DNA Amplification for Forensic Analysts

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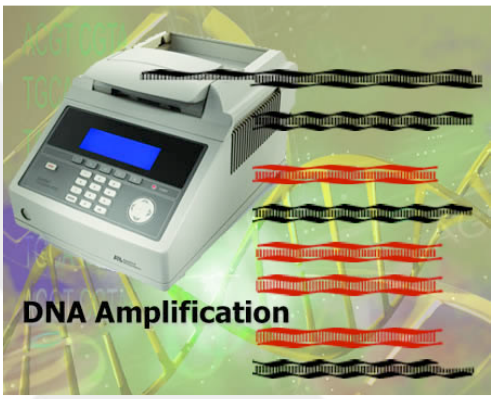

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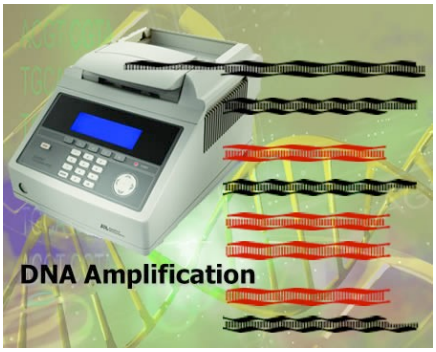
DNA Amplification for Forensic Analysts



This course provides information in four lessons.

Amplification Overview. Learn the amplification process, from primer design to methods for optimizing the amplification process.

Locus Selection. Learn the established core STR and Y-STR loci used in the U.S., how those loci were selected, and common List STR kit manufacturers.

Multiplexing. Learn how multiplexing affects forensic DNA analysis; the components, instruments, and kits that make it feasible; and the historical development of forensic multiplex DNA kits.

Contamination. Learn to recognize the sources and minimize the risk of laboratory contamination, the processes that aid in the detection of contamination, and how to implement corrective action processes.

Overview and Introduction

The Polymerase Chain Reaction (PCR) is a process by which a portion of a DNA strand can be replicated to yield multiple copies. Kary Mullis and members of the Human Genetics group at the Cetus Corporation (now Roche Molecular Systems) first described the PCR process in 1985. In 1993, Kary Mullis received the Nobel Prize in Chemistry for his work.

Visit the Applied Biosystems site for more information on PCR licensing, patents and trademarks.

The quality and quantity of DNA recovered from forensic samples are often limited, and characterization would not be possible without the PCR method. The PCR process has dramatically improved the capability of DNA analysis to obtain valuable evidence in a wide range of forensic applications.

Objectives

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

- Explain the amplification process to include primer design
- Describe how a thermal cycler works
- Describe the reagents needed for amplification
- Explain methods for optimizing the amplification process

Amplification

All methods for DNA amplification have rested on the concept of DNA strand complementarity discovered by James Watson and Francis Crick. The polymerase chain reaction is an enzymatic process analogous to the replication process used by cells to copy their own DNA. It targets a specific region of DNA, using two oligonucleotide primers that are designed to flank the template DNA segment to be amplified. Amplification of the template DNA is achieved by repeating a 3-step process through 25-30 cycles in a thermal cycler.

Each cycle has three steps:

1. The two DNA strands are denatured by heat.
2. The sample is then cooled to allow the primers to anneal to the DNA segments.
3. The temperature is raised to allow the DNA polymerase to add nucleotides to extend the primers to produce a copy of each DNA template strand.

This PCR product is sometimes referred to as an amplicon. Each cycle results in the doubling of amplicons. The result is an exponential accumulation of the specific target fragment, approximately 2^n, where n is the number of cycles of amplification performed. However, the process loses efficiency at higher cycle numbers. After 30 cycles, approximately a billion copies of the target DNA template are generated.

View an animation on the PCR process.

More than one region can be copied simultaneously by adding more than one primer set to the reaction;01 this is known as multiplexing. Primer design and the optimization of thermal cycling parameters are more complex with multiplex reactions than for a single-locus reaction.

Primer Design

Perhaps the most vital step in the development of a PCR method is the design of suitable primers. A PCR primer consists of two oligonucleotides that hybridize to complementary stands of the DNA template, and thus identify the region to be copied. A set of primers is used to amplify each DNA target region identified for the reaction.

The following are considerations for optimal primer design. Each item will be discussed in more detail throughout this module.02-08

Primer Design Considerations

Consideration	Comment
Primer Length	18-30 bases
Primer Melting Temperature (T_m)	55°-72°C
Primer Annealing Temperature (T_a)	~5°C < the lowest T_m of the of primers

T_m difference between forward and reverse primers	≤ 5°C
Max 3' Stability	ΔG value for five bases from 3' end
Percentage GC content	40-60%
No Secondary Structures	Identify primer pairs which do not assume secondary structure
No self-complementarity	< 4 contiguous bases
No complementarity to other primer(s)	< 4 contiguous bases
No long runs with the same base	< 4 contiguous bases
Distance between two primers on target sequence	< 2000 bases apart
Plateau Effect	accumulation of product ≤0.3 to 1 pmol

Primer Length

Uniquely designed primers will lead to generation of specific amplicons. The length of the primers is usually 18-30 bases, because the random combination of this size of a primer will hit less than once per total genomic sequence (3 billion base pairs).04
For example,

There is a $1/4$ chance (4^{-1}) of finding an A, G, C or T in any given DNA sequence.

There is a $1/16$ chance (4^{-2}) of finding any di-nucleotide sequence (e.g. AG).

There is a $1/256$ chance (4^{-4}) of finding a given 4-base sequence (e.g. AGCT).

So, a seventeen base sequence will statistically be present only once in every 4^{17} bases or approximately 17 billion.

A 17-mer or longer primer should be complex enough so that the likelihood of annealing to sequences other than the chosen target is very low. Primers of this length generally are unique sequences in the human genome; however, it is important to ensure that portions of the primer do not have sequence or cross-homology with the target. Computer programs such as BLAST can be used to find regions of local similarity between sequences. The program compares nucleotide sequences to sequence databases and calculates the statistical significance of matches.

Primers longer than 30 bases do not demonstrate higher specificity. Additionally, long amplicons are more likely to cross-hybridize with other primers and sequences in the reaction mixture, and this can terminate the DNA polymerization.09

Primer Melting Temperature

The melting temperature (T_m) is the temperature at which one-half of a particular DNA duplex will dissociate and become single-strand DNA. The stability of a primer-template DNA duplex can be measured by its T_m. Primers with melting temperatures in the range of 52-58°C generally produce better results than primers with lower melting temperatures. While the annealing temperature can go as high as 72°C, primers with melting temperatures above 65°C have a higher potential for secondary annealing.

Primer length and sequence are of critical importance in designing the parameters of a successful amplification. The melting temperature of a nucleic acid duplex increases both with its length and with increasing GC content. A simple formula for calculation of the (T_m) is:

$T_m = 4(G + C) + 2(A + T)$ °C

Note:

The actual (T_m) is influenced by the concentration of Mg^{2+}, K^+, and cosolvents. There are numerous computer programs to assist in primer design. The formula given above for (T_m) is simplistic; there are many primer design programs which use more complex nearest-neighbor thermodynamics values.01, 10 Also see www.basic.northwestern.edu/biotools/oligocalc.html for more complex nearest-neighbor thermodynamics values.

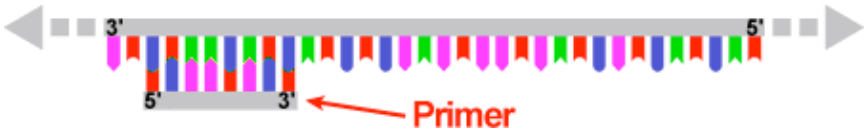
Primer Annealing Temperature

The **annealing temperature (T_a)** chosen for a PCR depends directly on length and composition of the primer(s). Generally, an annealing temperature about 5°C below the lowest T_m of the pair of primers is used.11 The optimal annealing temperature for any given primer pair on a particular target can be calculated as follows:12

T_a Opt = 0.3 x(T_m of primer) + 0.7 x(T_m of product) - 25

T_m of primer is the melting temperature of the less stable primer-template pair.
T_m of product is the melting temperature of the PCR product.

Polymerases require the binding of nucleotides at the 3' end of the primer to begin elongation, and because of this, any nonspecific binding at the 3' end will adversely affect amplification. Non-specific binding that occurs at the 5' end of the primer does not necessarily adversely impact amplifications since polymerases cannot begin elongation until the 3' end binds.



One consequence of having too low a T_a is that one or both primers will anneal to sequences other than the true target, as internal single-base mismatches or partial annealing may be tolerated. This can lead to nonspecific amplification and will consequently reduce the yield of the desired product if the 3' -most base is paired with a target. Conversely, too high a T_a may yield little product, as the likelihood of primer annealing is reduced.

Use of this calculated optimal T_a in the annealing step of the PCR cycle usually results in optimal PCR product yield with minimum false product production.

Values for T_m

An important consideration in primer design is to ensure that the 3' and 5' primers do not have very different values for T_m. Primers with a greater than 5°C difference in T_m may result in inadvertent preferential amplification of the most efficiently primed product strand. Large differences in T_m can lead to reduction in yield or even no amplification.

Maximum 3' Stability

Free energy, ΔG, can be used to measure the thermodynamic stability of the primer-binding site. The maximum ΔG value for the five bases from the 3' end is used for this calculation.

For primers shorter than 30 base pairs, ΔG is computed using the following equation:

$\Delta G = \Delta H - T \cdot \Delta S$

ΔH = enthalpy

ΔS = entropy

T = the user-defined melting temperature or T_m

When ΔG is negative, the reaction is spontaneous. When choosing between various primer pairs, a lower ΔG value is favorable.10, 13, 14

GC Content Percentage

Primers with a 40-60% GC content ensure stable binding of primer/template. G-C bonds contribute more to the stability (increased melting temperatures) of primer/template binding than do A-T bonds. However, two primer/template complexes with identical GC content can have different melting temperatures because base order influences the overall stability.

It has been reported that GC-rich regions of the target DNA are difficult to amplify, so these regions are generally avoided when choosing a target DNA sequence. This is also true for stretches of polypurines or polypyrimidines, which should be avoided.15

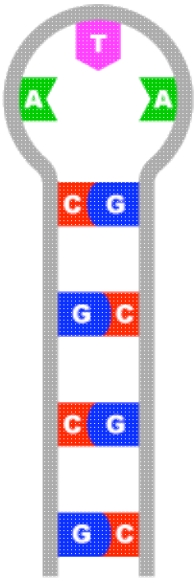
The presence of G or C bases at the 3' end of primers (GC clamp) helps to promote correct binding at the 3' end due to the stronger hydrogen bonding of G and C bases.06 However, strings of G and of C can form internal, non-Watson-Crick base pairs that disrupt stable primer binding. Generally, sequences containing more than three repeats of G or of C in sequence should be avoided in the first five bases from the 3' end of the primer, due to the higher probability of primer-dimer formation. A short run of G's at or near the 5' end of a primer will not disrupt stable binding because the 5' positioning does not lead to involvement in disruptive secondary structures. It is best to select primers with a random base distribution.

No Secondary Structures

A successful PCR reaction requires efficient amplification of the product. At normal temperatures nucleic acids fold into conformations (secondary structures) which have high negative free energy (ΔG). The stability of these template secondary structures depends largely on their free energy and melting temperatures (T_m), and is extremely important for designing primers. If these secondary structures in the nucleic acids are stable even above the annealing temperatures, then the primers are unable to bind to the template DNA, and the yield of PCR product is significantly reduced. It is desirable to design specific primer pairs which do not assume secondary structures during the reaction, and this can be determined from a program called mfold server.

Visit a website that uses the mfold server.

No self-complementarity



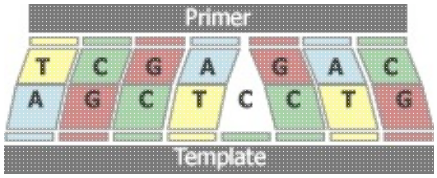
Self-complementarity can lead to stable hairpin formation with just four GC base pairs in the stem and three bases in the loop. If oligonucleotides form hairpins (intramolecular hybridization), they are not available for hybridization to the target regions. Any kind of hairpin structure should be avoided in a primer.

View an animation on hairpins.

The available nucleotides can be thought of as forming a smaller, and therefore less specific, primer because the entire primer is not used to discriminate among target sequences. If the 3' nucleotides bind strongly, any template sequences that are complementary to the 3' end are amplified. Conversely, if binding is strongest at the 5' end, the typing binding event on the template DNA begins at the 5' end. Polymerases, however, cannot begin elongation until the 3' end binds. Therefore, the entire primer is used to distinguish among target sequences.

AutoDimer is a program that was originally created to assist in the development of multiplex PCR assays for probing STR and SNP markers for forensic science purposes. The program rapidly screens previously selected PCR primers for primer-dimer and hairpin interactions in short DNA oligomers (< 30 nucleotides).13

Visit the AutoDimer homepage at the National Institute of Science and Technology.



In extreme cases of complementarity, a gapped duplex can form when the primer and target are completely complementary except for a few bases.

No Complementarity to other primers

Primers should contain fewer than 4 complementary bases especially at the 3' end. Complementarity between two primers, especially at the 3' ends, can lead to the formation of product artifacts arising from amplified primer-dimers and primer-oligomers. The concentration of primers is much higher than that of target DNA in PCR. Therefore, if the primers exhibit self-complementarity they may hybridize to each other and form homo-dimers or hetero-dimers.

Avoiding primers with 3' overlaps is extremely important in multiplex reactions.

A self-dimer is formed due to intermolecular interactions of the same primer.
A cross dimer is formed due to intermolecular interaction between sense and antisense primers.

In order to detect cross dimers, the sense primer in 5' - 3' direction is compared with the antisense primer in 3' - 5' direction for homology.

No Long Runs With The Same Base

Primers with long runs of a single base (more than three) should generally be avoided as it can cause breathing of the primer and aid in mispriming. For example, the sequence AGCGGGGGATGGGT has run of five for the base G.

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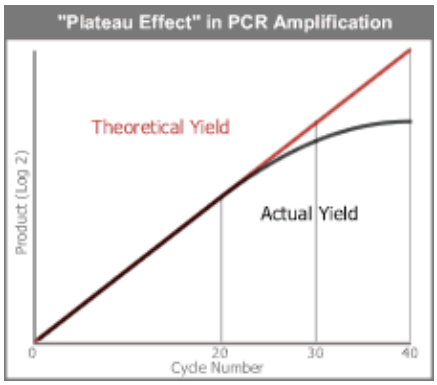
Distance Between Two Primers On Target Sequence

The distance between the 5' ends of the two primers defines the length of the amplicon. In general, primers distanced < 2000 bases apart are used. This allows for sufficient amplification of the target region.

The amplicon length is calculated as:

Product length = (Position of antisense primer - Position of sense primer) +1

Note:
The "+1" represents the non-template +A addition by the Taq polymerase
Plateau Effect



The term plateau effect is used to describe the attenuation of the normally exponential rate of product accumulation in PCR. The attenuation occurs during the late PCR cycles when the accumulation of product reaches 0.3 to 1 picomole. Depending on reaction conditions and thermal cycling, one or more of the following may influence when the plateau is reached:

Depletion of substrates (dNTPs or primers)
Stability of the reactants (dNTPs or enzyme) particularly at the denaturation temperature
End-product inhibition (pyrophosphate, duplex DNA)
Competition for reactants by nonspecific products or primer-dimer
Reannealing of specific product at concentration above 10^{-8} M (may decrease the extension rate or processivity of *Taq* DNA polymerase or change branch-migration of product strands and displacement of primers)
Incomplete denaturation/strand separation of product at high product concentration11

An important consequence of reaching plateau is that nonspecific products resulting from mispriming events, initially present at low concentration, may continue to amplify preferentially. Optimizing the number of PCR cycles is the best way to avoid amplifying background products.

Summary

PCR yield is directly affected by the primer design. There are commercially available primer design software packages that are commonly used to assist scientists in designing primers.

Read about primer design software packages at the Chemical Science and Technology home page of NIST.

Thermal Cyclers

A thermal cycler heats and cools a DNA sample in order to perform the PCR. There are numerous thermal cyclers available from a variety of manufacturers. The instruments vary in the number of samples that can be handled, the size of reaction tube used, the speed of temperature change, and the cost.

Thermal cyclers provided by Applied Biosystems have been the most widely used in the forensic community. Their model 480 was one of the first to be used in forensic DNA analysis, but it did not come with a heated lid. The process using the 480 required sample reaction mixtures to have an overlay with a drop of mineral oil in order to prevent evaporation. Later models such as the GeneAmp® PCR systems 2400 and 9600 have heated lids, but they have also been discontinued. (Note: some forensic laboratories still use them).

Currently, one of the most popular thermal cyclers in forensic science laboratories is the Applied Biosystems GeneAmp® PCR system 9700. The 9700 has interchangeable blocks, a 96-well format for 0.2mL tubes, and is fully programmable.

Calibration and Maintenance

Similar to other laboratory instrumentation, thermal cyclers must be calibrated and maintained.

Routine calibration and maintenance may include:

- Cleaning
- Temperature calibration verification test
- Temperature non-uniformity test
- Performance diagnostics tests

Cleaning

Prior to using a thermal cycler, sample wells should be examined to ensure that no debris has settled into the wells. Debris in the wells can prevent reaction tubes from achieving an optimal fit and may prevent the required heat transfer from the block to the tube. Changes in heat transfer may adversely affect the PCR.

The block and sample wells, trays, and the heat cover can be cleaned with a 10% bleach solution. This should be followed by the use of isopropanol or other appropriate alcohol to remove any sodium hypochlorite

residue. Bleach can be corrosive and damaging to the thermal cycler. Cotton swabs work well for the cleaning process.

Temperature Calibration Verification Test

The temperature calibration verification test is used to verify the temperature calibration of the sample block and that the instrument meets the manufacturer's factory calibration specifications. A Temperature Verification System kit (TVS) is required for this test. The manufacturer's user manual(s) outlines which kit model is needed for the specific thermal cycler and provides corresponding instructions.

Temperature Non-Uniformity Test

The temperature non-uniformity test is used to verify the temperature uniformity of the sample block and that the instrument meets the manufacturer's factory calibration specifications. This test is used to find wells within the sample block that exhibit unacceptable temperature variation. A Temperature Verification System kit (TVS) is required for this test. The manufacturer's user manual(s) outlines which kit is needed for the specific thermal cycler and provides corresponding instructions.

Note:

Temperature Verification System kits should be returned to the vendor for calibration annually or as specified by the manufacturer.

Performance Diagnostics Test

The performance diagnostic tests may include a cycle and rate test. These tests are used to verify the integrity of the cooling and heating system, incorporated into the instrument's software, and described in detail in the thermal cycler user manual(s).

Note:

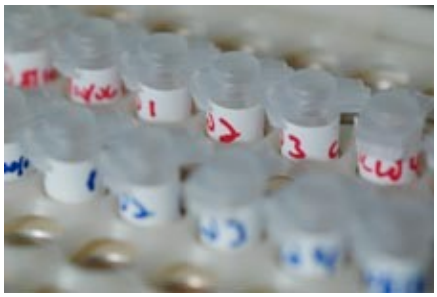
Any instrument that fails one or all of these tests may require service by a manufacturer's qualified repair specialist.

Reagents / Supplies

The necessary components for PCR are:

- Reaction tubes
- Buffer
- Mg^{2+}
- Deoxynucleotide triphosphates (dNTPs)
- DNA polymerase
- Primers
- DNA Template

Reaction Tubes



Prior to setting up an amplification reaction, the analyst must ensure that reaction tubes are compatible with the thermal cycler used. Each thermal cycler manufacturer can provide information regarding the size and type of reaction tubes that are recommended. Sample reaction volumes generally range from 25-100µL, although many forensic laboratories have successfully validated reactions with less than 25µL. The thermal cycler used may limit the choices of reaction tubes and sample reaction volumes (e.g., the 480 thermal cycler requires PCR reaction tubes with no lid to allow a mineral oil layer).

Deoxynucleotide Triphosphates (dNTPs)

The four dNTPs should be used at equivalent concentrations to minimize misincorporation errors. Lower dNTP concentrations minimize the mispairing at non-target sites and reduce the likelihood of extending misincorporated nucleotides.

Misincorporated bases cannot be proofread since Taq lacks a 3' to 5' exonuclease activity and mismatched bases are inefficiently extended. Misincorporation errors that do occur during PCR promote chain termination. Chain termination restricts the amplification of defective molecules and helps to maintain fidelity.

Stock dNTP solutions are generally neutralized to a pH 7.0. The stability of dNTPs during repeated cycles of PCR is such that approximately 50% of the dNTPs remain after 50 cycles.11 The concentration is usually between 20 and 200µM each.

Buffer and Mg^{2+}

Buffer

Reaction pH can alter the fidelity of an insertion. A 10-50µM Tris-HCl buffer with a pH between 8.3 and 8.8 (when measured at 20°C) is used.11

Mg^{2+}

The magnesium concentration may affect all of the following:11

- Primer annealing
- Strand disassociation temperatures of template
- Strand disassociation temperatures of PCR product
- Product specificity
- Formation of primer-dimer artifacts
- Enzyme activity and fidelity

In general, increasing the free magnesium concentration increases yield and decreases specificity and fidelity.

Taq DNA polymerase requires free magnesium (0.5 to 2.5mM) additional to that bound by template DNA, primers, and dNTPs. The presence of EDTA or other chelators in the primer stock or template DNA might disturb the apparent magnesium optimum.

DNA polymerase

DNA polymerases strongly favor incorporation of proper Watson-Crick base pairs. At each template position, there is one correct and three incorrect dNTPs competing for insertion. Nucleotide mis-insertion frequencies are, on average, between 10^{-3} and 10^{-5}. The three major factors affecting insertion error rates are:

> The type of mispair formed; G(primer)T and T(primer)G transition mispairs can generally be made more easily than CC or GG transversion mispairs.05
> The sequence context where the mispair occurs
> The identity of the polymerase

The PCR process was originally performed manually. The thermolabile Klenow DNA polymerase was used and had to be replenished at the beginning of each cycle. The subsequent introduction of *Thermus aquaticus* (Taq) polymerase, a thermostable DNA polymerase, represented a considerable advance. Taq DNA polymerase is the most widely used polymerase in forensic DNA analysis and is available from multiple vendors.

Taq DNA polymerase lacks endonuclease and 3' - 5' exonuclease (proofreading) activities, but has 5' - 3' exonuclease activity. Ampli *Taq* Gold® DNA Polymerase (supplied by Applied Biosystems) is a chemically modified form of Taq DNA Polymerase, which is delivered in an inactive state. A pre-PCR heating step is used to activate the enzyme. Loss of specificity and sensitivity can be caused by competing side reactions that usually occur during the pre-PCR setup period. At this time, all reactants are present in the reaction tube at permissive temperatures, which can result in the amplification of non-target sequences in background DNA either due to mispriming or to primer oligometization.

Enzyme requirements may vary with respect to individual target templates or primers. The recommended concentration range for Taq DNA polymerase is between 1 and 2.5 units per 100µl reaction. However, when conducting studies to optimize a PCR assay, 0.5 to 5 units/100µl is an appropriate range. If the enzyme concentration is too high, nonspecific background products may accumulate; if too low, an insufficient amount of desired product is made.11

Protocols can be enhanced with the addition of proteins (gelatin or bovine serum albumin) and/or nonionic detergents (Tween 20 or Laureth 12). These chemicals are included to help stabilize the enzyme.11

Taq DNA polymerase has the ability to add a single nucleotide to the blunt end DNA fragment, creating a single nucleotide overhang at the 3' end of a product. Although any of the four nucleotides can be added, dATP is preferred. This non-template directed dATP addition occurs during the extension step of the PCR process. The extension step of the process is designed to drive the addition of dATP to ensure that all amplicons are the same length.11

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Primers

Primer concentrations between 0.1 and 0.5µM are generally used. This relatively high concentration compared to the DNA template helps to drive the PCR reaction. Higher primer concentrations may promote mispriming

and accumulation of nonspecific product and may increase the probability of generating primer-dimer template-independent artifacts. Because nonspecific products and primer-dimer artifacts are substrates for PCR, they compete with the desired product for enzyme, dNTPs, and primers, resulting in a lower yield.

Mutations in the primer binding site region can result in null alleles. When common primer binding site mutations are identified, it is common practice to use **degenerate primers** for these loci. Degenerate primers are a set of primers that have a number of options at several positions in the sequence, which allows annealing to and amplification of a variety of related sequences. The use of degenerate primers can reduce the specificity of the primer(s), increasing both the likelihood of mismatches and the level of background noise. However, use of degenerate primers allows the amplification of those null alleles without significantly altering the overall performance of the amplification.

DNA Template

The concentration of template in a sample can affect the success of PCR amplification in a variety of ways. Too much template promotes nonspecific binding of primers to secondary sites or changes the pH of the reaction mix. Too little template can result in poor yields and/or preferential amplification, especially if the template is degraded. It should be noted that very low template concentration (e.g. 10 copies) can be sufficient for successful PCR amplification.

Thermal Cycling Parameters & Optimization

Primer Annealing

The temperature and length of time required for primer annealing depends upon the base composition, length, and concentration of the amplification primers. In general, the annealing temperature is 5°C below the true T_m of the amplification primers. Because Taq DNA polymerase is active over a broad range of temperatures, primer extension will occur at low temperatures, including those used during the annealing step. The range of enzyme activity varies by two orders of magnitude between 20 and 85°C.

Annealing temperatures in the range of 55 to 72°C generally yield the best results. The higher annealing temperatures enhance discrimination against incorrectly annealed primers and reduce mis-extension of incorrect nucleotides at the 3' end of primers. Stringent annealing temperatures, especially during the first several cycles, will help increase specificity. The reaction temperature is briefly cooled to 40 to 60°C and then raised to 70 to 75°C. Primers anneal to the complementary sequence during the cooling phase and extend the primers with Taq polymerase during the heating phase.

Primer Extension

Extension time depends upon the length and concentration of the target sequence and temperature. Primer extensions are usually performed at 72°C. Estimates for the rate of nucleotide incorporation at 72°C vary from 35 to 100 nucleotides per second , depending upon the buffer, pH, salt concentration, and the nature of the DNA template. An extension time of one minute at 72°C is considered sufficient for products up to 2 kb in

length.

The most likely cause for failure of a PCR is incomplete denaturation of the target template and/or the PCR product.11 Typical denaturation conditions are 95°C for 30 seconds or 97°C for 15 seconds; higher temperatures may be appropriate especially for targets containing a high GC percentage. Incomplete denaturation allows the DNA strand to snapback, reducing the product yield. Denaturation steps that are conducted at too high a temperature and for too long lead to unnecessary loss of enzyme activity.

The half-life of Taq DNA polymerase activity varies with temperature:

Less than 2 hours at 92.5°C
40 minutes at 95°C
5 minutes at 97.5°C

Finally, the double-stranded DNA is denatured by briefly heating the samples to 90 – 95°C.

Cycle Number

The optimum number of cycles depends mainly on the starting concentration of the target DNA when other parameters are optimized. Too many cycles can increase the amount and complexity of nonspecific background products (plateau effect) and too few cycles can produce low product yield.

Works Cited & Online Links

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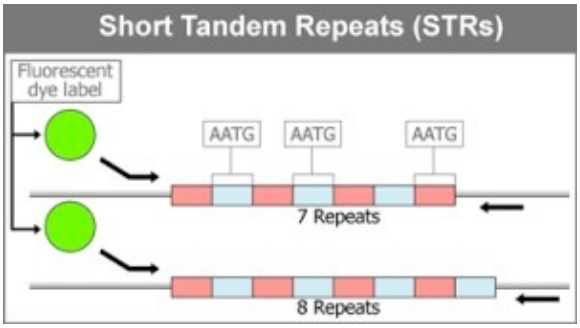
14. Edwards, M., and R. Gibbs. *PCR Methods and Applications*, 3, S65-S75.
15. Mitsuhashi, M. 1996. Technical report: Part 2; Basic requirements for designing optimal PCR primers. *J Clin Lab Anal* 10 (5): 285–93.

Online Links

Applied Biosystems
http://www.appliedbiosystems.com/
AutoDimer homepage
http://www.cstl.nist.gov/div831/strbase/AutoDimerHomepage/ AutoDimerProgramHomepage.htm
BLAST
http://www.ncbi.nlm.nih.gov/blast
mfold Server Site
http://www.bioinfo.rpi.edu/applications/mfold/dna/form1.cgi
Oligonucleotide Properties Calculator
http://www.basic.northwestern.edu/biotools/oligocalc.html
Primer Design Software Packages
http://www.cstl.nist.gov/div831/strbase/primer.htm

Locus Selection



There have been several technological developments since DNA testing was introduced into forensic science laboratories in the United States during the late 1980s. The historical developments are described in Crime Scene and DNA Basics for Forensic Analysts. The current technology is short tandem repeat (STR) analysis using the polymerase chain reaction (PCR).

The Human Genome Project has shown that there are tens of thousands of STR loci in human DNA. The selection of specific loci from the many available for use in forensic science laboratories is important for many reasons, but primarily to ensure that:

The loci have a high discriminating power.
The loci are stable in evidence samples.

There is a consensus panel of core loci for use in DNA databases.

Objectives

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

Describe the established core STR loci used in the United States.
Describe how loci are selected for use in forensic STR analysis in the United States.
List STR kit manufacturers.
Describe the established core Y-STR loci in the United States.

Overview

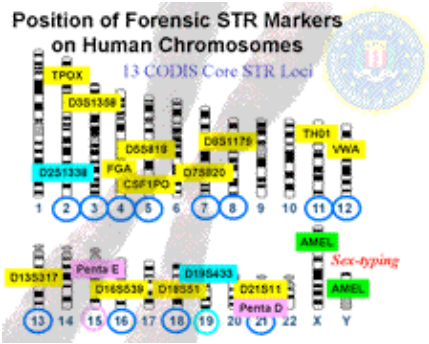
In the United States, the Federal Bureau of Investigation (FBI) bears the responsibility for maintenance of the national DNA database. The FBI worked with the relevant scientific communities to identify appropriate markers for STR DNA analysis. The European Network of Forensic Science Institutes (ENFSI) performed a similar role in Europe.

Visit the FBI's Laboratory Services website.

Visit ENFSI's website.

The steps involved in the selection of loci are:

Identify loci that have been published and characterized and are not linked (see Population Genetics & Statistics, Population Theory)
Select the best candidates for use in forensic science.
Run numerous population database samples to establish discriminating power (see Forensic Biology, Testing of Bodily Fluids and Tissues) and population frequency data.
Conduct method development (e.g., multiplex kits).
Conduct validation studies.



The selection of a core set of loci by the forensic science community must include consideration of method development. Multiplex systems are complex, and while certain loci may be more desirable than others, loci are selected based on their suitability in a multiplex system and their discriminating power.

Currently, a few manufacturers develop STR multiplex kits for mainstream forensic science use. Historically, the manufacturers and the forensic science community have collaborated to develop suitable end products. This has greatly benefited the forensic science community because it is unlikely that these laboratories would be able to devote the resources required for multiplex method development.

In 1994, the U.S. Congress passed the DNA Identification Act (Public Law No. 103-322 -1994).01 This law, in part, mandated that the FBI was responsible for the Combined DNA Index System (CODIS).

In 1996, the Department of Justice, through the FBI and the Technical Working Group on DNA Analysis Methods (TWGDAM), funded the CODIS STR Standardization Project.

DNA Identification Act

Requires the Director of the Federal Bureau of Investigation (FBI) to develop a voluntary plan to assist State and local forensic laboratories in performing deoxyribonucleic acid (DNA) analysis of DNA samples collected from convicted offenders, aimed at eliminating the backlog of convicted offender DNA samples awaiting analysis in State or local forensic laboratory storage in an efficient, expeditious manner that will provide for their entry into the Combined DNA Indexing System (CODIS). Sets forth provisions regarding plan conditions and plan implementation. Authorizes appropriations

The purpose of this project was to evaluate various STR loci and to establish core loci for the CODIS. Crime laboratories from Alabama, Arizona, California, Florida, Michigan, New York, North Carolina, Oregon, Texas, and Virginia, together with the Armed Forces DNA Identification Laboratory (AFDIL), the FBI, the National Institute of Standards and Technology (NIST), and the Royal Mounted Canadian Police (RCMP) participated in the study. Two manufacturers, Applied Biosystems and Promega, also participated in the STR Standardization Project.

Prior to this project, each manufacturer had forensic multiplex STR kits available:

Applied Biosystems AmpFSTR® Profiler™

- Amelogenin
- CSF1PO
- D3S1358
- D5S818

- ♦D7S820
- ♦D13S317
- ♦FGA
- ♦TH01
- ♦TPOX
- ♦vWA

Applied Biosystems AmpFSTR® Green II™

- ♦Amelogenin
- ♦D8S1179
- ♦D18S51
- ♦D21S11

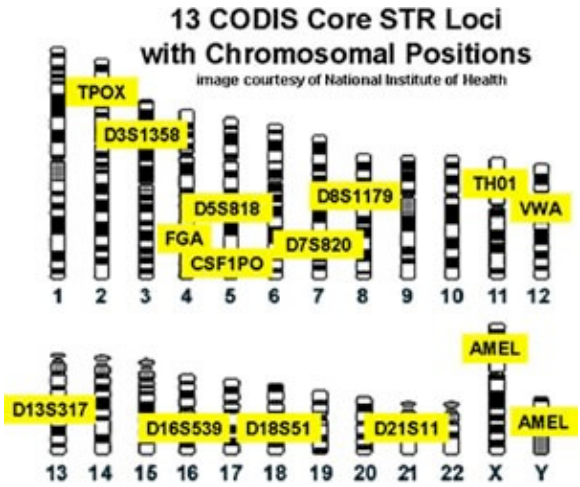
Promega PowerPlex®

- ♦CSF1PO
- ♦D5S818
- ♦D7S820
- ♦D13S317
- ♦D16S539
- ♦TH01
- ♦TPOX
- ♦vWA

Applied Biosystems

Although other kits were available, Applied Biosystem's AmpFSTR® and Promega's PowerPlex® kits were the main kits used during the CODIS STR Standardization Project.

The CODIS STR Standardization Project identified thirteen core loci, together with amelogenin:

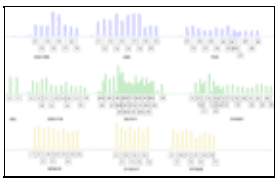


CSF1PO
D3S1358
D5S818
D7S820
D8S1179
D13S317

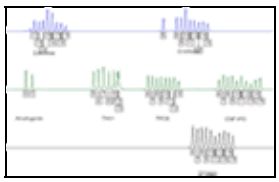
D16S539
D18S51
D21S11
FGA
TH01
TPOX
vWA

The two manufacturers responded rapidly by developing new kits that included the thirteen core loci and amelogenin. The manufacturers involved the forensic science community in the beta testing of the kits, requested feedback, and responded to numerous suggested changes. The cooperation was mutually beneficial. The industry was able to develop technology that would be readily accepted by the community; the community enabled industry to build a robust technology tailored to forensic STR testing.

Applied Biosystems retooled AmpFℓSTR® Profiler™ and developed AmpFℓSTR® Profiler Plus™ and AmpFℓSTR® COfiler™.



Applied Biosystems Profiler Plus



Applied Biosystems COfiler

The loci included in these Biosystems are: (highlighted loci are included in both kits)

Comparison of Applied Biosystems Kits	
AmpFℓSTR® Profiler Plus™	AmpFℓSTR® COfiler™
D3S1358	D3S1358
D5S818	
D7S820	D7S820
D8S1179	D16S539
D13S317	CSF1PO
D18S51	TH01
FGA	TPOX
vWA	
Amelogenin	Amelogenin

The combination of these two kits allows laboratories to test all core loci; the overlapping loci serve as a quality control measure in the testing process.

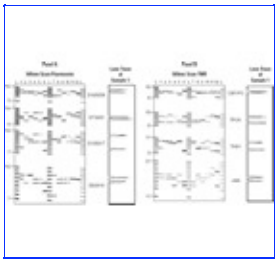
Promega

Promega continued to offer their original PowerPlex® 1.X kit and developed a second kit, PowerPlex® 2.1 (highlighted loci are included in both kits).

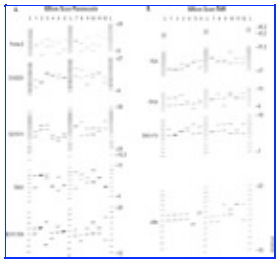
Comparison of Promega Kits
PowerPlex® 1.X

	PowerPlex® 2.1
D5S818	D3S1358
D7S820	D8S1179
D13S317	D18S51
D16S539	D21S11
CSF1PO	FGA
TH01	TH01
TPOX	TPOX
vWA	vWA
	Penta E
	Amelogenin

The combination of these two PowerPlex® kits provides a degree of overlap (vWA, TPOX, THO1) and adds an additional locus, Penta E.



Promega PowerPlex 1.1

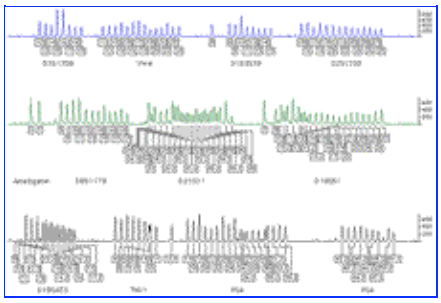


Promega PowerPlex 2.1

Applied Biosystems and Promega use different primer sets in their kits. This can be an advantage in detecting apparent null alleles caused by primer site mutations.02 Although the occurrence of discordance between the two manufacturers' kits is rare, it is something that analysts must consider when comparing profiles. An analyst can differentiate between a discordance event and a true exclusion between two samples.

Prior to uploading a sample, any laboratory participating in NDIS must attempt to develop results at all 13 core loci and must obtain results at 10 in order to upload the profile. Requiring a minimum of 10 loci reduces the chance of adventitious hits, allowing agencies to focus their resources on more discriminating investigative leads.

Other STR Loci



There are some differences between the international community and the U.S. regarding which STR loci are used. Nevertheless, there are sufficient common loci to permit the sharing of meaningful profile information.

The United Kingdom (U.K.) pioneered forensic DNA testing with programs that began more than 20 years ago. The Forensic Science Service (FSS) began STR testing in 1994 with the Quadruplex system, which consisted of vWA, THO1, FES, and F13A1. This kit was manufactured in-house at the FSS but was not very discriminating. The FSS quickly replaced it with the AmplFSTR® SGM™ (Second Generation Multiplex) kit. This kit consisted of six STR loci (D8S1179, THO1, vWA, D21S11, FGA, and D18S51) and amelogenin, with an estimated discriminating power of 1 in 50 million.03, 04

Visit the Forensic Science Service's website.

The AmplFSTR® SGM™ provided the core loci used when the U.K. established its National DNA Database in 1995. Further STR/database developments included the extension of the six STR loci used for crime stain analysis in 1996 and the subsequent adoption of the six STR loci by ENSFI.05

As the number of samples in the database increased to well over a million, the chances of a coincidental match between unrelated individuals became too great, as was illustrated in a 1999 case. A man with advanced Parkinson's disease, (unable to drive a car or dress himself without assistance) was linked to a burglary that occurred a great distance from his home. After he was arrested, it was apparent that his physical condition would have made it impossible for him to have committed the crime.

Researchers were already evaluating the possibility of adding more STR loci to the core group and their work resulted in the introduction of AmplFSTR® SGM Plus™. The kit contains the same loci as the AmplFSTR® SGM™ kit together with D3S1358, D19S433, D16S539, and D2S1338. The four additional loci increase the discriminating power to 1 in 33 trillion. Retesting the case samples with the additional loci subsequently exonerated the man.06

Across Europe, ENFSI (through the European DNA Profiling Group, or EDNAP) decided on the use of seven common loci for their core STRs:

D3S1358
TH01
D21S11
D18S51
vWA
D8S1179
FGA

Recently, ENFSI and EDNAP have increased the core loci to include:

D10S1248
D14S1434
D22S104507

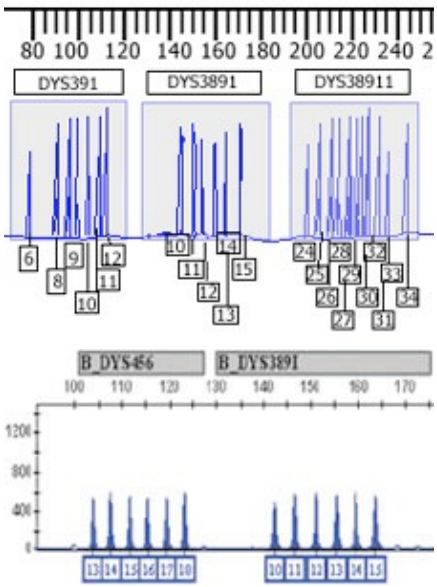
The addition of core loci has been implemented in anticipation of a significant increase in the size of the database. The International Criminal Police Organization (INTERPOL) has adopted the core European loci, enabling governments within Europe to compare samples across countries.

Visit INTERPOL's website.

Y-STRs

In the United States, the development of Y-STR core loci has followed a pattern similar to the development of the CODIS core STR loci. The Scientific Working Group for DNA Analysis Methods (SWGDAM) created a subcommittee to investigate Y-STRs. The subcommittee reviewed published data and ran several hundred population database samples to evaluate various loci.

The subcommittee established the following 11 loci as the core Y-STR loci:



DYS19
DYS385 A/B
DYS389I
DYS389II
DYS390
DYS391
DYS392
DYS393
DYS438
DYS439
DYS1908

With the establishment of the core loci, manufacturers have the specifications needed for the development of Y-STR kits

European laboratories have also established core Y-STR loci for forensic testing. ENFSI decided to use the same loci as the United States, minus DYS438 and DYS439.09

International Community

Most countries develop and implement their DNA testing programs, including databases, through coordinating bodies or other consensus working groups. For instance, the FBI and SWGDAM are responsible for setting standards, for training, and for development for the United States. Similarly, in Europe the ENFSI and EDNAP represent government institutions and coordinate efforts to develop European DNA databases, in part by recommending markers.

Individual countries vary on the STR loci that they use. It is important to remember that different countries developed their technologies at different times and have differing legislative requirements.

Works Cited & Online Links

1. U.S. Congress. House. 1994. *The Violent Crime Control and Law Enforcement Act of 1994*. Public Law 103-322.
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3. Sparkes, R., C. Kimpton, S. Watson, N. Oldroyd, T. Clayton, L. Barnett, and J. Arnold, et al. 1996. The validation of a 7-locus multiplex STR test for use in forensic casework. (I). Mixtures, ageing, degradation and species studies. *Int J Legal Med* 109 (4): 186–94.
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6. Applied Biosystems. 2005. AmpFlSTR® SGM Plus® PCR Amplification Kit User's Manual.
7. Gill, P., L. Fereday, N. Morling, and P. M. Schneider. 2006. The evolution of DNA databases: Recommendations for new European STR loci. *Forensic Sci Int* 156 (2–3): 242–44.
8. Scientific Working Group on DNA Analysis Methods (SWGDAM) Y-STR Subcommittee. 2004. Report on the current activities of the Scientific Working Group on DNA Analysis Methods Y-STR Subcommittee. *Forensic Science Communications* 6 (3). http://www.fbi.gov/hq/lab/fsc/backissu/july2004/standards/ 2004_03_standards03.htm.
9. Kayser, M., A. Caglia, D. Corach, N. Fretwell, C. Gehrig, G. Graziosi, F. Heidorn, et al. 1997. Evaluation of Y-chromosomal STRs: A multicenter study. *Int J Legal Med* 110 (3): 125–33, 141–49.

Online Links

ENFSI
http://www.enfsi.org/
FBI Laboratory Services
http://www.fbi.gov/hq/lab/org/labchart.htm
Forensic Science Service
http://www.forensic.gov.uk/forensic_t/index.htm
Human Genome Project
http://ornl.gov/swci/techresources/Human_Genome/home.shtml
INTERPOL
http://www.interpol.int/

Introduction

Multiplexing is the simultaneous amplification of more than one locus. This greatly improves the laboratory's casework throughput because the loci are analyzed concurrently rather than sequentially. However, for multiplexing to be effective, the loci must be capable of being analyzed on the same gel or the same capillary electrophoresis run. Factors such as technology and optimal inter-locus spacing have to be considered.

Objectives

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

Describe how multiplexing has affected the process of forensic DNA analysis.
Describe the components, instruments, and amplification kits that make multiplexing feasible.
Explain the historical development of forensic multiplex DNA kits in the United States.

Factors

Designing successful multiplexes can be challenging. The process begins by deciding which STR loci are suitable to incorporate into a multiplex.

Factors in deciding the loci include:

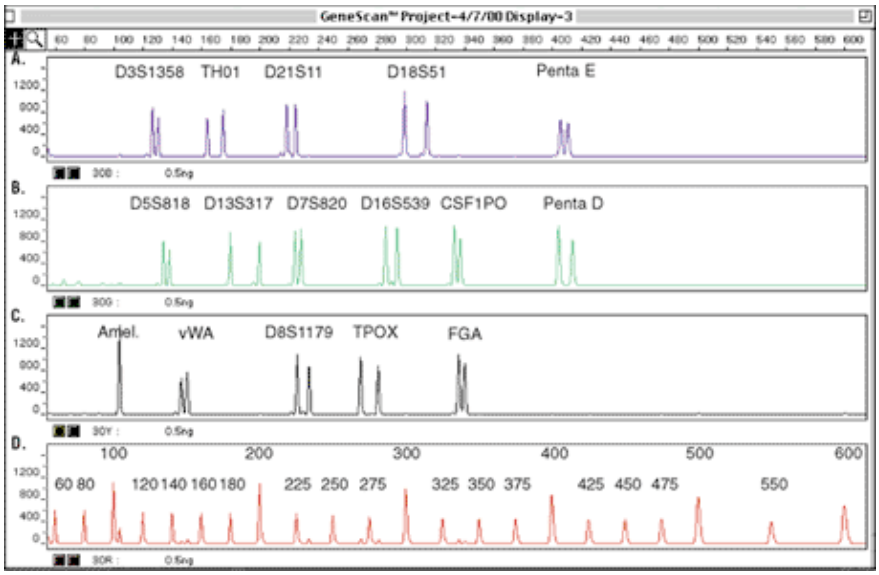
Locus size and overlap
Number of alleles

Thermal cycling parameters
Primers
Dyes

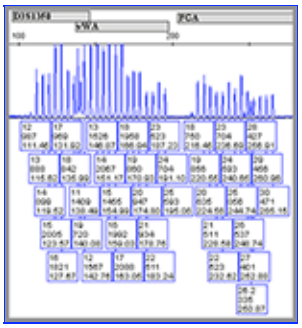
Locus Size and Overlap

The STR loci of interest fall into a size range of 75-400 base pairs. Designing the multiplex requires consideration of the competing demands of locus size. A narrow size range minimizes allelic dropout and permits balanced amplification; a broader size range minimizes overlap between the loci, permitting more reliable allele calls.

Many STR loci have been well characterized and population data are available. These data provide information on the allele size range of the locus. Multiplexes are designed to minimize overlap so that the smallest allele for one locus is larger than the largest allele of the adjacent locus within the same dye.



Number of Alleles



For single locus analysis, the general rule is, the more alleles, the better, as it will increase the discriminating power. However, when designing multiplexes, the number of alleles at each locus has to be considered along with the size of the STR product. If there are too many alleles, some may have sizes that overlap with product from adjacent loci.

Thermal Cycling Parameters

Primers have a theoretical optimal annealing temperature based on their sequence. Each primer intended for inclusion in the multiplex may have a different optimal annealing temperature. Therefore, it is important to select primers that are known to be robust and amplify successfully at less than optimal temperatures. Optimized multiplexes containing well-designed primers provide high yields of amplified product.

Read more about amplification elsewhere in this PDF file.

Primers

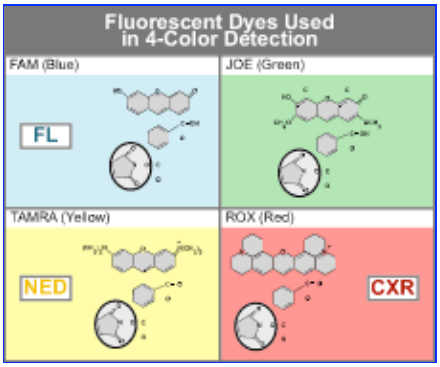
The most complex step in multiplex design is the selection of primers. Primer-primer interaction can result in decreased yield and artifacts. Computer programs are used to design primers and determine primer interaction. A set of primers must perform under a single amplification environment. It is important that the primers selected for the multiplex are designed based upon melting temperatures and locus specificity.

Read more about Locus Selection elsewhere in this PDF file.

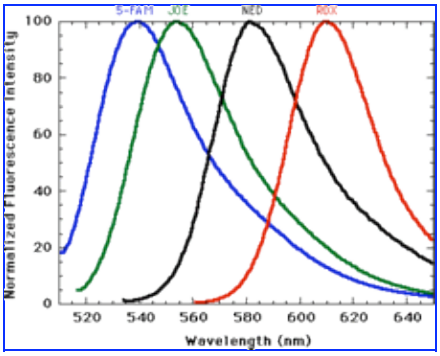
To achieve balance (product yield) across all loci, the quantity of an individual primer can be adjusted within the multiplex set. This part of the process is highly empirical and can be very time consuming.

Dyes

The use of dyes in STR product analysis is dealt with in course: Amplified DNA Product Separation, Fluorescent Detection Module. Selection of dyes is generally based upon instrument compatibility and spectral overlap. The effective use and development of different colored dyes (that can be used together) results in an output consisting of organized peaks in a variety of colors, rather than of a series of indistinguishable peaks. In general, the dyes used to label primers for forensic DNA analysis are fluorescent



and emit color at different wavelengths.



Although the dyes are different colors, their spectra can overlap because the wavelengths that determine color occur simultaneously, not discretely.

Due to spectral overlaps, a matrix must be run with each instrument. A matrix is created by running each color separately on the instrument. The instrument then compares the individual colors to one another. The matrix file is represented in table format.

310-F Matrix

REACTIONS

	B	G	Y	R
B	1.0000	0.5423	0.0428	0.0010
G	0.6601	1.0000	0.5460	0.0059
Y	0.3459	0.5735	1.0000	0.0893
R	0.1693	0.3236	0.5922	1.0000

The matrix file allows the instrument software to normalize the fluorescence between the colored dyes and creates a virtual filter, separating the colors for sample analysis.01

Proprietary Kits

Applied Biosystems and Promega manufacture most forensic STR DNA amplification kits. Each manufacturer has modified their kits to meet the changing needs of the forensic science community. While other manufacturers have developed DNA STR kits, this module focuses on Applied Biosystems and Promega kits because they are currently the most widely used in the forensic science community.

Read more about Locus Selection elsewhere in this PDF file.

Each manufacturer has independently developed different primer sets for the Combined DNA Index System (CODIS) core loci. It is possible, albeit rare, to have one set of primers successfully amplify a locus while another fails. The failures are primarily due to primer binding site mutations and the result is a null allele.02

In general, a primer binding site mutation results in a homozygous profile for an actual heterozygote. Both manufacturers have introduced additional primer sets for their kits in an effort to minimize the occurrences of null alleles. When comparing DNA profiles developed from the different manufacturers' kits, it is important to take this into account.

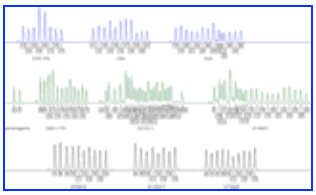
For example, if a kit from manufacturer A types a locus as "12, 14" and a kit from manufacturer B types the same locus as "12" this difference may likely be due to a null allele.

AmpFℓSTR (Applied Biosystems)

Applied Biosystems was the first kit manufacturer to produce an STR multiplex kit for DNA testing.

Applied Biosystems Kit Chronology

Kit	Loci	Dye	Comments
AmpFℓSTR® Blue™	D3S1358 vWA FGA	Blue 5-FAM™	Lower discrimination power compared to the larger multiplexes currently used in forensic laboratories Important precursors in the evolution of forensic STR multiplex testing
AmpFℓSTR® Green I™	Amelogenin THO1 TPOX CSF1PO	Green JOE™	
AmpFℓSTR® Profiler Plus™	D3S1358 vWA FGA	Blue 5-FAM™	Used in combination with AmpFℓSTR® COfiler™, meets the national requirements for analyzing the 13 CODIS core loci Incorporates a quality control measure by ensuring concordance of the repeated loci between the two kits (D3S1358, D7S820, and amelogenin), which can assist the laboratory in detecting tube mix-ups.
	Amelogenin D8S1179 D21S11 D18S51	Green JOE™	
	D5S818 D13S317 D7S820	Yellow NED™	
AmpFℓSTR® COfiler™	D3S1358 D16S539	Blue 5-FAM™	Used in combination with AmpFℓSTR® Profiler Plus™, meets the national requirements for analyzing the 13 CODIS core loci Incorporates a quality control measure by ensuring concordance of the repeated loci between the
	Amelogenin THO1 TPOX CSF1PO	Green JOE™	



	D7S820	Yellow NED™	two kits (D3S1358, D7S820, and amelogenin), which can assist the laboratory in detecting tube mix-ups.03, 04
AmpFℓSTR® Identifiler™	D8S1179 D21S11 D7S820 CSF1PO	6-FAM™	Faster and less costly Tests for all of the core loci with a single amplification Also amplifies D2S1338 and D19S433, which increases the power of discrimination05
	D3S1358 THO1 D13S317 D16S539 D2S1338	Green VIC™	
	D19S433 vWA TPOX D18S51	Yellow NED™	
	Amelogenin D5S818 FGA	Red PET™	

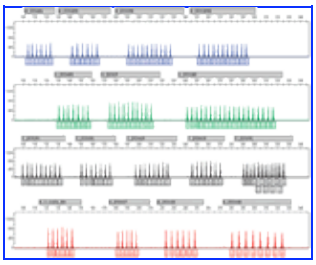
To establish a suitable layout for the sample run, Applied Biosystems introduced the use of non-nucleotide linkers to the following primers: CSF1PO, D2S1338, D13S317, D16S539 and TPOX. Non-nucleotide linkers are placed between the primer and the fluorescent dye during oligonucleotide synthesis.06, 07 This allows for reproducible typing of alleles and provides sufficient spacing between the loci in the multiplex.

Applied Biosystems introduced a Y-STR kit (AmpFℓSTR® Yfiler™) to test for the core Y-STR loci established by the Scientific Working Group on DNA Analysis Methods (SWGDAM).

Applied Biosystems AmpFℓSTR® Yfiler™ Kit

Loci	Dye	Comments
	Blue 6-FAM™	
DYS456 DYS389I DYS390 DYS389II		Core Y-STR loci recommended by the SWGDAM Y chromosome subcommittee in 2003 included DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS438, DYS439, DYS385 (DYS385 is multi-copy) Tests for 17 Y-STR loci08
	Green VIC™	
DYS458 DYS19 DYS385		
	Yellow NED™	
DYS393 DYS391		

DYS439
DYS635
DYS392



Red PET™

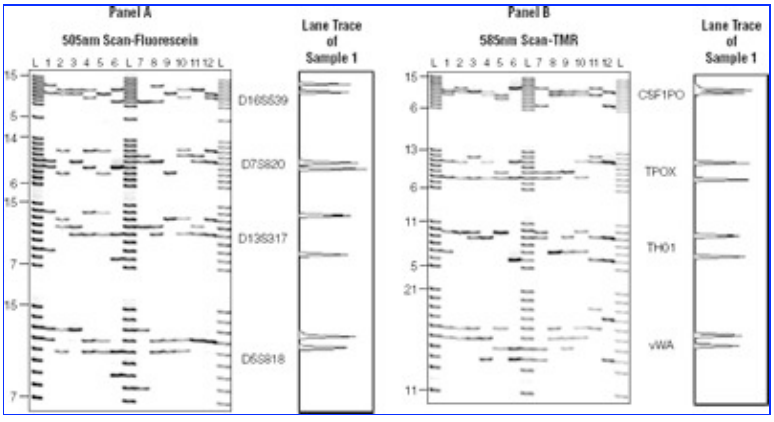
H4
DYS437
DYS438
DYS448

PowerPlex® (Promega GenePrint®)

Promega was the first manufacturer to produce kits for STR testing. Although these kits were initially offered as monoplexes, they were instrumental in setting the foundation for forensic STR DNA analysis. Promega went on to produce several multiplexes for STR DNA analysis. One of the first multiplex amplification kits offered by Promega was the GenePrint® CTTv Multiplex amplification, which amplified CSF1PO, TPOX, THO1, and vWA using a blue fluorescein (FL) dye. In addition, Promega produced both the GenePrint® FFFL Multiplex kit, which amplified F13A1, FES/FPS, F13B, and LPL, and the GenePrint® GammaSTR Multiplex kit, which amplified D16S539, D13S317, D7S820, and D5S818. Both of these kits also used the blue FL dye. When used together these kits provided the most powerful combination of multiplexes offered for forensic STR DNA analysis at that time.

As with Profiler and Cofiler, the combination of these two kits (GenePrint® PowerPlex™ 1.1 System and GenePrint® PowerPlex™ 2.1 System) provides laboratories with a means to meet the national requirements for analyzing the 13 CODIS core loci. They incorporate a quality control measure by ensuring concordance of the repeated loci. The first kit was PowerPlex® 1.1, for Hitachi FMBIO users, or the PowerPlex® 1.2 System, for ABI 377, 310 and 3100 users. The two dye kits amplified eight STR loci and amelogenin in the following configuration:

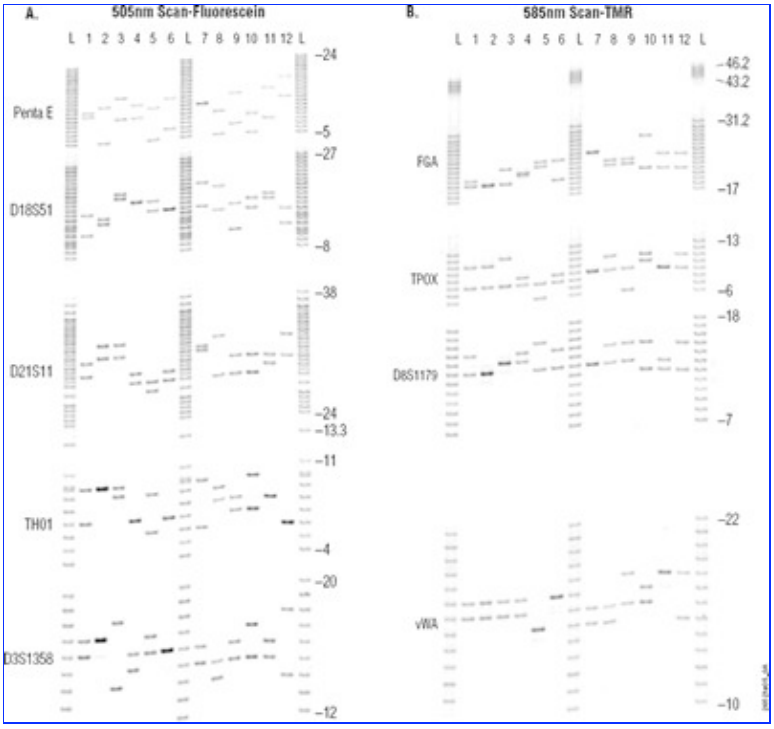
D5S818, D13S317, D7S820, and D16S539 labeled with a blue (FL) dye
vWA , THO1, TPOX, and CSF1PO labeled with a yellow carboxy-tetramethylrhodamide (TMR) dye.09, 10



Later Promega introduced the PowerPlex® 2.1 System, which is designed for Hitachi FMBIO users. The two dye kit amplified nine STR loci and amelogenin in the following configuration:

D3S1358, THO1, D21S11, D18S51, and Penta E labeled with a blue FL dye.
Amelogenin, vWA, D8S1179, TPOX, and FGA labeled with a yellow TMR dye

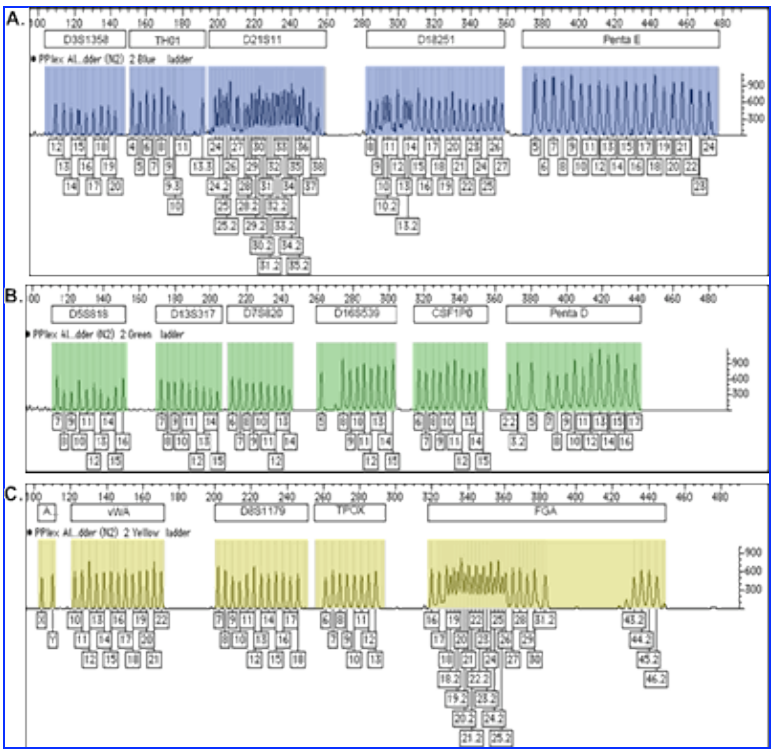
As with Profiler and Cofiler, combining these two kits provided laboratories with a means to meet the national requirements for analyzing the 13 CODIS core loci and incorporate a quality control measure by ensuring concordance of the repeated loci (THO1, vWA TPOX, and amelogenin). An additional locus, Penta E, was added to this set providing for increased discrimination power.[11]



Promega was the first commercial manufacturer in the country to offer a STR multiplex that typed all of the CODIS core loci with a single amplification. The kit, PowerPlex® 16, is a three-dye kit that amplifies 15 STR loci and amelogenin in the following configuration:

D3S1358, THO1, D21S11, D18S51, and Penta E labeled with a blue FL dye
D5S818, D13S317, D7S820, D16S539, CSF1PO, and Penta D labeled with a green 6-carboxy-4',5'-dichloro-2',7'-dimethoxy-fluorescein (JOE) dye
Amelogenin, vWA, D8S1179, TPOX, and FGA labeled with a yellow TMR dye

In addition to the core loci, PowerPlex® 16 also amplifies two penta-nucleotide repeats for increased discrimination power.[12]



Promega was the first commercial manufacturer to produce a Y-STR kit that amplified all of the core Y-STR loci, as outlined by SWGDAM. The PowerPlex® Y amplification kit is a three-dye chemistry kit that amplifies 12 Y-STR loci in the following configuration:

DYS391, DYS389I, DYS439, and DYS389II are labeled with a blue FL dye
DYS438, DYS437, DYS19, and DYS392 are labeled with a green JOE dye
DYS393, DYS390, andDYS385 A/B are labeled with a yellow TMR dye
The PowerPlex® Y kit amplifies all of the required core Y-STR loci in addition to DYS437.

The quantity of DNA added to an amplification reaction, along with the overall volume of the amplification itself, impacts the quality of the results. The multiplex kit manufacturers vary on the suggested input template DNA as well as the suggested reaction volume size. For example, the Profiler Plus User's Manual recommends 1.0-2.5ng of DNA in a 50µl reaction.13 while the PowerPlex® 16 kit recommends 0.5-1.0ng of DNA in a 25µl reaction.14

Read more about DNA amplification elsewhere in this PDF file.

Some laboratories have successfully validated reduced reaction volumes in part to limit the consumption of DNA evidence and reduce analysis costs.15 These validation studies have included assessment of preferential amplification, heterozygous peak height ratio, and an overall comparison between the reduced reactions to that specified by the manufacturer. As with the implementation of any new methods, laboratories must conduct appropriate validation studies.

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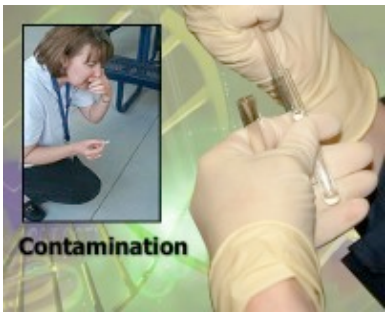
Introduction



Current methods used by forensic DNA laboratories are sensitive and capable of amplifying and detecting low quantities of DNA. With the ability to detect minute quantities of DNA, comes an increased chance of detecting contaminant DNA in both samples and controls. Contamination can happen during any step of the process and can result from a variety of substances, including plant material, bacteria, and human genomic DNA. There are occasions when the source of contamination cannot be determined and/or the incidences are sporadic in nature.

It is not always possible to prevent contamination; however, laboratories should ensure that procedures are in place to:

- Minimize the risk of contamination
- Detect contamination
- Document and implement corrective measures for incidents of contamination

This module focuses on extraneous human genomic DNA deposited after the crime event (during the collection and/or analysis process).

Objectives

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

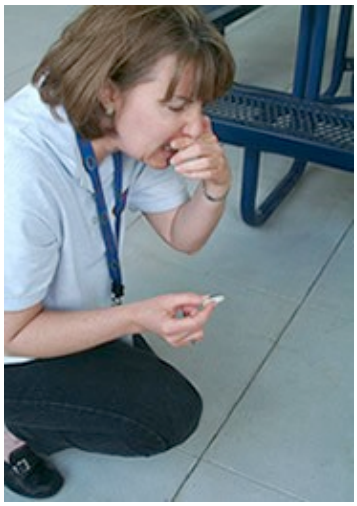
- Recognize the sources of laboratory contamination
- Describe how to minimize the risk of contamination
- Describe the processes that aid in the detection of contamination
- Describe how to implement corrective action processes

Sources

The most common sources of extraneous DNA into the collection and analysis processes are:01-04

- Investigators and laboratory staff
- Reagents and consumables
- Sample cross contamination during analysis

Investigators and Laboratory Staff



Investigators and laboratory staff can deposit their own DNA during collection, handling, and analysis of samples. One of the most common sample contamination events is the transfer of DNA from an individual who comes into contact with the evidence. The most probable means of depositing DNA from investigators and laboratory staff onto a sample is from sloughed skin cells and saliva spray. Individuals handling evidence should avoid talking or sneezing over evidence, and gloves should be worn so that sloughing of cells onto the evidence is prevented.

Reagents and Consumables

Reagents and consumable supplies can introduce exogenous DNA into the analysis process. Negative controls and reagent blanks will assist in detecting contamination associated with reagents. However, instances of contamination introduced by consumables (e.g., plastic ware, pipette tips) are commonly single tube events and may or may not be detected through the use of controls. 01-04

Reagent contamination is generally more easily detected than that from consumable products. Negative controls and reagent blanks provide a good way to monitor reagent contamination that can adversely affect an entire analysis batch. This is not true for contamination events from consumable products. These are usually single tube events and the level of contamination is low. This type of event is primarily detected in negative controls, reagent blanks, and evidentiary samples with low levels of DNA.

A growing concern in the forensic science community is the possibility that consumables, namely plastic ware, may be contaminated during the manufacturing and/or packaging process. In general, laboratories routinely purchase sterile consumables and have thought them to be free of DNA. However, contamination events have shown that these sterilized products can carry DNA from individuals working in the manufacturing and/or packaging process.

The Forensic Science Service (FSS) has reported incidents of casework-related STR contamination from staff of plastic ware manufacturers.05 Investigations carried out by the FSS prompted the novel establishment of a vendor database consisting of DNA profiles from individuals employed by various vendors of consumable products. The database has subsequently sourced unknown profiles developed in the laboratory to the manufacturing process.

Visit the Forensic Science Service website.

Note:

The names of the individuals are not included in the vendor database maintained by the FSS.

The first incident in the United States was reported after DNA profiles were uploaded into the Combined DNA Index System (CODIS) and subsequently linked multiple crimes across multiple states. After it was determined that the FSS had also observed this same profile (with SGM+) on more than one occasion, it was believed that the profile must have originated from a consumable used in the analysis process.

Sample Cross Contamination

Many laboratories process samples in batches to streamline analyses. One risk of batch analysis is the inadvertent cross contamination of DNA from one sample to another sample that was processed concurrently. Most detected sample-to-sample sample contamination will be from samples with higher concentrations of DNA to those with lower concentration.

The online version of this course contains a multimedia [or downloadable] file. Visit this URL to view the file: http://beta.amplification.dna.devis.com/m04/01/c/

Prevention

There are numerous processes that laboratories can establish to minimize the risk of contamination. It is important for each laboratory to assess their specific needs both technically and administratively prior to establishing a process.

Laboratories must demonstrate that they have a facility that is designed to minimize contamination. This may include restricting the movement of staff, equipment, and consumables between pre- and post-amplification areas.

Read Section 6, Facilities, of the QAS.

Some additional processes include:

- Staff training
- Quality control testing of reagents and consumables
- Storage and treatment of consumables
- Implementation of clean techniques05

Read about detailed prevention practices in the Laboratory Orientation and Testing of Body Fluids and Tissues for Forensic Analysts PDF file.

Training

Laboratory managers should ensure that all laboratory personnel are appropriately trained in the handling and processing of evidence and offender samples, in regard to DNA analysis. Providing biological evidence collection training to crime scene response staff and investigators can help ensure that they have an appreciation of DNA contamination issues.

The training should stress that the most effective means of protecting evidence from contamination from investigators and laboratory staff is to use personal protective equipment (PPE), such as gloves, gowns, and masks. In general, universal precaution methods not only protect the investigator and analyst but also ensure that the evidence is protected from contamination by handlers.

Read more about safety in the Safety PDF file.

Quality Control Testing of Reagents and Consumables

Reagents

Negative controls and reagent blanks provide a means to detect contamination from reagents. Laboratories should run quality control checks on reagents prior to use in casework. These checks assist in determining if a reagent is free of contamination at that time. Negative controls can then be assessed on an ongoing basis to demonstrate that they remain contaminant free.

Including and assessing negative controls and reagents blanks are critical quality control steps. These controls provide a means of detecting reagent contamination and, on occasion, sporadic contamination. Because many contamination events are sporadic, negative results in these controls do not necessarily mean that samples from the same batch are contaminant free. Additionally, the detection of contamination in these controls does not mean that all batch samples have been affected.

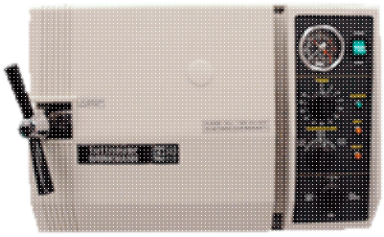
Consumables

Some consumables can be treated with ultraviolet (UV) light and/or autoclaved. These preventive measures may be useful in limiting contamination events. Other consumables, such as centrifugal filter units and filtered pipette tips, cannot be pretreated. In these instances, establishing a method for detecting contamination from these items is very important.

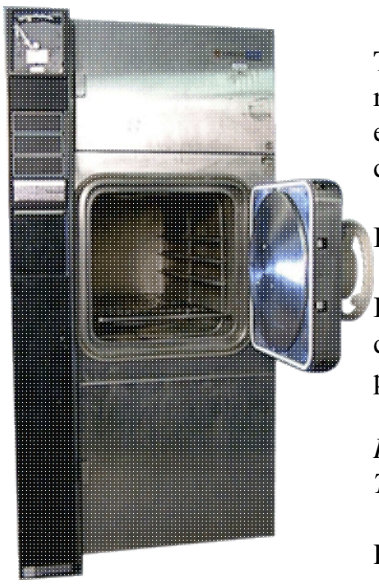
Laboratories can perform quality control checks of consumables similarly to those for reagents, but the process is not as clear-cut. Some laboratories have established procedures whereby a percentage of consumables from each lot number is evaluated prior to use in casework. This may be especially useful for laboratories that have observed contamination suspected to be from consumable products. While this approach will not prevent contamination, it can provide data from any profile(s) developed during these

checks, which can be used for future evaluation of potential contamination events.

Storage and Treatment of Consumables



It is recommended that laboratories store their consumables in such a way as to limit exposure to the environment and consider effective pretreatment. Current pretreatment methods, such as autoclaving and UV cross-linking, may not be entirely effective since they may not penetrate all surfaces of the consumable.



The FSS has reported ethylene oxide gas to be extremely effective in removing DNA. Ethylene oxide is commonly used to sterilize medical equipment, but this technology is not currently used by manufacturers of the consumables used in forensic DNA analysis.

Implementation of Clean Techniques

Implementation of clean techniques to reduce contamination is covered in course: Forensic Biology, Module 1. More information about detailed prevention practices can be found in this module.

Read about detailed prevention practices in the Laboratory Orientation and Testing of Body Fluids and Tissues for Forensic Analysts PDF file.

Reference and Evidentiary Sample Processing

One concern with contamination is that an individual may be falsely linked to a crime. Reference samples are generally good quality DNA samples and result in high quantities of extracted DNA. Many laboratories process samples in a way that isolates evidentiary samples from reference samples during the screening, extraction, and PCR stages. Therefore, the possibility of contaminating an evidentiary sample with reference DNA is avoided.

Detection

Contamination introduced in the laboratory analysis process may not be avoidable, making implementation of procedures to detect these incidences crucial. DNA profiles derived from evidentiary samples and reference samples are uploaded into CODIS and are used to associate individuals with a crime. It is imperative that laboratory procedures are in place to ensure the integrity of the data generated for these functions.

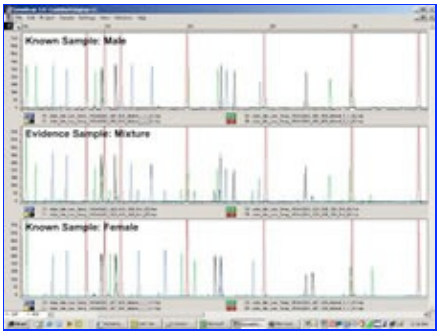
Most manufacturer's multiplexes demonstrate optimum efficiency when approximately 1ng of template DNA is analyzed for 28-30 amplification cycles.06 However, the lower limit of detection can be less than 250 pg. Most contamination events involve small quantities of DNA and therefore will be detected at lower threshold values. Laboratories establish reporting thresholds based on their validation studies. Because most contamination is below that threshold, it will not be reported; analysts should assess any allelic activity under

the reporting threshold to determine if it could be from contamination.

Some additional detection processes include:

- Assessment of controls
- Review of batch profiles
- Maintenance of unsourced contamination profiles
- Establishment of reference databases

Assessment of Controls



As stated above, negative controls and reagent blanks can greatly assist in the detection of contamination. Positive controls and samples from known sources may also aid in the detection of contamination. Positive controls are single-source samples of a known type; the detection of additional alleles may indicate contamination. Reference samples are expected to be from a single-source, and while the DNA profile may be unknown, results that indicate a mixture could be a sign of contamination.

Review of Batch Profiles

Many laboratories analyze samples using a batch process. Incorporating the assessment of samples against other profiles developed from the same batch processes can be used to detect sample-to-sample contamination events.

Maintenance of Unsourced Contamination Profiles

There may be occasions when laboratories detect contamination but are unable to determine the source. These instances will most likely occur in negative controls and/or reagent blanks. Unsourced contaminant profiles can be maintained by a laboratory for future comparison and can provide a mechanism for a laboratory to troubleshoot multiple occurrences of the same contaminant DNA profile.

For example, the laboratory may be able to:

- Isolate the profile to a specific consumable product
- Isolate the profile to a specific investigator(s)
- Isolate the profile to a specific staff member

Establishment of Reference Databases

A concern in the forensic science community is that a contaminant profile would be mistakenly reported. The most likely cause of contamination of evidence is from the staff involved in handling of samples. Based on this knowledge, it is highly desirable that the laboratory maintains a staff DNA database. Ideally, this would

include everyone involved from collection to completion of analysis. This database can be assessed against any unknown profiles developed from evidentiary samples as part of data interpretation to ensure that no contamination from a staff member is mistakenly reported.

Due to legal or departmental constraints, it is recognized that some laboratories may not be able to maintain a staff database of this scope, if at all. An alternative is for laboratories to obtain volunteer samples on a case-by-case basis where contamination is suspected.

This procedure can also be expanded to the following:

DNA profiles from contractors who work in the laboratory area
DNA profiles from visitors to the laboratory
DNA profiles from employees of subcontract vendor laboratories

Corrective Actions

There are four steps to taking corrective actions:

1. Identify the problem
2. Determine the root cause
3. Implement preventive measures
4. Document the event

Identify Problem

Step One

If contamination is suspected, the first step of the investigation should be to determine where the contaminant profile has arisen. In many cases, it can be traced back to a particular step in the analysis by working backwards in a step-by-step process.

View an animation on identifying the problem.

It is important to compare the contaminant profiles to:

Other samples from the same batch
Samples from other batches processed in the same time frame
Staff profiles
Previously detected contaminant profiles
Other persons involved in the collection and handling of the evidence

If the profile contains too few alleles to effectively screen against the above, consideration can be given to boosting the signal strength by using one or more of the following:

Amplifying additional extract
Extending the injection time
Concentrating the extract or amplicon
Increasing the number of PCR cycles

These troubleshooting procedures should not be used for processing, interpreting, or reporting samples unless validated by the laboratory.

Determine Root Cause

Step Two

The point at which the contamination has occurred may be determined by reworking the samples in reverse in a step-by-step manner (see Step One: Identify Problem).

Although alleles under the threshold are not reported in casework, these should be considered when performing investigations/corrective actions to assist in the determination of the source.

Implement Preventive Measures

Assuming that the above measures are in place, the following areas may be addressed:

- Staff training / awareness
- Laboratory decontamination
- Modification of procedures

If preventive measures are ineffective, the laboratory may need to implement additional or alternative measures

Document the Event

Each event should be documented. The following should be included in the documentation:

- Description of deficiency
- Description of root cause of deficiency
- Description of the impact of deficiency on past work and remedial action taken
- Description of resolution/completion

View an example of a Corrective Action Report

Note:

This process can be applied to any quality issue requiring a corrective action.

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Online Links

Online Links

Forensic Science Service
http://www.forensic.gov.uk/
Section 6, Facilities, of the QAS
http://www.fbi.gov/hq/lab/codis/forensic.htm

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Debbie Figarelli serves as DNA Technical Leader at the National Forensic Science Technology Center. Debbie assists with the development of DNA training programs and participates in compliance audits of DNA laboratories.

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Debbie Figarelli serves as DNA Technical Leader at the National Forensic Science Technology Center. Debbie assists with the development of DNA training programs and participates in compliance audits of DNA laboratories.

Course Completion

Congratulations! You have completed this course. Please click the "Forward" arrow to return to the course homepage.

If you have made it through all of the lessons within this course, then a link will appear on the homepage that takes you to your Course Certificate. This certificate verifies that you have completed all the necessary material in this course, and it can be printed out and kept for your records.

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General DNA Questions

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