DNA Extraction & Quantitation for Forensic Analysts

This course is provided free of charge and is part of a series designed to teach about DNA and forensic DNA use and analysis.

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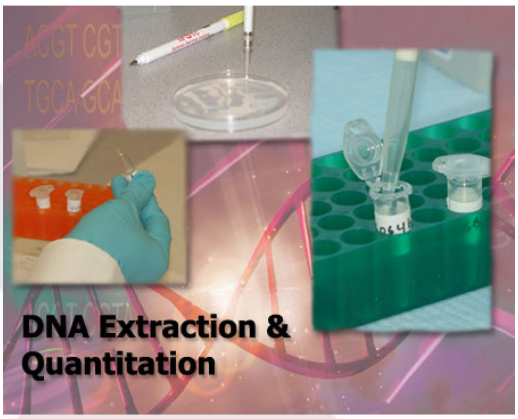
Updated: October 8, 2008



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DNA Analysis Considerations

Introduction

The nature of the wide variety of substrates on which forensic biological samples are deposited can create problems for the DNA analyst. For example, the substrate may cause collection problems or it may contain substances that interfere with the Polymerase Chain Reaction (PCR) assay. The main problem is inhibition; the efficiency of amplification of forensic samples is often significantly decreased because of inhibitors present in the sample substrate. Inhibition can result in partial DNA profiles and, in severe instances, false negative reactions.

Even when sample collection and PCR have worked properly, difficulties may arise in the interpretation of samples from multiple donors.

Read more about mixtures in the STR Data Analysis & Interpretation PDF File.

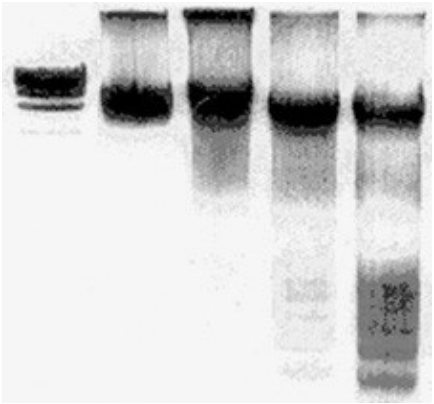
Objectives

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

- Identify potential obstacles in collection, extraction, and amplification
- Describe potential PCR inhibitors
- Identify techniques to overcome inhibition

In some instances, collection of samples poses a significant challenge. In general, the best collection strategy is to submit the entire item. This can be easily accomplished for items such as a small rock, clothing, or a knife, but is more difficult in the case of larger, fixed objects. In cases where the samples can be collected using a slightly moistened swab, such as from a window, the collection process is fairly straightforward. Some problems can arise when a hard porous surface, such as pavement or concrete, is encountered. In these cases, it can be difficult to collect small bloodstains or other biological fluids by swabbing. There are occasions where scraping the substrate containing the biological sample is a viable option. However, care must be taken. For example, if the crime scene is outdoors, wind could cause loss of sample or contamination of another item.

Inhibitors



It is hard to determine all of the causes of inhibition on the PCR reaction. The PCR process can be affected by compounds that interfere with the interaction between DNA and Taq polymerase, and thus inhibit the reaction. Many inhibitors are removed during the extraction process through ethanol precipitation or the use of a Microcon® or Centricon® centrifugal filter unit. However, some inhibitors co-elute with the DNA, which may lead to PCR inhibition. A number of inhibitors are contained in the samples themselves, while others can be introduced by the substrate or the analysis process. The presence of inhibitors can result in loss of data or results that could be mistaken for degradation. We can classify sources of inhibition into three groups:

- Internal, or those found in body fluids.
- Substrates, or those arising from the materials on which the blood stain or other source of DNA has been deposited.
- Other sources, such as reagents and materials used in the analysis.

Internal

Many body fluids contain substances that can inhibit PCR:01

Table of Biological Substances and Inhibitors

Biological Substance	Inhibitor	Comment
Blood	Heme	The amplification of blood samples can be significantly reduced or blocked by natural components of blood,

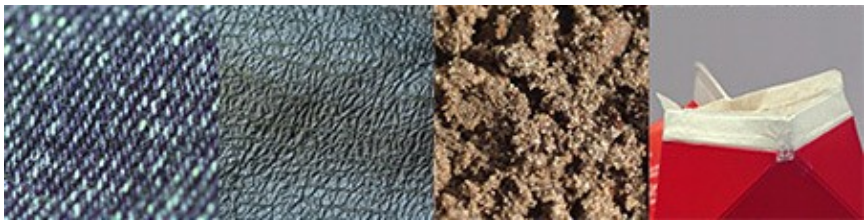
	Immunoglobulin G	such as heme, and immunoglobulin G. Hemin, a hemoglobin derivative, and its breakdown products, bilirubin and bile salts, are also found to be PCR inhibitors.
Vaginal Samples Buccal Samples Fecal Samples	Bacteria Microorganisms	Bacteria and microorganisms are commonly found in vaginal, fecal, and buccal samples. Note: these can also be found in other biological samples found at crime scenes.
Hair	melanin	Melanin, a pigment that affects skin, eye, and hair color, can inhibit PCR.
Tissue Bone Teeth	Ca^{2+}	Ca^{2+} is commonly found in bone and teeth and is known to interfere with Mg^{2+} concentration, which in turn may affect the activity of Taq polymerase.
Semen	Polyamines	Spermine and spermidine (polyamines originally isolated from semen) are found in ribosomes and living tissues, and can inhibit PCR.
Urine	Urea	Urea, a chemical found in urine, can inhibit PCR.

Substrates

Some of the substrates that contain inhibitors are:

Table of Substrates and Inhibitors

Substrate	Inhibitor	Comment/Example
Textile dyes	Textile dyes	Indigo dye used to color denim
Fabrics	Tannic Acid	Leather
Environmental	Humic compounds	Soil02
Samples	Heavy metals	
Food	Organic compounds	Many food products, such as milk, contain inhibitors, like Ca^{2+}. However, forensic scientists have had success in developing DNA profiles from saliva left on food or drink containers.01
Constituents	Phenolic compounds	
	Glycogen	
	Fats	
	Ca^{2+}	



Examples of substrates with inhibitive properties

Other Inhibitors

Inhibitors can be introduced in the collection and analysis processes or at the crime scene

Table of Other Inhibitors

Source	Inhibitor	Comment
Extraction Chemicals	Phenolic compounds from the organic extraction	Reagents commonly used in the purification of nucleic acids are inactivators of DNA polymerases. Phenol or Chelex resin left with the extracted DNA can inhibit the PCR process.
	Chelex resin	
	Salts	
	Guanidine	
	Proteases	
	Organic solvents	
	Phosphate buffers Detergents (such as Sodium Dodecyl Sulfate (SDS))	
Anticoagulants	EDTA and heparin	Known blood reference samples are collected in tubes containing anticoagulants.
Powder	Glove powder	Many forensics scientists use powdered gloves.
Laboratory plastic ware	PCR tubes	It has been reported that an inhibitory substance can be released from polystyrene or polypropylene upon exposure to ultraviolet light.
Plant and food products	Pollen	Biological material can be deposited on plants and food.
	Cellulose	
	Plant polysaccharides	
	Ca^{2+}	

Overcoming Inhibition

Not all of the factors affecting inhibition are known, and most of the methods used to overcome inhibition are specific to the inhibiting compound. In contrast, Bovine serum albumin (BSA) is included in both Applied Biosystems and Promega STR typing kits as a more general means of overcoming the effect of enzyme inhibitors.

Amplification test gels are a long-established technique to evaluate samples for inhibition. More recently, Quantitative PCR gives analysts the ability to assess inhibition during the quantitation process. However, not all inhibition will be detected. One certain way to assess a sample for inhibition is to add a portion of the extract to a control sample and attempt amplification. Failure of amplification demonstrates the presence of an inhibitor.

More about this in the Quantitation Module of this course.

The following are techniques that can be used to overcome inhibition:

One of the easiest approaches taken to overcome inhibition is to dilute the template DNA sample and reamplify. This dilutes out the inhibitor, allowing successful amplification to occur. In general, the quality of DNA and purity of the sample is more important to amplification than the quantity of DNA. Usually, a 1:10 or greater dilution is sufficient to overcome inhibition.

BSA has been shown to reduce inhibition in samples affected by hemin as well as other inhibiting compounds. BSA is a general stabilizing agent, as is gelatin, which has also been used to overcome inhibition.03,02,04

Reextraction & Additional Sample Preparation

An analyst can take additional steps to clean up the sample by reextraction using Chelex resin, phenol chloroform, Thiopropyl Sepharose 6B (Sigma)04 extraction beads, or magnetic beads (DNA IQ™). Sample preparation using a Centricon® or Microcon® device may also assist in removing inhibitors.

Increased Taq Polymerase

The interaction between Taq polymerase and the template DNA is affected by inhibitors. Increasing the concentration of Taq polymerase in the reaction can overcome the effects of some inhibitors.04

Non-ionic detergents, such as Tween® -20, NP-40 and Triton® X-100, can assist in overcoming inhibition, having the specific benefit of overcoming the inhibitory effects of trace amounts of strong ionic detergents, such as SDS.05

Miscellaneous Chemicals

Ammonium ions and dimethyl sulfoxide (DMSO) have been used to overcome inhibition.

One study cites a method for neutralization of Taq polymerase inhibitors by denaturing and washing with NaOH in Microcon® -100 filtration units. The study speculates that many inhibitors bind the template DNA and possibly intercalate into the double stranded DNA. Denaturizing the DNA could reduce the substance's affinity for it. One other hypothesis is that the NaOH may directly inactivate the inhibitor.06

The effects of some inhibitors can be overcome with the use of shorter primers.07

Summary

This module has briefly dealt with collection of DNA from scenes, and with one of the main difficulties encountered with the PCR process, namely the presence of inhibitors. The module describes the nature of inhibitors and techniques for their removal or neutralization.

Organic Extraction

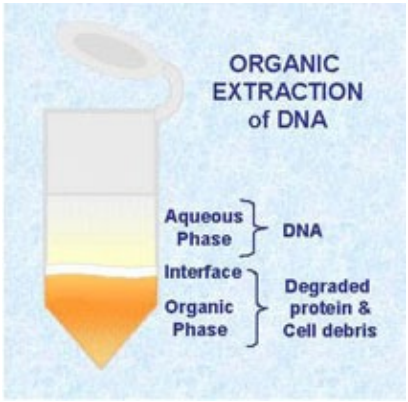
Introduction

Techniques using organic reagents for DNA extraction are well accepted in the forensic science community. Organic extraction methods are often preferred for the extraction of biological stains containing small amounts of DNA or degraded DNA.01-05 These methods could be considered less harsh than other methods, such as the use of Chelex beads, because no boiling step is required.

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

- List steps involved in extracting DNA using an organic extraction method
- Identify the advantages and disadvantages for use of organic extraction

Organic Extraction Process



Historically, DNA extraction was accomplished by mechanical or chemical disruption of cells to release their organelles and contents. This works well in samples containing many cells, but has required adaptation for use with the much smaller biological samples collected at crime scenes.

Organic extraction is a conventional method that uses organic chemicals to isolate genomic DNA. The procedure can be described in four steps:

1. solubilization of the stain components
2. denaturation and hydrolysis of proteins
3. removal of denatured proteins
4. purification of DNA06

Step One: Solubilization of Stain Components

Water must be replaced so that dried stains can be resolubilized for DNA extraction procedures. The DNA is protected from unnecessary degradation in this process by adding EDTA, a magnesium chelator, to the lysis buffer. EDTA prevents nucleases from degrading the DNA. Tris (a component of the buffer) interacts with the lipopolysaccharides present on the outer cell membrane, which helps to make it permeable. This effect is enhanced with the addition of EDTA.06

Step One Reagents

Stain extraction buffers are usually slightly alkaline and generally contain Tris base or Tris-HCl (pH between 7.5-8.0), EDTA, and sodium chloride (NaCl).

Step Two: Denaturation and Hydrolysis of Proteins

Cells are lysed using a detergent, Proteinase K, and dithiothreitol (DTT). Extractions must use appropriate salt concentration and pH to ensure that proteins and other contaminants are separated into the organic phase and that DNA remains in the aqueous phase.

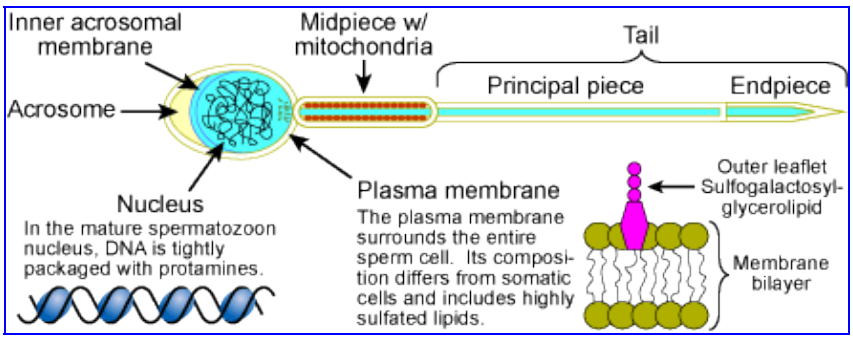
Detergents, which are included in the stain extraction buffer, have the following functions:

- They lyse cell membranes.
- They separate histone proteins from DNA.
- They denature histone proteins.
- They destroy secondary and tertiary structures of proteins, which decreases their solubility in aqueous solution.

Proteinase K is used to hydrolyze histone proteins and is well suited to the extraction process for the following reasons:

- It is active over a wide pH range (4-12.5).
- It is active in the presence of SDS.
- It is not affected by EDTA.

Dithiothreitol (DTT) reduces disulfides to dithiols, allowing release of the DNA from its protective proteins and further degradation of the proteins by Proteinase K. DTT is an essential component for sperm cell lysis because the cell membrane contains a high concentration of disulfides.06



Step Two Reagents

- **Detergents**

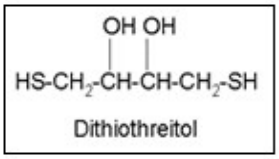
Sarkosyl is generally used if a lysis procedure is conducted under refrigerated conditions (less than room temperature) because sodium dodecyl sulfate (SDS) precipitates out of solution at these temperatures. SDS is the detergent of choice when lysis procedures are conducted at room temperature.

- **Proteinase K**

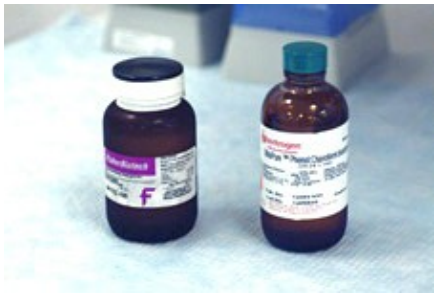
Proteinase K is produced by the fungus Tritirachium album Limber. It is an endolytic protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic, or hydrophobic amino acids. Proteinase K is classified as a serine protease. Proteinase K in the extraction buffer inactivates nucleases and aids in lysis of

epithelial and white blood cells to free nuclear DNA. Nuclease inactivation is a very important step in DNA isolation. Nucleases naturally exist in the cell to break down the nucleic acids after they serve their functions in protein manufacture, thus allowing the individual building blocks of the DNA and RNA to be recycled by the cell. Inactivating these nucleases preserves the DNA so that it can be extracted and purified.06

- **DTT**



DTT Construction. The DTT reduces disulfides to dithiols, allowing release of the DNA from its protective proteins and further degradation of the proteins by Proteinase K.
Step Three: Removal of Denatured Proteins



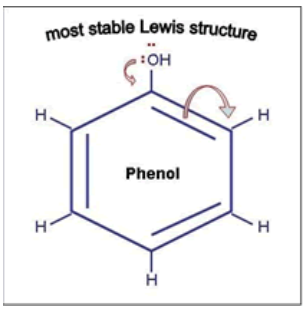
Denatured proteins are removed during the extraction using a phenol chloroform isoamyl alcohol (PCI) combination. Phenol denatures proteins that are subsequently hydrolyzed with Proteinase K. Due to their negative charge, DNA molecules can be separated from other cellular components. Addition of PCI to a sample promotes separation of non-polar (organic) and polar (aqueous) phases. The phenol is not miscible with water, denatures protein, and sequesters the denatured hydrolyzed protein in the organic phase. During this process, the DNA remains in the aqueous phase in its double-stranded state.

PCI may be purchased commercially or prepared in a ratio of 25:24:1. Because the pH of phenol (approximately 7.0) would generally be too acidic for purposes of DNA extraction, the phenol is buffered by saturation with TE buffer.

Note:
Care must be taken to isolate only the aqueous phase during this procedure. Any residual PCI can compromise the filters in the filtration devices and also acts as a PCR inhibitor.

Step Three Reagents

- **Phenol**

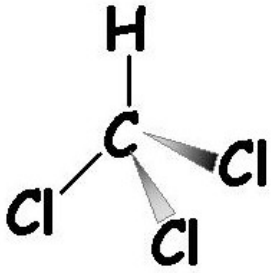


Phenol is a member of a class of organic compounds containing a hydroxyl group fixed to an unsaturated carbon in a benzene ring. Phenols are not true alcohols; they are more acidic than alcohols but less so than carboxylic acids. In most reactions they behave as nucleophiles. Phenols are also readily oxidized, more so than alcohols. This property is employed in DNA extraction. The addition of hydroxyquinoline to the reagent gives the organic phase an orange color, making it easier to differentiate the aqueous phase containing DNA. Polysaccharides and proteins are soluble in phenol, allowing for their separation from DNA.

Safety Note: CAS# 108-95-2

Although originally used for its antiseptic properties, phenol is highly toxic and should be handled in a fume hood while wearing personal protective equipment. Skin contact and inhalation should be avoided.

- **Chloroform**

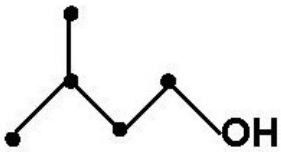


Chloroform ($CHCl_3$), or trichloro-methane, is a colorless liquid that is slightly water-soluble and miscible with organic solvents such as phenol. It is more dense than water or buffer (in which DNA is soluble), yet less dense than phenol. As it increases the phenol phase density, it promotes a sharp interface between the organic and aqueous layers. Chloroform also solubilizes lipids. During the extraction procedure, cellular debris that is not totally digested can be observed at the interface.

Safety Note: CAS# 67-66-3

Previously widely used as an anesthetic, inhalation of chloroform depresses the central nervous system. It is also a suspected teratogen and known carcinogen and should never be handled outside of a fume hood.

- **Isoamyl Alcohol**



Isoamyl alcohol, or 3-methyl-1-butanol, is a primary alcohol. A liquid solvent, it is often included in genomic extraction protocols to help prevent foaming of the reagents, making it easier to detect the interface between the organic and aqueous phases. It is included in the protocol in very small concentration, compared to the other reagents.06

Safety Note: CAS# 123-51-3

A component of some paint strippers and other solvents, isoamyl alcohol causes irritation upon skin contact or inhalation. Vapors can also cause ocular discomfort and effects. This reagent should be handled with care and in a fume hood.

Step Four: DNA Purification

DNA can be recovered from the aqueous phase with an ethanol precipitation or using a centrifugal filter unit (Centricon® or Microcon®).

Centrifugal filter units are used to purify and concentrate DNA. When extracting DNA from small or degraded forensic samples, the final concentration of DNA may be too low for subsequent amplification. Although 1ng is a target quantity of DNA for amplification, if 1ng is suspended in 100 µl of fluid, it would be impossible to transfer 100 µl of this solution into an amplification reaction optimized for 50 µl or less. Centrifugal filter units increase the concentration of DNA in solution by retaining the DNA while eliminating a portion of the fluid from the sample. Another benefit of the unit is the ability to secure DNA while contaminants (possibly PCR inhibitors) are washed from the sample.

Centrifugal filter units separate molecules by size through a series of washing and centrifugation steps. The Millipore Corporation produces two centrifugal filter units under the names Centricon® and Microcon®.

Visit the Millipore Corporation website.

Attributes of Centricon® and Microcon® filter units include:

- Both employ Amicon® filters to retain the DNA.
- Filter porosity varies.
- Proteins flow through, rather than sticking to, the surface during centrifugation.
- Filters are anisotrophic, with increasingly smaller interstitial spaces in the direction of filtration, allowing for better retention of smaller molecules.
- Filters are composed of regenerated cellulose, which can be sterilized.
- Filters exhibit sufficient strength when wet.

Note:

It is important not to spin the unit at rates higher than recommended by the manufacturer because the unit may become compromised, resulting in reduced sample recovery.

Textile dye molecules, such as indigo, are known PCR inhibitors and are readily washed through the filter. Salts introduced by buffers are removed from the sample during the process. Salts carried over from the process may interfere with capillary electrophoresis.

Read about capillary electrophoresis in the Amplified DNA Product Separation PDF File.

Note:

Care must be taken to avoid introducing phenol from the organic extraction into the unit because it will break down both the cellulose filter and its supporting silicone rubber o-ring.

Step Four Reagents

The only reagents used in this process are buffers used in the DNA purification process.

Techniques

Organic extraction techniques are used on all types of forensic samples. The basic organic extraction method is used on samples that do not contain spermatozoa, whereas a modified differential extraction is used on those samples containing spermatozoa.

Microcentrifuge tubes with spin baskets can be used for both basic and differential extractions, allowing the substrate (e.g. fabric, cotton swab, etc.) to be removed without difficulty during the extraction process.

This section outlines:

- Use of microcentrifuge tubes with spin baskets, which can be incorporated into most extraction methods
- Basic organic extraction
- Differential organic extraction

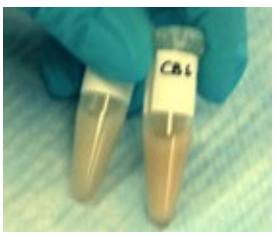
Microcentrifuge Tubes with Spin Baskets

Constructed with an open end and a woven end, spin baskets hold a cutting of substrate and are inserted into a common microcentrifuge tube. This allows for the retention of liquid carrying the biological material to be separated from the substrate during centrifugation steps. Any cells that may not be completely freed from the substrate are forced into the tube, along with the excess fluid. Spin baskets may be incorporated into most extraction methods; however, their composition prohibits immersion in phenol/chloroform/isoamyl alcohol (PCI).

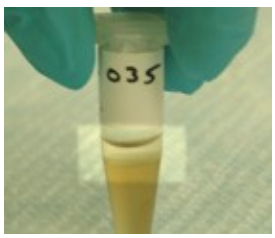
Basic Organic Extraction

The basic organic extraction method can be used for most forensic samples, which includes bloodstains, saliva stains, tissue and hair. Details of the method are given in the laboratory manual.

A stain extraction buffer containing a buffer, detergent, DTT, and Proteinase K is added to the sample. This mixture is incubated, and the sample substrate is removed. Then, the mixture is extracted with a phenol/chloroform/isoamyl alcohol combination. The extracted DNA is generally purified and concentrated with a filtration device.07



Vortexed PCI



Aqueous Phase

Differential Method

Differential extraction methods are used to separate spermatozoa from other cell types. Spermatozoa are more difficult to lyse than other cells and conditions can be set so that all cells except spermatozoa are lysed. The supernatant containing the DNA from these cells is removed from the sperm cells, which can then be lysed separately.

The differential extraction steps are:

- Optional wash step
    - Some laboratories have incorporated an optional wash step at the beginning of the procedure to remove cellular debris and contaminants. The sample is gently washed in a buffer and detergent and the supernatant is removed (wash fraction). This can be done under refrigerated conditions or at room temperature.
- Non-sperm cell lysis
    - An extraction buffer containing a buffer, detergent, and Proteinase K is added to the sample and incubated. This step lyses all cells except spermatozoa. The supernatant containing the DNA from the lysed cells (fraction 1) is removed after pelleting the spermatozoa. The sperm pellet is often washed numerous times with a buffer to remove excess DNA from this lysis step. If this wash is not done, it is not unusual to see a low level of fraction 1 DNA in fraction 2. If any of the sperm cells are weak or otherwise compromised, these may lyse in the first step, leaving a low level of fraction 2 DNA in fraction 1.
- Sperm cell lysis
    - The pelleted sperm cells are lysed under more stringent conditions, using a buffer, detergent, DTT, and a higher concentration of Proteinase K (fraction 2), and are subsequently incubated.
    - Both fractions (including the wash fraction, if appropriate) are extracted separately with the phenol/chloroform/isoamyl alcohol combination and purified.08

The success of differential extraction depends on the sperm head resisting the processes that readily lyse epithelial and white blood cells. Separating the sources of DNA from different contributors to a stain, namely a male donor and female victim, lessens the difficulty associated with mixture interpretation during data analysis, source attribution, and/or statistical calculations. The value of differential extraction is demonstrated by the requirement in the Quality Assurance Standards (QAS) for inclusion in the laboratory's recorded procedures (see Standard 9.1.3). Details of the method are provided in the laboratory manual.

The online version of this course contains a multimedia [or downloadable] file. Visit this URL to view the file: http://beta.extraction.dna.devis.com/m02/03/default_page

A commercial kit for differential extraction is available from Promega. The Differex™ System, includes a proprietary separation solution and the use of a spin basket. The procedure begins with a Proteinase K digestion to lyse non-sperm cells. The sample and buffer are placed into a spin basket within the tube containing the proprietary solution (which is not miscible with the aqueous buffer). Through centrifugation, the sperm cells are isolated as a pellet, while the non-sperm DNA remain in solution. The difference between this system and routine differential extraction is that the separation solution acts as a selective membrane (without the risk of clogging). After the removal of the solution containing non-sperm DNA, the sperm DNA is isolated using the DNA IQ™ System, described later in this subject.

Go to the Differex™ System website.

Chelex®100 Extraction

Extraction procedures using Chelex® 100 (Bio-Rad Laboratories) are popular in the forensic science community because they save time, reduce costs, simplify extractions, reduce safety risks, and minimize potential for contamination. Chelex® 100, a chelating resin, is used to successfully extract DNA from many forensic samples, including bloodstains, tissue, hair, and bone. 01,02, 03, 04, 05

Visit Bio-Rad's website and learn more about Chelex® 100.

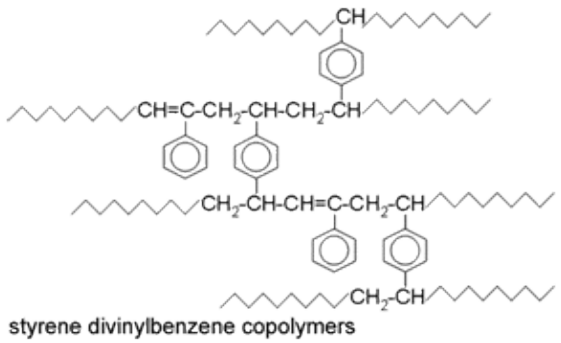
Objectives

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

- List steps involved in extracting DNA using the Chelex® method.
- Identify the advantages and disadvantages of the Chelex® method for use of organic extraction in order to be consistent with the organic module.

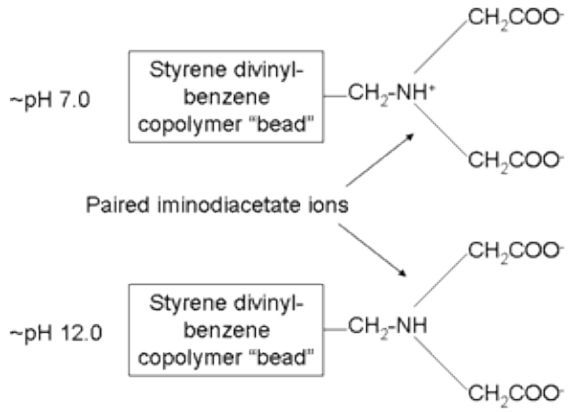
Chelex® 100 Extraction Process

Chelating resins have been used in ion-exchange columns, trace metal removal, metal analysis, and water testing in environmental and agricultural laboratories. In clinical applications and biomedical research, chelating resins can be used to remove or assay cations in whole blood or urine, to remove contaminants from buffers and stock solutions, and to prepare samples for nuclear magnetic resonance spectroscopy.



styrene divinylbenzene copolymers

Chelex®, like most chemicals, is supplied in various grades of purity. Analytical grade Chelex®100 resin is highly purified and most suitable for forensic DNA applications.



Chelex®100 resin is composed of styrene divinylbenzene copolymers with paired iminodiacetate ions. The iminodiacetate ions act as chelators for binding polyvalent metal ions. Chelex®100 is very effective in binding metal contaminants with a high selectivity for divalent ions, without altering the concentration on non-metal ions.06

The extraction is set up under aqueous alkaline conditions. In this environment, Chelex®100 has an increased affinity for heavy metal cations such as Ca^{2+}, Mn^{2+}, and Mg^{2+}, in contrast to its affinity for Na^{+}. One benefit of using the Chelex extraction is that divalent heavy metals can introduce DNA damage at high temperature (e.g. 100oC) and removing the ions can diminish this concern. In addition, magnesium is necessary for **nuclease** activation, and binding these ions inactivates the enzyme.

Read more later about PCR in the Quantitation module of this course.

The extraction process involves boiling a sample in a 5% suspension of deionized water and Chelex®100. The alkalinity of the suspension and the boiling process disrupts the cell membranes, destroys cell proteins, and denatures the DNA. The suspension is then centrifuged, separating the resin and cellular debris from the supernatant containing the denatured DNA. The DNA can then be amplified.05

Advantages and Disadvantages

Extraction methods are evaluated based on their yield and the quality of results. Early studies using HLA DQ-alpha compared Chelex® 100 with traditional organic extraction methods, and demonstrated the method's suitability for forensic samples.01, 02, 04 An advantage of this method is that there are fewer tube manipulations required when compared to traditional organic extraction methods, reducing contamination risks. Automated extraction procedures will diminish this concern even further.

Read more about Other Extraction Techniques in this PDF file.

Chelex® 100 extraction methods are performed in a harsh environment that may not be suitable for highly degraded or low-level DNA samples.07 The alkaline environment and high temperatures could further degrade the DNA, which may result in an inability to obtain results.

One study assessed DNA samples that were isolated with Chelex ® 100, stored long-term (approximately 1 year), and subjected to several freeze-thaw cycles. When these samples were characterized using STR amplification, they showed decreased signal, peak imbalance, and allelic drop out. The authors speculated that

the unbuffered suspension of the DNA, coupled with multiple freeze-thaw cycles, could accelerate the degradation process of isolated DNA samples.08

DNA isolated using the Chelex® 100 extraction is denatured. This was problematic when laboratories used restriction fragment length polymorphism (RFLP) for characterization since double-stranded DNA is required (RFLP). This is no longer a concern with current STR methods.

The Chelex® 100 procedure does not include a purification step. If the sample contains inhibitors and contaminants, increasing the sample size increases their concentration, which can inhibit PCR. Some laboratories have incorporated the use of a filter unit such as a Microcon® or Centricon® prior to amplification so that some PCR inhibitors can be removed and the sample can be concentrated. Because the resin is a PCR inhibitor, it is important to ensure that when removing the supernatant, no resin is removed from the tube.09

Read more about DNA Analysis Consideration later in this PDF file.

Chelex®100 Resin Extraction Method

Advantages and Disadvantages

Advantages	Disadvantages
Fast	Harsh extraction environment (pH between 10-11 and temperature approximately 100° C)
Simple	Potential degradation concerns for long-term storage of isolated DNA samples
Inexpensive	Resin remaining in extracted DNA sample can inhibit PCR process
No hazardous chemicals	Less effective extraction of some sample types
Few tube manipulations	Isolated DNA is single-stranded
Binds heavy metal cations	Resin loses its chelating capacity after a few hours in suspension
Removes some PCR inhibitors	

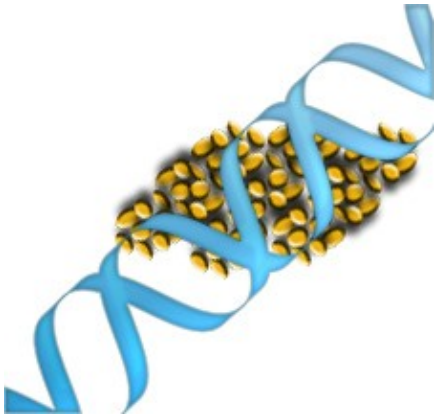
Non-differential

Chelex® 100 extraction methods are straightforward, with little variation between procedures. Some examples are:

- Laboratories may differ with respect to their inclusion of Proteinase K, a serine protease that aids in protein digestion.
- For extraction from blood, the sample may be incubated in sterile deionized water that promotes hemolysis of red blood cells. This aids in the removal of heme, a known PCR inhibitor.

- Some laboratories wash the sample in phosphate buffered saline prior to extraction. The sample may be washed one or more times and pelleted through centrifugation before resuspending it to carry out the Chelex® 100 extraction procedure.

Differential



Generally, sexual assault evidence is a mixture of epithelial cells and semen. The QAS Standard 9.1.3 (FO) requires the laboratory to have a procedure for the differential extraction of stains that contain semen. Many laboratories have adopted a Chelex differential extraction procedure for these sample types. 09

Proteinase K, mentioned in the non-differential procedure, lyses epithelial and white blood cells but not sperm cells under the extraction conditions. It is particularly useful in organic extraction procedures as it maintains activity in the presence of denaturing agents such as sodium dodecyl sulfate (SDS), a component of extraction or digest buffers. Similarly, Proteinase K is not inactivated by metal chelating agents.

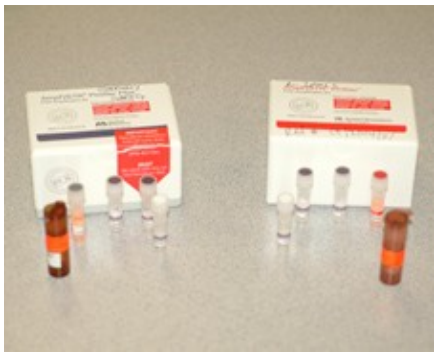
Read more about Organic Extraction later in this PDF file.

After extracting the sample in digest buffer and Proteinase K, the sample is centrifuged. The supernatant contains DNA from the lysed epithelial and white blood cells. The supernatant containing the cell lysate is subjected to extraction using 5% Chelex® 100. This fraction is treated as a single sample and referred to as the F1 fraction, the epithelial cell fraction, or the female fraction, depending upon laboratory procedures

Sperm DNA is wound around proteins called protamines, which are analogous to histones. Protamines contain a high concentration of cysteine residues and disulfide bonds, making the sperm head and its DNA more resistant to the effects of Proteinase K. The pellet from the original extraction contains the sperm components from the sample and is subjected to a second incubation in buffer containing Proteinase K and dithiothreitol (DTT). This fraction is treated as a second sample referred to as the F2, sperm fraction, or male fraction. DTT prevents oxidation of thiol (SH) groups and reduces disulfides to dithiols.

Other Extraction Techniques

Introduction



This module focuses on the modifications to common extraction methods as well as commercially available extraction systems. While several techniques are presented, this module does not cover all available methods represented in forensic DNA laboratories.

Objectives

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

- List several commercially available extraction kits and accessories
- Identify the advantages and disadvantages in the use of various types of proprietary reagents and materials
- Determine the appropriate application of various techniques to different sample types and substrates
- Describe the use of commercially obtained materials in automated systems

Many extraction procedures incorporate the use of a microcentrifuge tube with a spin basket. Costar Spin-X® centrifuge tube filters are one example. These centrifuge tube inserts contain no membrane, but have a bonded fritted bottom on the 500µL insert that fits into the microcentrifuge tube.1,2

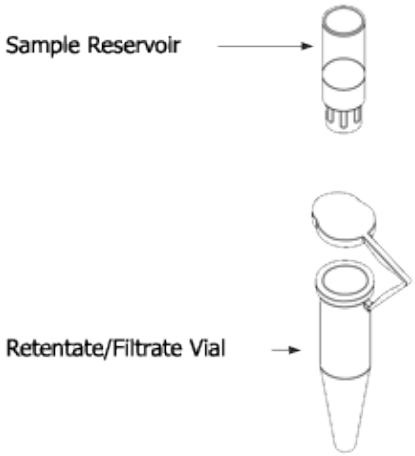
This allows for the separation of liquid carrying the biological material from the substrate during centrifugation steps. Another modification is the use of centrifugal filter units. These allow for extracted DNA to be purified and concentrated after extraction and prior to amplification. Millipore Microcons® are one example of centrifugal filter units.2 Both of these modifications are routinely used in DNA extraction procedures.

Microcentrifuge Tubes with Spin Baskets

Constructed with an open end and a woven end, spin baskets hold a cutting of substrate and are inserted into a common microcentrifuge tube. This allows for the retention of liquid carrying the biological material to be separated from the substrate during centrifugation steps. Any cells that may not be completely freed from the substrate are forced into the tube, along with the excess fluid. Spin baskets may also be used in organic extractions, although their composition prohibits immersion in phenol/chloroform/isoamyl alcohol (PCI).

For example, Promega offers the Differex™ System, which includes a proprietary separation solution and the use of a spin basket, increasing the efficiency of the traditional method of differential extraction from sperm versus non-sperm cells. The procedure begins with Proteinase K digestion to lyse non-sperm cells. The sample and buffer are placed into a spin basket within the tube containing the proprietary solution. This solution is not miscible with the aqueous buffer. Through centrifugation, the sperm cells form pellets, while the non-sperm DNA remains in solution. The difference between this system and routine differential extraction is that the separation solution acts as a selective membrane (without the risk of clogging), which is purported to reduce the chance of sperm loss. After the removal of the solution containing non-sperm DNA, the sperm DNA is isolated using the DN™ System, described later in this module.

Centrifugal Filter Units



Centrifugal filter units are used to purify and concentrate DNA. When extracting DNA from small or degraded forensic samples, the final concentration of DNA may be too low for subsequent amplification. Although 1ng is a target quantity of DNA for amplification, if 1ng is suspended in 100µl of fluid, it would be impossible to transfer 100µl of this solution into an amplification reaction optimized for 50µl or less. Centrifugal filter units increase the concentration of DNA in solution by retaining the DNA while eliminating a portion of the fluid from the sample. Another benefit of the unit is the ability to secure DNA while contaminants (possibly PCR inhibitors) are washed from the sample.

Centrifugal filter units separate molecules by size through a series of washing and centrifugation steps. The Millipore Corporation produces two centrifugal filter units under the names Centricon® and Microcon®.

Visit The Millipore Corporation website.

Attributes of Centricon® and Microcon® filter units:

- Both employ Amicon® filters to retain the DNA.
- Filter porosity varies.
- Proteins flow through rather than sticking to the surface during centrifugation.
- Filters are anisotrophic, with increasingly smaller interstitial spaces in the direction of filtration allowing for better retention of smaller molecules.

- Filters are composed of regenerated cellulose, which can be sterilized.
- Filters exhibit sufficient strength when wet.

Note:

It is important not to spin the unit at rates higher than recommended by the manufacturer because the unit may become compromised, resulting in reduced sample recovery.

Textile dye molecules, such as indigo, are known PCR inhibitors and are readily washed through the filter. Salts introduced by buffers are removed from the sample during the process. Salts carried over from the process may interfere with capillary electrophoresis.

Read more about capillary electrophoresis in the Amplified DNA Product Separation PDF file.

Note:

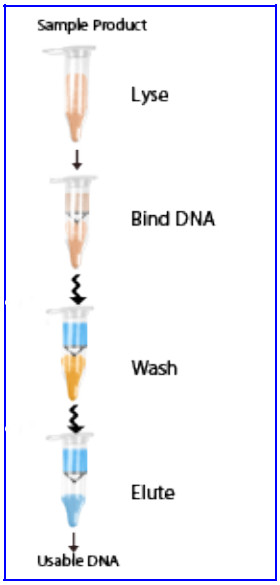
Care must be taken to avoid introducing phenol from the organic extraction into the unit because it will break down both the cellulose filter and its supporting silicone rubber o-ring. Chelex® beads carried over from extraction may clog or damage the filter.

Other Techniques

The Chelex® and organic extraction methods have been mainstays in the forensic science community, but are not without their limitations. The community continues to assess other extraction techniques that are automatable, safe, and demonstrate high performance. Automated extraction methods are rapidly replacing Chelex® and organic extraction techniques in forensic laboratories.

Read more about Organic Extraction earlier in this PDF file.

Spin Column



The Qiagen QIAamp® DNA Micro Kit, a spin column using a silica-based extraction method, is used in forensic DNA laboratories. This kit does not require the use of hazardous chemicals. Nucleic acids are attracted to the silica bead under high chaotropic salt concentrations. The sample and lysis buffer (provided in the kit) are added to a sterile tube. The lysate is combined with alcohol and placed into the spin column, which is inserted into a tube. The removal of proteins and divalent cations is accomplished using multiple buffer washes and centrifugation steps. Removal of cations, such as Mg^{2+}, prevents nucleases from further

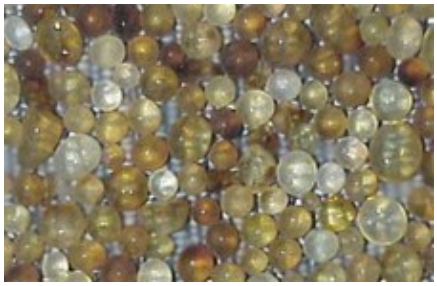
degrading the DNA. Pure DNA is eluted from the membrane into sterile water or TE buffer.

Qiagen produces several types of kits under the QIAamp® name, including kits specific to the extraction of whole liquid blood, feces, hair, dried stains, and swabs. The primary difference in these kits is the type of wash buffers provided.[3,4,5]

There are other proprietary spin columns that use glass fiber, silica, and paramagnetic particles. These include, but are not limited to, the following:

- Amersham Biosciences (part of GE Healthcare) produces kits, such as the GFX™ Genomic Blood DNA Purification Kit, that rely upon a glass fiber matrix packed into a spin column.
- UBI Life Sciences markets the Omega Bio-Tek E.Z.N.A.® Forensic DNA and E.Z.N.A.® Mag-Bind® Forensic DNA Kits.
    - ♦ The E.Z.N.A.® Forensic DNA Isolation Kits use a silica-based material, combined mini-column spin technology — HiBind®.
    - ♦ The E.Z.N.A.® Mag-Bind® Forensic DNA Kit uses paramagnetic particles, Mag-Bind® particles for DNA isolation.

Silica Beads



Silica bead extractions (another solid phase extraction method) have gained popularity in research and clinical arenas; however, it is the combination of magnetic technologies and silica coatings that are being implemented in forensic laboratories.

Kits and materials for DNA extraction involving the use of silica beads are available from many companies. Similar to the Chelex® procedure, these beads are suspended in an aqueous medium and mixed with samples. Using this procedure, proteins and contaminants, which would otherwise interfere with the isolation of purified DNA, are removed. DNA binds to silica in the presence of high salt concentrations.

MO BIO offers a selection of kits under the name UltraClean ™, which are designed to extract and purify DNA samples from various sources such as blood, tissue, fecal matter, and soil.

Visit the UltraClean™ page on MO BIO's website.

The following are the extraction steps:

1. Sample and buffer are added to a tube (provided in the kit) containing a bead solution.

2. A typical extraction buffer containing sodium dodecyl sulfate (SDS) is added, followed by another solution designed to precipitate out the humic acid components and other PCR inhibitors.
3. The tubes are vortexed extensively. Chemical lysis, using SDS, breaks down cell membranes and proteins, while mechanical lysis occurs as the beads physically beat up the cells.
4. Centrifugation pellets the debris while the DNA remains in the aqueous supernatant.
5. A DNA binding salt solution, which promotes binding of the DNA to the silica beads, is added.
6. The solution of beads and DNA (in aqueous buffer) is added to a spin device. The DNA remains bound and is impeded by a membrane in the spin filter. Other molecules and contaminants pass through.
7. The DNA can be washed with an ethanol solution for further purification.
8. An elution buffer is added to remove the salt, thus allowing the DNA to be washed through the membrane while the silica beads are retained. The resulting filtrate is purified DNA in an aqueous buffer, suitable for quantitation.

Note:
The kits for extracting DNA from soil and feces contain reagents that reportedly break down humic acids.
Read more about DNA Analysis Considerations elsewhere in this PDF file.

Magnetic Beads

The use of magnetic beads is another solid phase extraction method.

These methods:

- Can be automated
- Increase throughput
- Demonstrate the ability to remove amplification inhibitors
- Use no hazardous chemicals.

These kits can also be used as adjuncts to traditional methods, when additional clean-up is warranted.

The principle underlying magnetic bead procedures involves attracting DNA to magnetic beads, holding the beads in place using a magnetized source, such as a rack or tube holder, and washing away other components of the sample.

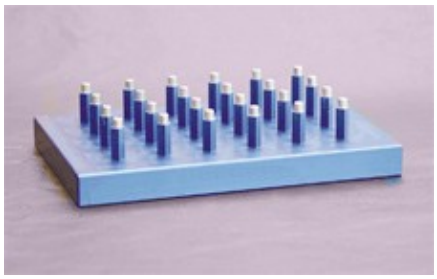
Invitrogen's ChargeSwitch® Technology (CST®) involves the use of magnetic beads whose surface bears a charge that can be switched based on the pH of the surrounding buffer environment. At low pH, the beads are positively charged, attracting the negatively charged DNA molecules and allowing proteins and contaminants to be removed by washing.

The ChargeSwitch® Forensic DNA Purification Kit includes:

- Lysis buffer
- Magnetic beads
- Proteinase K
- Purification buffer
- Wash buffer
- Elution buffer

View an animation on magnetic beads.

The DNA IQ™ System by Promega uses silica-coated magnetic beads. DN™ was designed for automation on robotic systems. The quantity of beads used in the procedure defines the binding capacity, which is approximately 100ng of DNA. This relative quantitative capability makes it ideal for databasing and paternity applications because consistent quantities of DNA are isolated.



The DNA IQ™ kit contains the proprietary resin and several specialized buffers, including lysis, wash, and elution. The method demonstrates efficient DNA extraction from small samples and the removal of inhibiting compounds. For example, internal validation studies of DN™ were successful in the extraction of DNA from small stains deposited on difficult substrates, such as denim, leather, and soil.

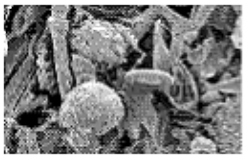
Promega also offers a special buffer for the extraction of DNA from bone and a procedure for differential extraction.

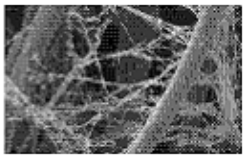
FTA® Technology

FTA® is an acronym for fast technology for analysis of nucleic acids. It was originally developed by Burgoyne and Fowler at Flinders University in Australia in the 1980s as a means of protecting nucleic acid samples from degradation by nucleases and other processes. The concept was to apply a weak base, chelating agent, anionic surfactant or detergent, and uric acid (or a urate salt) to a cellulose based matrix (filter paper). A sample containing DNA could then be applied to the treated filter paper for preservation and long-term storage.

Whatman® licenses the FTA® technology from Flinders University. They offer a line of products using this technology, most notably filter paper cards.

Read more about filter paper cards on Whatman's website.





Biological samples, such as blood and saliva, adhere to the paper through the mechanism of entanglement, while the mixture of chemicals lyses cells and denatures proteins. Because nucleases are inactivated, the DNA is essentially stable when the sample is properly dried and stored. Nucleic acid damage from nucleases, oxidation, ultraviolet light (UV) damage, microbes, and fungus is reduced when samples are stored on the

FTA card.09,10

A marketable advantage of the FTA® technology is that samples spotted on treated cards may be stored at room temperature. The chemicals on the FTA cards enhance the preservation of the DNA and inactivate many dangerous pathogens that may be found in liquid blood samples or dried biological stains. Because the cards are small in size (approximately 3.5" x 5"), they are easily packaged, shipped, and stored for databasing.

Quantitation

Introduction

Quantitation refers to determining the quantity or concentration of extracted DNA in a biological sample. It is required by the quality assurance standards (QAS) as a step in the analysis of evidence samples.

Objectives

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

- List the reasons quantitation is necessary
- List the advantages and disadvantages of the principal DNA quantitation methods
- Understand the basic underlying chemistry involved in several quantitation methods including slot blots, luminescent reactions, and quantitative PCR
- Discuss proprietary kits available for DNA quantitation commonly used in forensic laboratories
- Perform calculations and dilutions to target specific amounts of DNA suitable for PCR

Overview

Current forensic DNA analysis uses polymerase chain reaction (PCR) based short tandem repeat (STR) testing. Many laboratories use commercially available STR amplification kits. Depending on the kit and reaction volume, the optimal concentration of input DNA will be in the range of 0.5ng – 2ng. Adding too much or too little DNA to the amplification reaction can result in problems in the analysis.

Read more about PCR in the Crime Scene and DNA Basics for Forensic Analysts PDF file.

In addition to the need for amplification optimization, quantitation of samples is a requirement of the QAS. Standards 9.3 and 9.4 require forensic laboratories to use a quantitation method that is specific to human DNA.

The quantitation process also serves as a quality control and/or troubleshooting procedure. If the STR results are not concordant with those from the quantitation, it may be an indication of a problem, such as inhibition, sample switching, or contamination.

Methods

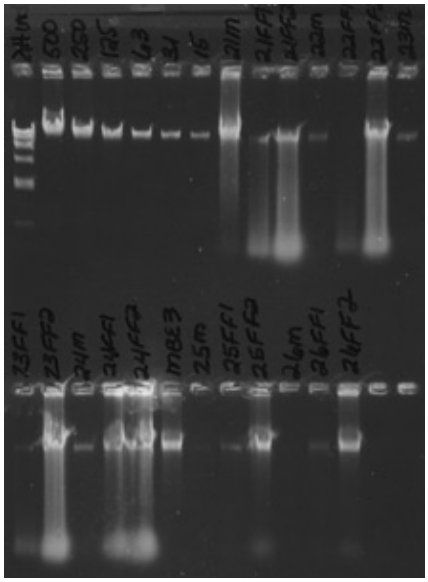
Commonly used quantitation methods include the following:

- Yield gels
- Spectrophotometry
- Fluorometry
- Slot blot hybridization
- AluQuant®

- Quantitative PCR (qPCR)

AluQuant® and Quantitative PCR are the most recent techniques and are becoming more widely used.

Yield Gels



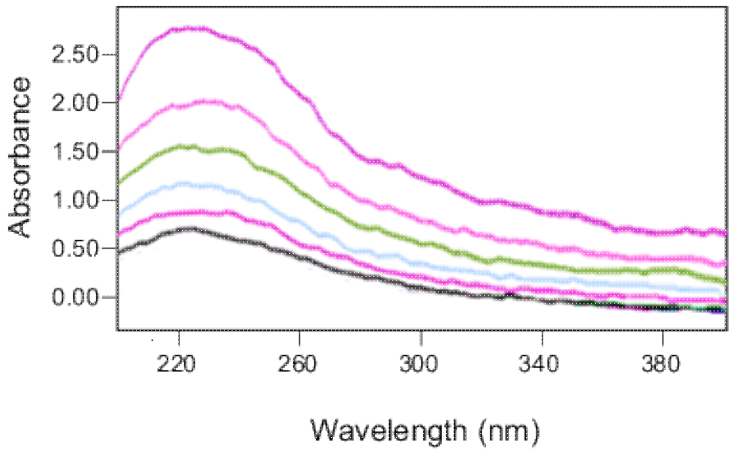
The yield gel technique is a semi-quantitative/qualitative assay that allows for the estimation of the concentration and quality of DNA in a specimen. The method consists of the electrophoresis of DNA in an agarose gel matrix incorporating a fluorescent intercalating dye such as ethidium bromide (EtBr). The concentration of a sample can be determined by comparing the intensity of the fluorescence of the sample to that of the calibration standards.

One of the benefits of yield gels is the ability to assess the quality of the DNA sample. Larger (undegraded) DNA fragments migrate at a slower rate than those of lower molecular weight. Degraded DNA will consist of lower molecular weight fragments.

Safety Note:

Ethidium bromide (EtBr) is a known carcinogen.

Read more about capillary electrophoresis in the Amplified DNA Product Separation PDF file.



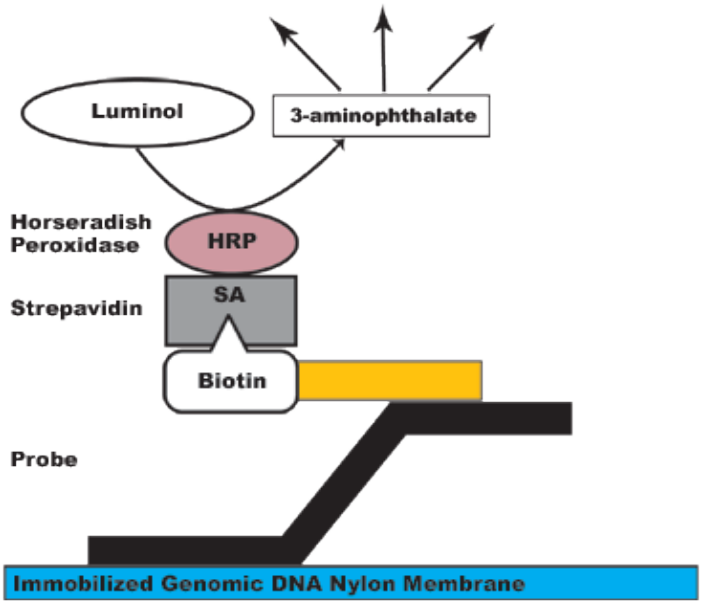
Absorption spectrophotometry is a widely used technique in analytical chemistry based on the property of molecules to absorb light at specific wavelengths. The optical density (O.D.) of a solution containing 50µg/µl of double-stranded DNA or 40 µg/µl of single-stranded DNA is 1.00 at a wavelength of 260nm.04 The quality or purity of the sample can be determined by comparing the measurements at 260 and at 280 nm (the wavelengths for which DNA and protein absorb).

Fluorometry is another spectrophotometric technique based on the absorption of light by molecules. In this case, however, the molecules lose some of the absorbed energy by irradiating light of a longer wavelength. This property is known as fluorescence and can be the basis for sensitive quantitative assays.02

There are several dyes that show significant fluorescence enhancement when bound to double-stranded DNA, providing a quantitative assay for the DNA. PicoGreen® is one of the more common fluorescent dyes that can be used in this way. The fluorescence is easily measured using a fluorometer.03

Slot blot hybridization was the most commonly used method until recently. The majority of laboratories used the commercially available **QuantiBlot®** kit, which employs the following procedures:

- Extracted DNA is denatured and the single-stranded DNA is bound to a positively charged nylon membrane.
- After the DNA is bound to the membrane, a probe complementary to the D17Z1 locus (present in high quantities in higher primates) is applied and allowed to hybridize to the DNA.
- The hybridized complex is detected by one of several methods.04
- The amount of DNA in the sample is estimated by comparison of the density of the band or bands observed to that of the standards.

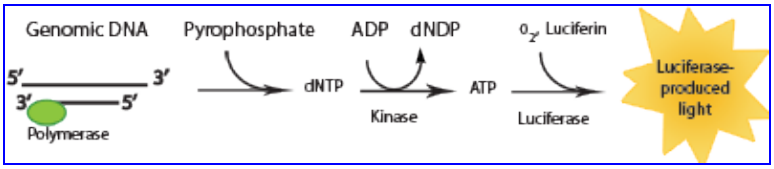


Detection methods include:

- Colorimetry, usually employing tetramethylbenzidine (TMB), which yields a blue color when oxidized by hydrogen peroxide.
- Chemiluminescence, for example ACES™ (Gibco BRL) and™ (Amersham Biosciences). The chemiluminescent reactions cause the release of photons that are captured on film or a digital imaging device. Chemiluminescence is more sensitive than colorimetry and can detect down to 10 to 20 pg of DNA.05

AluQuant™

Alu sequences are abundant in the human genome numbering approximately 500,000 to 1,000,000 copies per genome. The **AluQuant**™ Human DNA Quantitation System probes Alu repeats. It is performed by denaturing the sample and incubating it with the AluQuant™ Enzyme and AluQ™ Probe solutions that are furnished with the kit. The method employs a series of reactions that ultimately result in the production of adenosine triphosphate (ATP), which correlates with the amount of DNA present. The amount of ATP is determined by using a luminometer.06



Read more about the AluQuant™ reaction at Promega.com

Quantitative PCR

Quantitative PCR (qPCR), sometimes refered to as real time PCR, is the most accurate, precise, and efficient method currently available for human DNA quantitation. It is an amplification process that detects and

measures the accumulation of fluorescent dyes as the reaction progresses.

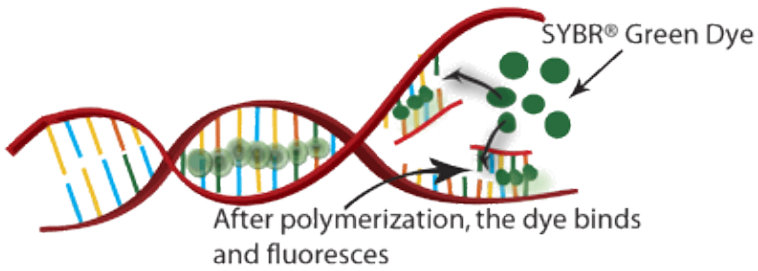
The initial quantity of DNA in the sample is detected by monitoring the exponential growth phase of the reaction and measuring the cycle number at which the fluorescent intensity of the sample overcomes the background noise or threshold. This cycle number is directly proportional to the quantity of DNA in the reaction. Analysis of the quantity of DNA in the sample is performed using software that compares the unknowns with the best fit regression line constructed from the standards.

The use of Quantitative PCR is discussed by reference to two methods:

- SYBR® Green detection
- Fluorescent resonance energy transfer (FRET)

Note:
Quantitative PCR instruments include: Applied Biosystems (AB) 7000 and AB 7500, Corbett Rotor-Gene™, Stratagene® Mx 4000®, Cepheid® SmartCycler®, and Roche LightCycler®).07

SYBR® Green Detection Method



The Quantitative PCR instrument consists of a thermal cycler housed together with a digital fluorometer detector. The principle behind the method is that as the PCR amplification process progresses, there is an increase in fluorescence from SYBR® Green dye. As the SYBR® Green dye binds to double-stranded amplicons, it undergoes a conformational change and emits fluorescence at a greater intensity.07 For forensic DNA analysis the most common SYBR® Green method is Alu-based, which targets human specific Alu sequences.08

Fluorescent Resonance Energy Transfer (FRET)

Methods of fluorescent resonance energy transfer (FRET) technology involve a single probe where two dyes (the quencher and the reporter) are in close proximity. Due to this close proximity, an energy transfer occurs between the two dyes, suppressing the fluorescence of the reporter dye.09

Read more about FRET at Wikipedia.com

Methods include:

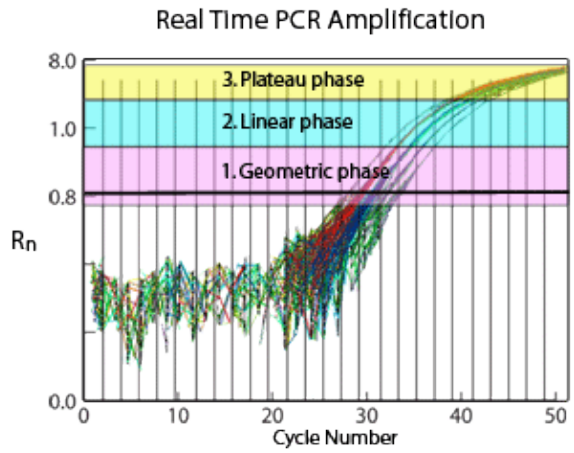
- TaqMan® kit from Applied Biosystems uses a probe that hybridizes to the complementary target on the DNA strand. Polymerization cleaves the probe, releasing the reporter dye and resulting in fluorescence.10

View an animation about TaqMan kits.

- Molecular beacons are probes that form a hairpin loop with attached fluorescent reporter and quencher dyes. The fluorescent dye is suppressed while the probe is in this confirmation, due to the proximity of the dyes. Upon heating the probe (during the denaturation cycle of the PCR process) the hairpin structure is disassociated. This allows the probe (beacon) to bind to the PCR product in the subsequent annealing cycle. Binding of the beacon inactivates the quencher and releases fluorescence into the reaction.11

View an animation about molecular beacons.

Data Analysis for Quantitative PCR



Quantitative PCR monitors the increase in fluorescent signal throughout the PCR cycling process. The change in fluorescence is monitored between samples and standards so that a comparison can be made.12

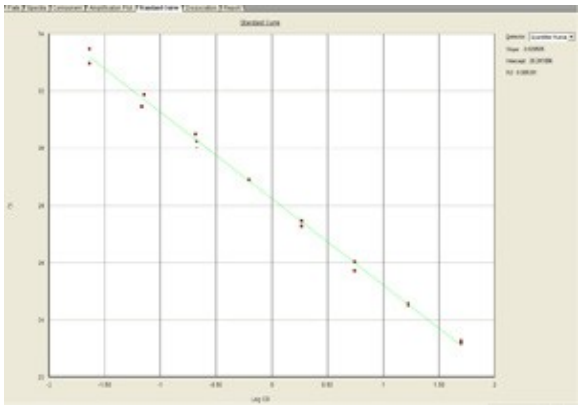
The amplification process can be summarized in three consecutive phases:

1. Exponential/ Geometric amplification
2. Linear amplification
3. Plateau region

Read more about the PCR process in the Crime Scene and DNA Basics for Forensic Analysts PDF file.

During the exponential phase of the PCR process, the reaction results in a theoretical doubling of amplicons with each cycle. While the ideal efficiency is not achieved, the doubling is close to 100%, yielding a consistent relationship of input DNA to product.13

At the beginning of the exponential phase, the baseline is determined by measuring the background fluorescence signal (noise). This baseline establishes the threshold. During the amplification process of a sample, the point (in terms of amplification cycles) in which the level of fluorescence exceeds the threshold is referred to as the Cycle Threshold (CT) . The CT value is lower for a sample with a higher initial concentration and is higher for a lower-concentration sample.



The standard curve is a regression line that is derived from the CT values of the standards plotted against the log of the concentration of the standards. The concentration of each sample is determined by comparing its CT value against the standard curve.

The consumption of one or more PCR reagents during the amplification process will impede the efficiency, causing the onset of the linear phase. Product amplification slows down yielding an inconsistent ratio of input DNA to product. Therefore, the linear phase is not commonly used in the data analysis process.

The final amplification phase signifies the depletion of critical reagents and is known as the plateau region. During this phase, the amplification process ceases.14

Quantitation Methods and Kits

There are several quantitative PCR methods currently is use. Quantitation kits are available through commercial sources or may be developed in-house. The National Institute of Standards and Technology (NIST) has developed a new standard that can be used as a quality control measure to assess the accuracy of quantitation results.

Learn more about SRM 2372-Human DNA Quantitation Standard from the NIST STRBase Web site.

Summary of Method Advantages and Disadvantages

Summary of Method Advantages and Disadvantages

Method	Advantages	Disadvantages
Yield Gel	• Quality of DNA can be assessed (level of degradation)	• Carcinogenic chemical (EtBr) • Not as sensitive as other methods • Uses intercalating dye requiring double-stranded DNA • Not human DNA specific • Not automatable
Absorption Spectrophotometry	• Rapid process • Quality of DNA can be assessed (level of degradation) • Relatively inexpensive	• Not human DNA specific

	• Automatable	
Fluorometry		
	• Semi-selective for double-stranded DNA • Relatively sensitive for DNA detection • Automatable	• Not human DNA specific • Quality of DNA cannot be assessed (level of degradation)
Slot Blot Hybridization		
	• Human DNA specific • Easy to analyze	• Not automatable • More labor intensive than some other methods • Semi-quantitative • Quality of DNA cannot be assessed (level of degradation)
AluQuant™		
	• Sensitive • Automatable • Human DNA specific	• Quality of DNA cannot be assessed
SYBR® Green		
	• Sensitive • Indications of inhibition14 • Less expensive - no requirement for probes	• No commercial kit available • Binds to double stranded DNA • Must be placed in a dedicated room due to amplified product
Fluorescent resonance energy transfer (FRET)	• Rapid process • Higher precision and accuracy compared to other methods • Automatable • Human DNA specific • Indications of inhibition • Sensitive	• Must be placed in a dedicated room due to amplified product • No commercial kit currently available for all methods (molecular beacons)

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Author: Leigh Clark

Leigh Clark is a DNA Analyst with the Florida Department of Law Enforcement. Previously, Leigh was Academic Program Specialist at the National Forensic Science Technology Center, where she authored this module.

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Resources by Module

- DNA Analysis Considerations
- Organic Extraction
- Chelex 100 Extraction
- Other Extraction Techniques
- Quantitation

DNA Analysis Considerations

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Online Links

- What Every Law Enforcement Officer Should Know About DNA Evidence
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Organic Extraction

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Read the Sam Baechtel paper.

Online Links

- Differex™ System web page
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- Millipore Corporation Website
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  http://www.whatman.com/products/?pageID=7.31
- UltraClean™ page on MO BIO's website
  http://www.mobio.com/products/categorylist.php?cat_id=5

Quantitation

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Online Links

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- FRET at Wikipedia
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