaded from the manufacturer's website or may be packaged with the item. They can be stored in binders or in computer folders or accessed just in time via the World Wide Web.

Safety data sheets have extensive information including information on the identity of the substance or mixture, the composition and ingredients, personal protection equipment for handling, handling and storage, first aid measures in case of exposure, precautions in case of accidental release, firefighting measures, supplier details and contact, physical and chemical properties, stability and reactivity, toxicological information, ecological information, disposal considerations, hazards information including classification of the substance or mixture, transport information and precautions, and regulator information.

Next let's talk about accuracy. Accuracy is the degree of conformity of a measured quantity to its actual (true) value. To maintain accuracy instruments and tools are calibrated using standards and pipette performance is assessed using balances. Accuracy is the ability to obtain the correct result with standards, competency, and proficiency test samples. Record instrumental readings as output without rounding. Rounding to the appropriate significant figures can be performed after computations are done.

Case reports are completed and filed. Reports should go to the requesting officer and officials of the court.

Documentation includes tracking reagents lot and product used, the case report results of analysis, contemporaneously recording by the person who performed the testing, completely, and with necessary supporting information. Documentation should be retained and provided as required by law.

Administrative review is an evaluation of documentation to check for consistency, accuracy, and completeness. Lab data, documentation, and reports are checked for accuracy and consistency with lab SOPs. Reviews are also checked for correctness.

Technical review is an evaluation of reports, notes, data, and other documents to ensure there is an appropriate and sufficient basis for the scientific conclusions. Technical review ensures that conclusions are concordant with data/results obtained.

As discussed before, the quality management system has many parts. Most forensic DNA laboratories have a quality assurance or QA program to ensure quality testing. Key elements of a well-designed QA program aim to ensure samples were protected from contamination and handled by trained personnel in accordance with documented SOPs. The FBI provides guidelines for laboratories conducting forensic DNA testing and convicted offender databasing.

Not all labs are accredited but achievement of accreditation signifies the quality of the lab and work product and that specific standards are documented to be followed. It is a sign that work is credible, structured, consistent, valid, reviewed, and grounded in science. Accreditation can be important in ensuring the results are legally defensible.

Non-conformance issues may arise in accredited and non-accredited labs and include issues with case registration, case management (within and between departments and agencies), contamination, interpretation, reporting, security, among other issues.

Some quality control issues include instances in which an extraction negative is contaminated and DNA is detected in quantitation or peaks in CE post-amplification, DNA extract quantities are low for buccal swabs or reference samples, and mixed profiles are detected in single source suspect sample profiles.

Non-conformance must be noted in reports and supervisors notified. Non-conformance is any aspect of testing, result or analysis that does not conform to SOPs or agreements with the customer. Non-conformance can include issues of training, consumables, reagents, and with instruments. Non-conformance may be escalated to a corrective action. A corrective action deals with errors as they occur and to prevent reoccurrences, improve practice, and solving and remediating issues. Non-conformance reporting can help to create and institute new policy to avoid such issues in the future. Non-conformance reporting and correction may be an accreditation requirement.

Non-conformance reporting and correction includes evaluative risk and the significance of an error or issue, determining how the error or issue occurred, logging errors and issues in log books and bench notes, and communicating and discussing issues with technical supervisors and lab management and teams for quality improvement.

Maintaining quality is integral to forensic science. Curiosity to solve problems that the lab encounters, professional development and continued learning, asking questions and providing input to supervisors and managers are all essential for maintaining quality.

That’s all for today regarding Quality control. Keep abreast of new approaches through professional development and course opportunities.

Some questions to consider as you review and study include: Define quality control. What is the purpose of a positive control? What is the purpose of a negative control. Give some examples of negative controls. What is the difference between a background test, a NTC and a negative control? What is an elimination sample? Know how you should report a nonconformance issue in your lab.

If you want to know more about the content in this video, review the ANSI/ASB Standards 115 and 117 available on the AAFS website and the FBI Quality Assurance Standards for DNA Testing and Databasing Laboratories and DNA Testing Laboratories.

This presentation is focused on the topic of Storage, Preservation, and Retention.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3h in ANSI/ASB Standard 115.

This presentation is made possible through award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST) and created by Dr. Kelly Elkins.

The learning objectives of this video include providing trainees with an understanding of: The storage, preservation, and retention of amplified DNA product according to laboratory policy.

First, let us recall the process of collecting evidence to analyzing it in the lab. The steps include: Evidence collection, Evidence sampling, DNA extraction, DNA quantitation, PCR amplification, Separation & detection, and Interpretation.

Separate storage areas shall exist for reagents, consumables, DNA extracts, and PCR products.It is important to properly handle, store and retain DNA extracts as the DNA may be the only source of material for future testing. Historically, extracted DNA has been stored in water, TE buffer, or in a preservative and then refrigerated for the short term or frozen for long term storage. The stability and recovery of DNA extracts is dependent on the quantity and quality of the extracted DNA prior to storage as well as the type of tube and temperature used for storage.

DNA extracts may be assigned their own sample numbers and barcodes or be considered work product for consumption in testing. Preservative may be added to protect the DNA integrity or the DNA may be eluted as lab protocol indicates. Extracted DNA samples are stored at in the refrigerator at -4 °C or in a -20 °C freezer in the pre-PCR area or discarded following testing and case resolution, if indicated. Label DNA extracts with 4-digit code or barcode or as indicated by your lab SOP. The location may be managed in the LIMS system via barcoded storage boxes.

Store applicable reagents, consumables, and PCR product separately in post-PCR areas. DNA extracts may be assigned a sample number or barcode or be discarded following testing and analysis based on the lab SOP. DNA Extracts should be stored in the freezer at –20 or –80 °C in the post-amp area. Retain samples until electrophoresis results are confirmed or no repeat testing is needed.

Evidence is stored until the case clears court or as required by law and laboratory policy. Store biological evidence in sealed, labeled breathable paper bags or in containers with desiccant at room temperature conditions for long periods. Homicide case evidence may be stored indefinitely until the parties are deceased. Evidence should be retained until final disposition to the owner or destruction. Final disposition should be documented.

That’s all today regarding Storage, Preservation, and Retention. Keep abreast of changes in the field as they arise through courses and professional development opportunities.

Some questions to consider as you review and study include: How and where should evidence be stored? How should extracted DNA be stored? How can extracted DNA be preserved? How should amplified DNA product be preserved? And, when must samples and evidence be retained and when can it be discarded?

If you want to know more about the content in this video, review The Biological Evidence Preservation Handbook: Best Practices for Evidence Handlers by Sue Ballou and colleagues and the FBI, Quality Assurance Standards for DNA Testing and Databasing Laboratories.

This presentation is focused on the topic of Troubleshooting.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3i in ANSI/ASB Standard 115.

This presentation is made possible through award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST) and created by Dr. Kelly Elkins.

The learning objectives of this video include providing trainees with an understanding of troubleshooting components including thermal cycling errors such as ramping and temperature control; DNA detection errors such as spectral calibration failure and resolution failure; and general equipment failure.

Before we get started, let us review some terms from the document. An artifact is a non-allelic product of the amplification process, for example, stutter, non-templated nucleotide addition, or other non-specific product, an anomaly of the detection process such as pull-up or a spike, or a byproduct of primer synthesis such as a “dye blob” that may be observed on an electropherogram; some artifacts may complicate the interpretation of DNA profiles when they cannot be distinguished from the actual allele or alleles from a particular sample.

Spectral calibration is an examination of the contribution of overlap in the emission spectrum of fluorescent dyes used for a specific DNA test on a capillary electrophoresis instrument and permits the color deconvolution necessary for multi-color STR typing or sequencing to be performed. A poor spectral calibration may cause artifact peaks or inaccurate peak height determinations.

Stochastic means chance, or random variation and, in DNA testing, refers to random sampling error from extracts containing low levels of DNA and/or random variation in selection of alleles amplified at a particular locus.

To interpret results and perform troubleshooting as necessary, always include positive and negative controls in sample testing. Controls will be used to determine if the method or instrument performed properly.

The DNA typing process includes several important steps including sampling, DNA extraction, DNA quantitation, DNA amplification, DNA separation, allele call interpretation, statistics and reporting steps. Instrumentation includes an ALS or alternate light source, DNA extraction robot, thermal cycling instrument, and a capillary electrophoresis instrument or genetic analyzer. Software such as GeneMapper is used for interpretation following the instrumental analysis.

The lab is a dynamic place, and errors can occur. Thermal cycling errors are one example of errors that can occur. Control or sample issues can signify thermal cycling errors. Positive controls and samples may fail to amplify or lead to an unexpected result. Amplified DNA may not be detected by the real time detection system and incomplete cycling may lead to low DNA yields. CE errors are other errors that may lead positive controls and sample STRs to fail to separate or be detected.

In order to address thermal cycling issues and errors, the following steps can be performed. If you detect thermal cycler failure, check the power, battery, and surge protector for voltage overload or failure. Check for an electronics failure which can occur in instruments cycling from

hot to cold. To do so, check the integrity of the circuit control board and check for a heater lid short. If reduced DNA amplification of yield is observed, check the heating and cooling rate. A low ramp rate may lead to low yields if a suboptimal temperature is maintained for a longer duration. A faulty heating element may necessitate a replacement Peltier thermoelectric thermal control assembly. If there is no screen display, check for a blown fuse.

On the thermal cycler, you can check calibration, settings for appropriate temperature attainment and cycling, run an instrument verification to check performance, and check sensor error codes if one is encountered. Note that an open or ajar lid can lead to an open circuit for the lid heater so check that it closes tightly. If contamination is detected, decontaminate the instrument. If sensitivity is reduced, use a cotton swab moistened with ethanol or isopropanol to clean the lenses to improve detection sensitivity.

Some thermal cycler errors are a result of issues with temperature control. Symptoms that may be observed include failure to amplify, low product yield or detection, and non-specific target amplification. Solutions include checking that the tubes or plate fits in the wells of the instrument, verifying that thin-walled and low-profile plastics were used, using clear plastics for fluorescence detection and not exceeding the fill volume. In general, increasing the annealing temperature will increase specificity but if the primers are optimized; loss of specificity may indicate that the thermal cycler is not reaching the intended temperature. Check that primers and your mastermix were mixed well to achieve expected yields. Insufficient primers or polymerase may also lead to low product yield and reduced product detected. Be sure to mix primers and your mastermix well prior to rerunning.

Other thermal cycler errors are a result of faulty ramp rates. The higher the ramp rate, the shorter the run time but errors can arise. Symptoms of ramping errors include non-specific priming or spurious annealing and overshooting the denaturation temperature. To troubleshoot ramping errors, set the ramp rate to the highest speed to avoid non-specific priming; in contrast, slow down the ramp rate to avoid overshooting the desired temperature.

Additional thermal cycler issues including using incompatible consumables can lead to melted plates or tubes. Fluorescent detection issues include improper sample position which may lead to a reduction in detected signal, cloudy or dusty windows or lenses which may lead to low detection and underestimating quantitation values, low laser intensity which may lead to lowered detection, and the gain set too high may lead to oversaturation while a gain set too low may lead to the signal being undetectable from the background noise.

DNA detection errors can arise due to spectral calibration failure. These include pull up and low intensity peaks. If you detect pull-up artifacts in dye channels at the same position as the expected peak as shown, rerun your samples or your matrix standards. To resolve low intensity, clean cloudy detection windows such as with a cotton swab. You may need to replace the laser or LED if this does not resolve the problem.

DNA detection errors can also lead to resolution failure. These issues include poorly resolved peaks and anomalous migration due to secondary structure formation. If peaks are poorly resolved, change to new polymer and rerun your samples. To address anomalous migration, be sure to run samples in Hi Di formamide and heat to denature and snap cool them prior to injection to avoid duplex formation.

Examples of secondary structure formation and CE run issues from not using Hi Di formamide are shown in the figure on the right on the next slide. With the Hi Di formamide, the peaks are clearly resolved but without the HiDi formamide, the peaks coelute together to yield broad and unseparated peaks.

CE run issues can also lead to DNA interpretation issues. The run issues include off ladder peaks and spikes in the electropherogram. To troubleshoot off-ladder peaks, ensure the correct size standard was used and mark the sizes in the GeneMapper or other software. Shown below is an electropherogram of a sizing run issue indicated by the red locus flag in GeneMapper and the OL designation instead of an allele number call under the peaks. Other issues arise from warm room environment temperatures. Check that the CE room temperature was within the desired range. Also check that sufficient buffer is available to complete the circuit. If necessary, rerun the allelic ladder so that the alleles are assigned correctly. If spikes are observed in the electropherogram, check for and remove bubbles and rerun. Check that polymer is available and that the capillary has not run dry. Spikes may also be caused by an electrical voltage spike; rerun the sample if this is suspected.

Additional CE run issues include peak broadening. Check for capillary failure and replace the capillary as needed.

Signal intensities can also lead to DNA interpretation issues. If the signal is too high, reduce DNA quantity inputted in the PCR reaction, reduce the quantity of DNA inputted for CE, and/or decrease the injection time from 25 seconds down to 15 seconds and 5 seconds and reinject. If the signal is too low, the analyst can purify the PCR products and reinject, concentrate the PCR product using ethanol precipitation, Microcons or another method or add more amplified DNA, perform whole genome amplification or WGA to enrich the template prior to PCR, decrease the quantity of size standard to peak heights of approximately 500 RFU and reinject, increase the injection time in the software, and/or input more ladder if the ladder peaks are below the detection value. If the signal is imbalanced, the incorrect volume of primer or master mix may have been used or the primer may not have been well vortexed or insufficient DNA may have been added. Check the settings and remake the reaction adding the appropriate input quantity of DNA and reamplify prior to reinjecting.

General equipment failure can include electrical, mechanical and optical failure. If electrical failure is suspected, check the wire connections and the power source or if there was a surge. The connections or power may need to be repaired or replaced. If mechanical failure is suspected, check for metal fatigue and parts that may need to be replaced and lubricant starvation for stuck parts that need lubricating. If optical failure is suspected, check for alignment of the lasers, bulbs or LEDs and chromatic aberration such as light source variations and reduce vibration.

For general equipment failure, call instrument repair service unless you are trained to service the instruments. Consult your technical leader or lab director for guidance. Your lab may have service plans that cover instrument repair and maintenance. In cases of instrument failure, laboratories may choose to replace rather than repair instruments.

That’s all for today regarding Troubleshooting. Keep abreast of changes in technology including potential problems and solutions through courses and professional development opportunities.

Some questions to consider as you review and study include: Why is it important to use controls and standards in DNA typing? List some issues that may lead to little to no amplification.What could happen if the stated ramp rate is not used? What are some symptoms of spectral failure? How can you overcome resolution failure? And, what can you do if the peaks are below the analytical threshold on an electropherogram?

If you want to know more about the content in this video, review the ANSI/ASB Standard 115 available on the AAFS website and the FBI Quality Assurance Standards for DNA Testing and Databasing Laboratories, Chapter 11 Low Level DNA Testing: Issues, Concerns and Solutions of Dr. John Butler's book Advanced Topics in Forensic DNA Typing: Methodology, and the FBI Quality Assurance Standards for DNA Databasing and Testing Laboratories documents.