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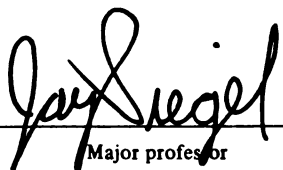
DNA TYPING OF TEN POSTMORTEM PSOAS MUSCLE SAMPLES
USING THE RESTRICTION FRAGMENT LENGTH POLYMORPHISM
TECHNIQUE AND CHEMILUMINESCENCE

presented by

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of the requirements for

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**DEOXYRIBOSE NUCLEIC ACID (DNA) TYPING OF TEN POSTMORTEM
PSOAS MUSCLE SAMPLES USING THE
RESTRICTION FRAGMENT LENGTH POLYMORPHISM
TECHNIQUE AND CHEMILUMINESCENCE**

By

Amelia Christy Harlukowicz

A THESIS

**Submitted to
Michigan State University
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1996

ABSTRACT

DNA TYPING OF TEN POSTMORTEM PSOAS MUSCLE SAMPLES USING THE RESTRICTION FRAGMENT LENGTH POLYMORPHISM TECHNIQUE AND CHEMILUMINESCENCE

by

Amelia Christy Harlukowicz

The task of identifying human remains at a crime scene, can be difficult due to the decomposition of biological tissues. Traditional means of identification, such as fingerprints, x-rays and dental records are useless if a body is badly decomposed. DNA evidence provides a strong statistical link between biological evidence and an individual from a crime scene. Unfortunately, the lack of sample and/or degradation of DNA produces poor or even no results using restriction fragment length polymorphism (RFLP) analysis. Several studies have evaluated the DNA recovered from various biological tissues. High molecular weight DNA has been successfully recovered from psoas muscle. The psoas muscle aids in flexing the thigh laterally. Muscle fibers carry out limited enzymatic reactions. Enzyme activity tends to shear DNA into small fragments and even shorten the DNA fragment lengths. Ten psoas muscle samples were evaluated using the Lifecodes DNA typing protocol. Chemiluminescent labeled probes reduce safety concerns and speed up result time. Nine of the ten samples tested provided useful DNA typing results within a 16 hour time period.

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To Elaine, Carrie, and Ann Marie Harlukowicz
“It’s a great life if you don’t weaken”

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INTRODUCTION

Often times the only evidence available at a crime or death scene is the presence of human remains. The identification of human remains using DNA testing offers many advantages and few disadvantages to law enforcement agencies and the legal system. Traditional genetic marker testing is not very successful in associating or disassociating suspects with a crime. DNA evidence provides a strong statistical link between biological evidence and an individual from a crime scene.

The primary function of forensic biology is to establish associations between biological samples and the individuals from which they originated (Waye et. al., 1993). Advances in deoxyribonucleic acid (DNA) technology over the past decade have given scientists the ability to better identify such individuals. DNA evidence has a very high probative value. Except for identical twins, the DNA of each individual is unique. More than 99% of the DNA genetic code is the same for all people. This 99% is what makes us human beings. Forensic scientists are interested in the remaining one percent which is responsible for the variations among individuals.

Forensic analysis of DNA is an extension of classical serology methods that have been performed for more than 50 years. Karl Landsteiner, in 1902, first suggested that ABO typing could be used to associate or exclude blood at a crime scene to a suspect. The ABO system was first used to determine family relationships. Later this technique was applied forensically. Conventional typing systems only provide limited statistical information to individualize crime scene samples. The ABO blood group system, for example, only discriminates between four types (A, AB, B and O). DNA typing provides the potential for greater statistical individualization. Some have claimed that the use of DNA profiling has been the biggest breakthrough in the forensic science community since fingerprinting was developed.

Although this paper focuses on forensic DNA analysis for identification purposes, this procedure has been successful in other forensic disciplines including medicine, paternity testing, animal and plant science and wildlife forensic science (Kirby, 1992). Forensic DNA typing is simply the application of DNA typing techniques from the medical research arena to matters concerning criminal justice and the forensic science community.

Forensic DNA analysis can assist in solving serial crimes, determine if more than one person was involved in a crime, place a suspect at a crime scene even if there are no eye witnesses, eliminate falsely accused individuals

and even confirm persons suspected in insurance fraud cases (Lifecodes, 1990).

The task of identifying human remains may prove to be difficult due to the decomposition of biological tissues. Traditional means of identification, such as fingerprints, x-rays and dental records are useless if a body is badly decomposed. In this instance, visual confirmation of a victim's identity is not possible.

A critical point in the development of useful DNA typing results is the condition of a sample's DNA. Contamination and degradation are two factors to consider. A problem discussed in the literature is that lack of sample and/or degradation of DNA produces poor or even no results using RFLP analysis. RFLP analysis requires a sufficient quantity (>50 ng) of relatively undegraded DNA. The FBI prefers at least 100 ng of relatively undegraded DNA for analysis (Adams, 1988).

Degradation can result from several factors including humidity, sunlight, bacteria and soil. The quality of results is dependent upon the degree of degradation. The more severe the degradation the lower the molecular weight of the DNA. Severely degraded DNA will produce no results. A valid DNA pattern can be identified if partially degraded DNA is of high enough quality to test. Inconclusive results are often the product of typing partially degraded DNA.

The purpose of this research is to determine the quality and quantity of DNA recovered from multiple postmortem psoas muscle samples. This will be accomplished using the RFLP technique and chemiluminescent development methods.

Due to the fact that high molecular weight DNA is a prerequisite to obtain successful DNA typing results, it is important to investigate the postmortem stability of DNA. Several articles have dealt with investigating the results of DNA analyzed from various biological tissues. A recurring problem in the literature (discussed in the next chapter) is the fact that not all tissues produce high-grade DNA results. Several studies have been performed to determine the stability of DNA in various human organ tissues. Muscle tissue, particularly psoas muscle, has proven successful in previous studies.

The psoas muscle aids in flexing and rotating the thigh laterally. The word psoa means muscle of loin (VanDeGraff, pg. 350). Skeletal muscle tissue is responsible for various body movements. This group of muscle contracts to carry out various body motions, produce heat, and provide good posture. Muscle fibers carry out very little enzymatic activity. Other biological tissue samples such as liver, carry out numerous enzymatic reactions. Enzyme activity tends to shear DNA into small fragments and even shorten the lengths of DNA fragments (Bar et. al., 1988). Therefore,

due to the fact that muscle tissue carries out only a limited number of enzymatic reactions, it should provide good DNA typing results.

DNA contains our genetic blueprint and is found in every nucleated cell in the body. The human genome is composed of 46 chromosomes. Twenty-three chromosomes are inherited from the father's sperm and 23 are inherited from the mother's egg. DNA has several features that make it useful for forensic purposes. One feature previously mentioned is the fact that every individual has unique DNA except identical twins. One's DNA is constant throughout their lifetime. In addition, DNA contained within an individual's blood, semen, saliva, bone and every other nucleated cell is the same.

Along with the numerous advantages of DNA evidence there are some limitations. For example, statistical data relayed to a jury can often be overwhelming and can overshadow other evidence. DNA evidence is not individual. Statistical evidence may show that only a limited number of individuals in the population share the same DNA pattern. However, DNA analysis cannot be used to individualize evidence left at a crime scene to exclusively one individual. DNA testing is very costly when analysis, legal fees and expert witness expenses are all considered. This may prove to be a disadvantage to some defendants. Finally, crimes are not solved solely from DNA evidence. Even though DNA evidence has become increasingly popular,

some types of physical evidence such as fingerprints, may be more probative (Genetic Witness, pg. 101).

There are two types of DNA analysis; restriction fragment length polymorphism (RFLP) and the polymerase chain reaction (PCR). The RFLP technique requires relatively high molecular weight DNA to produce useful results. When a sample is of insufficient quantity or is degraded, PCR would prove useful.

The PCR method is actually the amplification of small amounts of DNA to produce a large quantity of useful DNA. The DQ-alpha typing method, in conjunction with PCR, provides limited discrimination between samples. The RFLP method requires a much larger sample than the PCR method, but is a better tool for individualization. Thus choosing between the two methods depends on whether sensitivity or specificity is the ultimate goal.

Forensic DNA typing currently relies on variable regions of DNA called variable number tandem repeats (VNTRs) or minisatellites. Tandem repeats are small sections of repeated DNA sequences. These regions are repeated a variable number of times. VNTRs vary greatly in size amongst the population. A core sequence of bases can be present as a single copy and repeated anywhere from 2 to 100 times. Jeffreys and his colleagues in 1985 used a sequence found in the myoglobin gene to describe properties of VNTR sequences. Highly variable or polymorphic regions of DNA are analyzed

using the RFLP procedure. A DNA “fingerprint” obtained from this procedure can prove to be a powerful investigative tool.

History

A Swedish biologist, Johann (Friedrich) Miescher, discovered an unusual phosphorous containing substance in 1868. Miescher was studying the nuclei isolated from the white blood cells from wounded soldiers bandages. He named this material “nuclein.” “Nuclein” contained both acidic and basic components. The acidic portion was DNA and the basic portion was made of histones. Miescher found a similar “nuclein” substance in a study he performed on salmon spermatozoa. This soon became the first realization the DNA was a universal substance (Kobilinsky, 1993).

DNA is a nucleic acid made up of subunits known as nucleotides. The chemical make up of DNA is phosphoric acid, deoxyribose (sugar) and four nitrogenous bases. The adenine (A) and guanine (G) bases are purines, cytosine (C) and thymine (T) are the pyrimidine bases.

Erwin Chargoff discovered in 1947 that the purine to pyrimidine ratio $(A+G)/(C+T)$ in DNA was always equal to 1.0. Each pair of bases is bound by a hydrogen bond and is referred to as a base pair (bp). In 1950 Chargoff and his colleagues discovered a critical point in determining the DNA structure. Their findings were that for every one mole of adenine there is one mole of thymine and for every one mole of cytosine there is one mole of guanine (Waye et. al., 1993).

In 1953, James Watson and Francis H. C. Crick presented a paper in Nature revealing the structure of the DNA molecule. Their model depicted the DNA molecule as a right-handed double helix. The double helix is formed by a phosphate sugar backbone. Along this backbone the four different nitrogenous bases are attached. This model somewhat resembles a ladder and its rungs. The bases can form any number of sequences within a single strand of DNA. The two chains of the DNA double helix run in opposite directions. If one chain runs from the 5' to the 3' end then the complementary chain containing the complementary base sequence will run 3' to 5'. For example, if one DNA strand was 5'-A-C-T-A-G-T-C-3' then the complementary chain would be 3'-T-G-A-T-C-A-G-5'. The 5' and 3' numbers indicate the numbering across the carbon atoms of the sugar molecules. Many of the techniques used to analyze DNA are based on the complementary base-pairing of the DNA molecule.

DNA analysis has a history that dates back before its use to identify crime scene samples. David Botstein and his colleagues were the first to use the RFLP procedure in 1980 to begin to construct a human gene map. There is no difference in the procedures used for clinical DNA analysis and forensic application.

The RFLP procedure was first applied forensically by Jeffreys in 1985. Jeffrey's procedure used a variable number tandem repeat (VNTR) probe That detected multiple VNTR loci simultaneously. This multilocus technique

produces results that are very complex. Although this is a good discriminating technique, it is oftentimes difficult to analyze mixed samples. Mixed samples contain DNA from more than one individual and are often encountered in forensic cases. Another potential problem is the fact that multilocus analysis is not a good technique to use on degraded DNA or limited amounts of sample (Waye et. al., 1993). A vast majority of laboratories in the USA and Europe use single locus probes. Single locus probes have proven to be more sensitive and their DNA profiles are easier to interpret (Balazs, 1992).

The United Kingdom, in 1985, was the first country to use DNA typing for forensic purposes. In the United States, DNA testing was introduced to private and state laboratories in late 1986. The Federal Bureau of Investigation (FBI) began DNA casework in 1988. Today, virtually all state and federal courts accept DNA evidence. The few instances in which DNA was inadmissible were due to disputes over statistical matters and not due to the validity of the DNA testing techniques themselves. The majority of the arguments concerning DNA testing laboratories are how certain testing is performed (Ohio, 1994).

Laboratory standards and guidelines have been developed by the Technical Working Group on DNA Analysis Methods (TWGDAM). TWGDAM meets several times a year and is made up of scientists from the industry, forensic science and academic fields. TWGDAM has published guidelines to

assure quality assurance among DNA laboratories. The admissibility of DNA test results into court often depend upon whether TWGDAM guidelines were followed.

The National Research Council (NRC) issued a report in 1992 detailing how various parties in the legal system could be affected by DNA evidence. Their major conclusions are as follows. The main purpose of any scientific analysis is to provide as much information as possible on which guilt or innocence can be determined by a jury. As with any expert testimony, the DNA expert must keep matters as simple as possible when addressing a jury. It is important that the jury does not become overwhelmed with statistics, scientific terms and techniques. Therefore, it is suggested that the DNA experts use charts and reports to thoroughly educate the jury about DNA typing evidence.

Prosecutors have the responsibility of making defense counsel and experts aware of all evidence necessary to perform DNA tests. The NRC also suggests that any materials such as the experts' data sheets and other materials be readily available for use in court to ensure thorough evaluation of all scientific evidence.

The prosecutor also has the responsibility of informing defense counsel early on that DNA testing is involved. This allows defense counsel to acquire any necessary expert assistance. A portion of the DNA sample

should be preserved for repeat testing whenever possible. This allows the defense to perform its own analysis of the evidence.

LITERATURE REVIEW

In 1985, Jeffreys, Wilson and Thein discovered that the human genome contains polymorphic “minisatellite” regions. These regions have a 10-15 base pair (bp) ‘core’ sequence. Probes were designed that detected multiple “minisatellite” regions simultaneously. Their research would later prove to be the beginning to DNA “fingerprinting” as it is known today.

As a follow-up to their previous study, Jeffreys, Wilson and Thein (1985) looked at the DNA fingerprints of twenty unrelated British Caucasians. They were successful in their attempt to illustrate the DNA patterns were highly specific to each individual.

Then in October 1985, Gill, Jeffreys and Werrett published a study that showed that DNA fingerprinting could be used for forensic purposes. This was the first major article to deal with the application of this technique to biological samples in forensic science. An attempt was made to show that DNA fingerprinting could prevail over conventional serologic methods used to analyze various biological samples such as blood and semen.

In 1986, Kanter, Baird, Shaler and Balazs analyzed RFLPs from DNA recovered from dried bloodstains. This study showed that high molecular weight (HMW) DNA could be recovered from dried bloodstains as old as three

years. Blood stains that enter a laboratory may be dried, aged or contaminated to the point where results obtained from traditional genetic typing may be unsuccessful.

A similar study was performed by Guisti, Baird, Pasquali, Balazs and Glassberg (1986). This study focused on DNA recovered from sperm. The authors presented evidence that DNA could successfully be extracted from spermatozoa to perform RFLP analysis. The RFLP patterns of both a subject's sperm and blood DNA were identical. The results of this study demonstrated the reproducibility of RFLP analysis and the possibility of excluding or including assailants in rape cases.

Gill, Lygo, Fowler and Werrett (1987) evaluated DNA fingerprinting for forensic purposes. The results of this study are fourfold: 1) HMW DNA can be isolated from blood and semen stains up to four years old. 2) The positive identification of rapists is possible. Sperm nuclei and vaginal debris are first separated from mixed stains. The two different fractions can then be analyzed separately. 3) Vaginal DNA can be isolated from penile swabs and then analyzed. 4) DNA can successfully be obtained from buccal swabs.

In 1987, Baird, Giusti, Meade, Glyne, Shaler, Bemm, Glassberg, and Balazs of Lifecodes Corporation applied DNA typing to forensic biological materials. RFLP analysis was performed on 170 forensic samples. Samples included kidney, liver, muscle, brain, blood and semen. They also reported the findings of some forensic cases. One case in particular involved a

homicide victim. An abandoned car was found with what appeared to be brain fragments on the front grill. The car's owner was missing and no body was recovered. The DNA recovered from the victim's parents' blood was consistent with having the same pattern as the victim's DNA. Statistical analysis showed that the DNA isolated from the brain tissue was 160,000 times more likely to have come from the offspring of the two parents than from any other individual.

Swarner, Reynolds and Sensabaugh (1989) evaluated the quantity of DNA from postmortem heart, spleen, liver, muscle, blood, hair and bone. The purpose of their research was to determine which postmortem tissue produced the best quantity and quality of DNA. The goal of this study was to determine if one type of tissue would be the best source of HMW DNA. However, all the tissues yielded DNA. No one tissue proved to be a reproducible source of HMW DNA. Another finding of this research was that no correlation could be drawn between the time of death and the quality of DNA results from each sample. They suggested that a collection of several tissues would be useful to ensure successful DNA typing results.

Bar, Kratzer, Machler and Schmid (1988) published an article on the postmortem stability of DNA. The major focus of DNA typing in this study was identification purposes. They concluded that, "the amount of degraded DNA correlated directly to the duration of the postmortem period." (Bar et. al. page 59). Due to the fact that relatively HMW DNA is required to obtain

reliable RFLP results they studied postmortem stability of DNA under various conditions. Changes of banding patterns at various postmortem intervals were studied using tissue specimens of brain, lymph nodes, liver, spleen, psoas muscle, kidney, thyroid gland and blood. They stated, "the discovery of DNA polymorphism's represents a novel and powerful means of identification of bodies by reconstructing familial relations." (Bar et. al. page 68). Relatively HMW DNA was recovered in high quantities from blood, psoas muscle, brain cortex and lymph nodes. Good DNA stability up to 5 days postmortem was observed in kidney, spleen and thyroid gland tissues.

In 1988, Higuchi, Von Beroldenger, Sensabaugh and Erlich typed the DNA from single hairs. Their approach was different from the previously discussed RFLP method of DNA typing. RFLP analysis requires a relatively large amount of undegraded DNA for typing. Often times such DNA cannot be obtained from such forensic evidence as blood stains and single hairs. This study utilized the polymerase chain reaction (PCR) to amplify the DNA from single hair roots. The findings were that any DNA from degraded biological samples, that are not suitable for RFLP analysis, can be analyzed using PCR. These samples would include old blood or semen stains and possible mummified tissue remains.

An important study was completed in 1989 by McNally, Shaler, Baird, Balazs, DeForest, and Kobilinsky. They analyzed the effects of various environmental insults on the RFLP patterns obtained from DNA. This study

evaluated results of DNA from bloodstains exposed to ultraviolet light, heat, humidity, and soil contamination. The quality of the DNA did not result in false RFLP patterns under the conditions studied. One significant effect observed however, was the weakening of the overall RFLP pattern produced.

In 1989, a study was performed by Honma, Yoshii, Ishiyana, Mitani, Kominami, and Maramatsu. This research used the DNA fingerprint technique to characterize semen samples. By comparing DNA results from blood and semen of several volunteers, the authors successfully determined the semen donors. The semen from a condom left at a crime scene was also analyzed in this study. A person was found dead in a Tokyo hotel room. Beside her body lay a condom. The DNA from the semen in the condom and the blood of a person suspected of the murder were the same. This was the first time the DNA typing technique was successfully used in Japan for investigative purposes.

Yokoi and Sagisaka (1990) presented an article on the postmortem stability of DNA retrieved from human bloodstains and various other biological tissues. DNA was obtained from liver, lung, heart, pancreas, spleen, kidney, adrenal gland, thyroid gland, psoas muscle, brain cortex and lymph node tissues. High molecular weight DNA was retrieved from the heart, thyroid gland, psoas muscle and brain cortex. The high molecular weight DNA from the liver, pancreas, spleen, lung, and kidney degraded

quite rapidly. The retrieval of high molecular weight DNA from bloodstains was successful for stains up to two years old.

A case report was published in 1990 on the identification of decomposed human remains using DNA profiling. Haglund, Reary, and Tipper were the first to report the use of DNA fingerprinting using DNA from the victim's parents to establish identity. The victim was in a moderate to advanced state of decomposition. Visual, x-ray and fingerprint confirmation were not possible. Successful DNA results were obtained from three known blood samples and the victim's psoas muscle. This case demonstrated that a "valid" option for identification was that of comparing DNA from the deceased to relatives.

In 1991, Lee, Pagliaro, Berha, Folk, Anderson, Ruano, Keeth, Phipps, Herren, Gainer and Gaensslen used DNA analysis to study genetic markers in human bone. The quantity and degree of DNA degradation were studied using spectrophotofluorometry and ethidium bromide. They found that the quantity of DNA recovered is dependent on bone type. It was estimated that the amount of DNA recovered from spongy tissue was 10 to 20 times greater than that recovered from compact bone. The ethidium-bromide method proved more useful when estimating the HMW DNA content than the flourometry method. This study established that bone tissue is suitable for both for RFLP and PCR analysis. The results from this research also

confirmed that DNA patterns from blood and bone of one individual were the same.

Schwartz, Schwartz, Mieszerski, McNally and Koblinsky (1991) studied the quality and quantity of DNA obtained from dental pulp of human teeth. The teeth were exposed to various environmental factors including soil, seawater, humidity, variation in pH, temperature and burial. RFLP analysis was performed on the samples and it was found that soil was the only environmental factor that affected the DNA results. DNA was also successfully isolated from a 19-year old tooth in this study.

Also, in 1991, Hochmeister, Budowle, Borer, Eggmann, Comey and Dirnhofer analyzed DNA extracted from compact bone tissue from badly decomposed bodies from known and unknown human remains. Successful RFLP results were obtained in some cases, but the DNA was often too degraded to produce successful RFLP patterns. PCR was successfully used on some bone tissue up to 11 years old. The DNA recovered from the decomposed remains was not correlated with the time of death. The DNA, in some samples was so degraded that it was not suitable for RFLP or PCR typing. This study provided useful data to support the potential reliability of PCR typing for identification purposes when RFLP analysis proved unsuccessful.

In 1993, Sprecher, Liss and Schumm published an article on Southern transfer and chemiluminescent detection. The authors discussed the

simplicity and speed of chemiluminescent detection over radioactive detection methods. Using a nonradioactive detection method they detected a 10 ng sample of human genomic DNA within seven hours.

METHODOLOGY

The experiments were conducted in several stages. Equal emphasis was placed on both qualitative and quantitative results. The goal in this research was to assess the reproducibility of the RFLP results from a number of different samples. The method was to use the RFLP typing technique on ten different psoas muscle specimens.

The RFLP procedure involves the following steps developed by Edwin Southern in 1975: 1) isolation of genomic DNA from biological samples, 2) determining the quantity and quality of DNA, 3) digesting the DNA with a suitable restriction enzyme, 4) separating the DNA fragments using gel electrophoresis, 5) transferring the DNA fragments from an agarose gel to a nylon membrane using Southern transfer, 6) exposing the membrane-bound DNA fragments to labeled probes, and finally, 7) developing the membrane with x-ray film (NRC pg. 38).

Genomic DNA can be isolated from many different substrates. The isolation process usually involves placing the cellular material in a buffered solution of salts, detergents and proteinase K. The cells are lysed by the detergents and the cellular proteins are digested by the proteinase K. Any

extraneous debris is removed and genomic DNA is precipitated out with ethanol.

The quantity and quality of genomic DNA is determined once isolated from the forensic specimen. An agarose “yield gel” is used in this step. Unknown DNA samples and control samples are loaded onto an agarose gel. The gel is stained with ethidium bromide. After electrophoresis, ultraviolet light is used to visualize the DNA. High molecular weight (HMW) DNA suitable for RFLP typing will exhibit a HMW band greater than 20 kilobases (kb). If the DNA has been degraded, a smear of DNA forms. The extent of the smear is proportional to the degree of degradation (Saferstein, 1993).

Restriction enzymes cleave the DNA at specific sequences. These enzymes are sometimes referred to as chemical scissors. There are numerous restriction enzymes but the two used most frequently in forensic DNA typing are HaeIII that recognizes the sequence 5'-GGCC-3' and PST I that recognizes the sequence CTGCAG. Restriction enzymes are also chosen based on what probes are compatible with that enzyme. The process of cleaving the DNA molecule is called digestion. An agarose “Re test gel” stained with ethidium bromide can determine the completeness of digestion.

Once the DNA has been digested, the fragments are separated according to size by electrophoresis in which an electrical current runs through the agarose or acrylamide gel forcing the smaller fragments to move faster through the gel while the larger fragments remain behind.

Agarose and acrylamide gels are very fragile and they can easily be damaged during the hybridization, wash and autoradiograph steps. A Southern transfer must be performed before handling the gels, to transfer the DNA fragments to a nylon membrane. This principle uses capillary action to transfer the agarose bound DNA to the nylon membrane. Southern transfer or Southern blotting, as it is sometimes referred to, was first described by Edwin Southern in 1975 (Kirby 1992). Once the Southern transfer is complete, the fragments are denatured. Denatured DNA is single stranded.

The hybridization step involves recognition of a particular DNA sequence with a complimentary sequence called a probe. Probes are single strands of DNA that contain a specific base sequence. For example, a probe with the sequence "ACTGA" will bind to the complementary sequence "TGACT". Probes can be either radioactively (^{32}P) labeled or nonradioactively labeled. Hybridization conditions need to be correct to permit the probe to bind to the membrane bound DNA fragments. A washing process follows the hybridization process. This allows excess unbound probe to be washed away. Once the membranes are washed they are placed next to a sheet of x-ray film in a cassette. Autoradiographs of membranes hybridized with ^{32}P can take one to five days to develop (Farley pg. 44). An advantage of using non-radioactively labeled probes is speed of development. Kirby states

that within one hour a signal from one ng of DNA can be detected with chemiluminescent labeled probes (Kirby, 1992).

The research was completed following the Lifecodes RFLP chemiluminescent procedure. Two single locus probes were chosen from the Lifecodes protocol. Only two were chosen because of research costs and, in addition, the two probes selected provided enough data to be meaningful. The particular probes chosen for this research detect small and large fragments of DNA.

As discussed earlier, probes are tagged pieces of DNA. They are genetically-engineered to bind to single stands of DNA that contain complementary base pair sequences. DNA probes are given specific names to identify the DNA region, locus or gene to which the probe binds. For example, probe D18S27 can be dissected as follows: D18 signifies the DNA sequence located on chromosome #18, the S indicates a single occurrence on the chromosome and the 27 signifies sequence #27 registered in the Human Gene Mapping database.

DNA probes can be polymorphic or monomorphic. Polymorphic probes detect regions in the DNA molecule that vary dramatically in every individual. Monomorphic probes detect DNA regions that are identical from person to person. Polymorphic probes are used to establish DNA profiles for identification purposes. The following is a list of some, but not all, of the

polymorphic probes used at Lifecodes: D2S44, D17S79, D14S13, D18S27, DXYS14, and D4S163.

For this research, two polymorphic probes were chosen based on the size of the DNA fragment each detected. The two probes selected were, D12S11 and D17S79. Identity probe D12S11 has an allelic size range of 3.0-26.0 kilobases (Kb) long. Identity probe D17S79 has an allelic size range of 2.0-7.0 Kb.

DNA isolation from muscle tissue

DNA was isolated from the postmortem muscle tissue samples using the Lifecodes protocol in Appendix A. The approximate age of the psoas muscle samples can be found in Table 1. The muscle tissue samples and reagents used during the isolation process were kept on ice during use.

Determination of DNA quantity and quality from yield gel

The concentration of DNA in each sample must be known for a successful restriction digest. Appendix B contains the steps performed during this step of the RFLP procedure. A yield gel was prepared by following the instructions in Appendix F, "Procedure for Preparation of Agarose Gels." Yield gel concentration results are found in Figure 1.

Restriction digestion of isolated DNA

Appendix C is the Lifecodes protocol that was followed during the restriction digestion step of the RFLP procedure.

Table 1-Age of psoas muscle samples

Sample number	Hours before specimen was collected
1	Within 24 hours between 6/25/95 and 6/26/95
2	Within 24 hours between 6/19/95 and 6/20/95
3	Approximately 24 hours
4	Approximately 24 hours
5	Approximately 24 hours
6	Approximately 24 hours
7	Approximately 48 hours
8	Approximately 24 hours
9	Approximately 30 hours
10	Approximately 30 hours

Determination of the completeness of digestion from test gel

A test gel is run to determine if the samples have enough DNA to be loaded onto the analytical gels in the next step of the procedure. Appendix F is the protocol for preparation of a test gel. Appendix D contains the detailed instructions that were followed during this stage of the procedure.

Analytical gel electrophoresis

The instructions for the preparation and assembly of analytical gels are contained in Appendix F. The analytical gel is electrophoresed during this step to separate the DNA fragments. Appendix E lists the detailed steps followed during this process.

Southern transfer of DNA onto nylon membrane

Appendix G contains the preparation instructions for transfer working solutions. After the electrophoresis of the analytical gels was complete, a Southern transfer was completed to transfer the DNA from the agarose gels onto nylon membranes. Appendix H contains detailed steps that were performed during this procedure.

Hybridization

After a Southern transfer was complete, the membranes were hybridized with the D12S11 and D17S79 probes. Appendix I lists the detailed steps followed during this procedure. After the hybridization procedure was complete, the nylon membranes were placed in development folders and sprayed with Lumi-Phos 480. The folders were placed into X-ray cassettes and exposed overnight at room temperature. The X-ray films were developed and the Lumigraphs were visually inspected.

Visual inspection of Lumigraphs

The Lumigraphs must be visually inspected to determine if the following four criteria are met. 1. Were the samples completely digested? There must be evidence of partialling. Extra alleles on the gel may be present if the DNA was partially cleaved by the endonuclease. Extra bands can also be present as a result of star activity. Star activity occurs as a result of extra activity of the endonuclease. 2. The Lumigraphs must be observed to determine if band shifting occurred because of salt concentration, proteinase K, improper dilutions, or contamination. 3. Only one or two or bands should be present in each lane. 4. Were the Lumigraphs exposed properly? The membranes may have to be developed several times to

distinguish between close bands or to visualize all the alleles.

Computer assisted Lumigraph analysis

Even though a visual inspection may be sufficient to determine the allele pattern of each sample, a computer is used to digitize the DNA bands and compare them to controls. The internal controls are of known size for DNA fragment size determination. The digitizing process has certain criteria that have to be met to ensure the accuracy of the operator. An operator reads the bands in a series of standards and controls with known band sizes. The standards must be within 2% of the known DNA fragment sized. The operator must re-read the standards if this criterion is not met. If the controls do not fit within an acceptable range, the Lumigraph is considered invalid and must be rerun. The control ranges for the Isolation control and Allelic control can be found in Table 5. The results of the computer assisted analysis for the psoas muscle samples can be found in Table 6. The computer assisted analysis is actually designed to visualize bands and perform calculations for paternity cases. Appendix J fully explains the theories behind the calculations used in the computer analysis.

Table 2-Isolation and allelic control ranges

Control Ranges	Isolation Control	Allelic Control
D12S11	12.75-12.25	16.08-15.45
	7.30-7.01	7.67-7.37
D17S79	3.92-3.77	4.12-3.96
	3.50-3.37	3.63-3.49

FINDINGS

This research produced four major findings. First, successful DNA typing results were obtained from nine of the ten psoas muscle samples. Figure 1 shows the lumigraph produced during the hybridization step of this research. Table 3 lists the results obtained from the computer assisted lumigraph analysis.

Second, even though the samples were fresh tissue samples, the quantity of DNA in each sample determined by the yield gel was significantly low. Figure 2 shows the yield gel photo produced during this research. Table 4 lists the results obtained from the yield gel interpretation. Refer to Appendix B, "Determination of DNA quantity and quality from yield gel", to see the calculations performed during this step.

Third, the test gel indicated that there was a significant amount of DNA in each sample to run an analytical gel. Only one sample produced no results at all. Figure 3 shows a photo of the test gel. Table 5 lists the results of the test gel analysis. Appendix D, "Determination of the completeness of digestion from test gel", contains the calculations performed during this step.

Fourth, the lumigraph was developed using a chemiluminescent detection method. A successful lumigraph was produced in only 16 hours.

Table 3-Results of computed assisted lumigraph analysis

Sample number	D12S11 Probe Allele sizes (bp)	D17S79 Probe Allele sizes (bp)
1	11.467	2.667
	5.636	2.667
2	10.546	3.675
	9.750	2.873
3	11.320	3.902
	9.913	3.391
4	12.249	4.092
	7.077	3.351
5	No results	No results
6	10.999	3.885
	7.047	3.286
7	11.129	3.814
	10.328	3.636
8	11.994	3.330
	11.268	2.824
9	9.793	3.622
	7.310	3.362
10	11.920	3.848
	6.900	3.699
Allelic control	15.952	4.074
	7.582	3.593
Isolation control	12.540	3.818
	7.204	3.433

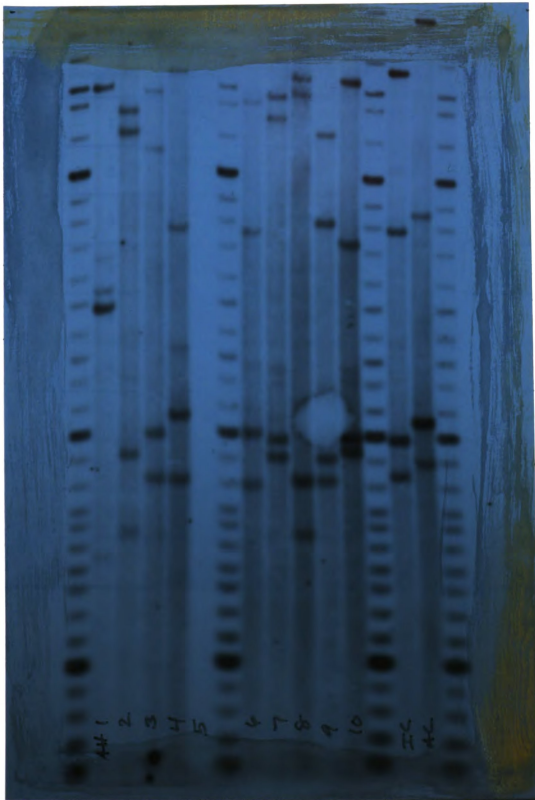


Figure 1-Lumigraph developed with LumiPhos 480

Table 4-Results of yield gel interpretation

Lane #	Sample #	NG/2UL	NG/UL	UL DNA	UL H ₂ O
7	1	50	25	200	-----
8	2	50	25	200	-----
9	3	50	25	200	-----
10	4	50	25	200	-----
11	5	50	25	200	-----
12	6	50	25	200	-----
13	7	50	25	200	-----
14	8	100	50	100	100
15	9	50	25	200	-----
16	10	50	25	200	-----

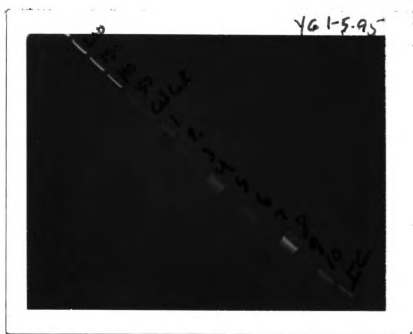


Figure 2-Yield gel photo

Table 5-Results from test gel interpretation

Lane number	Sample number	Amount of loading buffer	
1	Pst Test Gel Standard (10 ul)	-----	
2	1	40	-----
3	2	20	-----
4	3	20	-----
5	4	45	45
6	5	20	-----
7	6	45	45
8	7	45	-----
9	8	45	90
10	9	45	-----
11	10	45	90
12	IC 1/3	45	-----
13	RC 1/12	45	-----
14	Pst Test Gel Standard (20 ul)	-----	



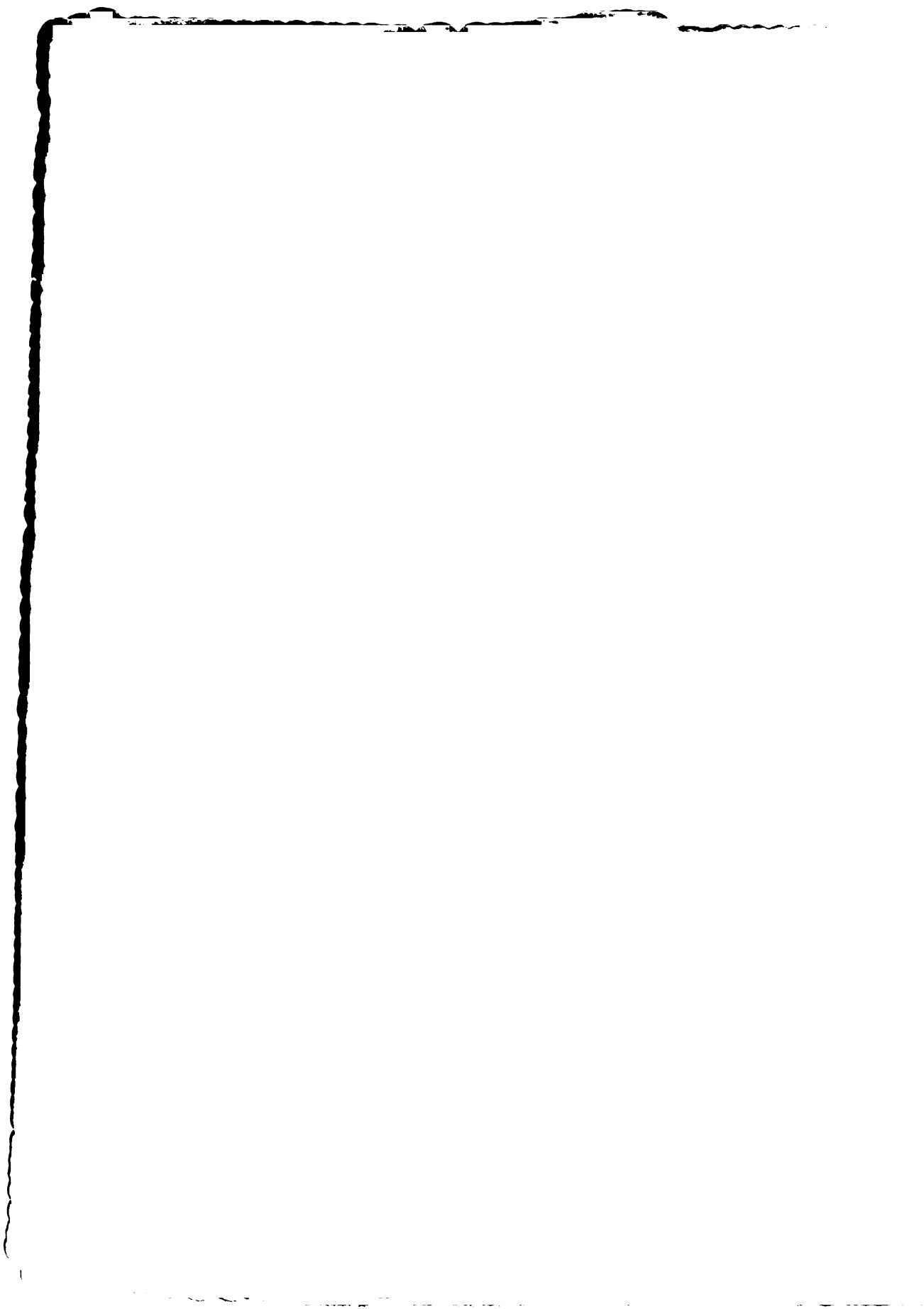




Figure 3-Test gel photo

CONCLUSIONS AND RECOMMENDATIONS

After a review of previous research, this project set out to determine if useful DNA typing results could be obtained from psoas muscle tissue. Although all but one of the samples provided usable results, further research is necessary to determine if postmortem psoas muscle provides useable results under extreme environmental conditions. The muscle tissue samples analyzed in this research were pristine. Each sample was collected approximately 24 hours after death and frozen immediately. They were not exposed to any harsh environmental conditions such as heat, ultraviolet light, soil or humidity.

Another goal of this research was to determine if useable quantities of good quality DNA could be extracted from each sample. Only six of the ten psoas muscle samples showed results on the yield gel. It was decided that the procedure should proceed as normal with all ten samples. A possible explanation for this is that the amount of DNA extracted from each sample may be too small to visualize utilizing the yield gel but, there still may have been a sufficient quantity to type. Fortunately, nine of the ten tissue samples resulted in useful RFLP patterns. Previous research findings indicate that the amount of degraded DNA correlated with the length of the postmortem period. However, some DNA degradation was prominent after

short periods of time in some cases. Early degradation is usually the result of high environmental temperature and/or infectious disease. Early degradation is a possible explanation for why only one of the ten samples in this research did not produce usable results. All the samples were frozen immediately after collection. Therefore, good results would be expected from all the muscle tissue samples.

The results obtained from the DNA yield gel indicated that all of the psoas muscle samples yielded $\geq 5\mu\text{g}$ of DNA. The literature indicated that greater than 50 ng of relatively undegraded DNA is necessary to produce useful RFLP results. Therefore, the amount of DNA extracted from the samples in the research exceeded the amount suggested by the to obtain useful RFLP results.

As with all research projects, further research suggestions are made. An alternative to RFLP typing is the polymerase chain reaction (PCR) typing method. Previous research has indicated that successful PCR results have been obtained from samples whose DNA was degraded and yielded no RFLP results. The application of the PCR technique has been successfully applied to very small blood or semen stains, stains that contained degraded DNA and even DNA extracted from single hairs. An attempt could possibly be made to use PCR typing to analyze the single psoas muscle that did not produce useful RFLP results. The RFLP technique is extremely sensitive, reliable and has been said to offer a higher power of discrimination than the PCR technique.

Another important aspect of this research was the use of

chemiluminescent detection of the RFLP patterns. Both analysis time and safety have been improved by the use of chemiluminescent labeled probes verses the ^{32}P -labeled radioactive probes. Analysis time utilizing the radioactively labeled probes can take weeks to complete. The analysis time using chemiluminescent labeled probes can be measured in hours. Only 16 hours were required to develop the lumigraph of the RFLP results produced in this research.

Further research suggestions include performing a comparison study of DNA extracted from different types of tissue, and perform a time line study to determine how the results from fresh psoas muscle samples compare to aged psoas muscle samples.

APPENDIX A

APPENDIX A

DNA ISOLATION FROM MUSCLE TISSUE

1. Label 2 sets of microcentrifuge tubes. The first set should be labeled with the specimen number and the date. The second set of tubes are labeled with just the specimen numbers.
2. Finely chop the muscle tissue (350 mg) in a disposable petri dish with saline, using a fresh scalpel blade.
3. Add 1.0 ml of the diced muscle tissue to a labeled centrifuge tube.
4. The LIFEPRINT CONTROL should be processed with the samples beginning at step 5.
5. Add 1.0 ml Cell Lysis Buffer. Cap and vortex 1 minute. Visually inspect specimens to ensure even suspension. Manual resuspension with pipeters and tips may be necessary to break up any clumps.
6. Centrifuge on high speed for 2 minutes. Decant supernatant. Blot each tube on kimwipe.
7. Repeat steps 5 and 6 two (2) times. An additional wash may be performed if red cells remain. (Heme will inhibit the reaction).
8. Add 1.0 ml Protein Lysis buffer. Let samples sit for approximately 20 minutes. Vortex to resuspend pellet, making sure there are no clumps of cells.
9. Centrifuge at high speed for 2 minutes. Decant supernatant. Blot. **PLACE SAMPLES ON ICE.**
10. Immediately before use, prepare a master mix of 225 ul Protein Lysis Buffer and 25 ul PRO-K per sample, **PLUS** one extra aliquot compensate for pipetting tolerances. Mastermix **MUST BE KEPT ON ICE AT ALL TIMES.**
11. Processing one sample at a time: Add 250 ul of Mastermix. Pipet up and down to **THOROUGHLY** resuspend pellet. **MIX WELL.** Place tube in 65° C heat block.

NOTE: It is very important that the cell pellet be completely resuspended when Mastermix is added. Do not rely on vortexing alone.

12. Begin two hour incubation after last tube is added to heat block.
Vortex every 15-20 minutes to INSURE nuclear pellet is resuspended.
13. Vortex vigorously for 30 seconds following complete incubation.
Microcentrifuge for 2 minutes.

NOTE: The procedure may be stopped at this point and samples may be stored at 4° C.

APPENDIX B

APPENDIX B

DETERINATION OF DNA QUANTITY AND QUALITY FROM YIELD GEL

1. Vortex samples for 15 seconds. Microcentriguge briefly to bring ontents to bottom of tube. Incubate 5 minutes at 65° C.
2. Microcentrifuge briefly to bring contents to bottom of tube.
3. Add 2.4 ul DNA sample and 9.6 ul Yield Gel Loading Buffer to second set of labeled microcentrifuge tubes. Store unused samples at 4° C.
4. Incubate all samples, Visual Marker, Calibration Control, and Yield Calibrators for 5 minutes at 65° C. Vortex briefly. Microcentrifuge all tubes briefly.
5. Load first row of Yield Gel in the following manner:

1.	30 ng/ul Calibrator	(10 ul)
2.	20 ng/ul Calibrator	(10 ul)
3.	15 ng/ul Calibrator	(10 ul)
4.	10 ng/ul Calibrator	(10 ul)
5.	5 ng/ul Calibrator	(10 ul)
6.	Calibration Control	(10 ul)
7.	Sample #	(10 ul)
8.	.	(10 ul)
9.	.	(10 ul)
.	.	(10 ul)
.	.	(10 ul)
17.	Sample #	(10 ul)
6. Subsequent rows in the SAME gel may be run minus the calibrators.
7. Electrophorese between 50-100 volts for 45-60 minutes. Photograph under UV on low setting.

INTERPRETATION OF YIELD GEL:

1. Estimate the quantity (ng) of high molecular weight DNA loaded for each sample be comparing the band size and the intensity of the unknowns with those of the Yield Calibrators. Since 10 ul of each

calibrator was loaded, the calibrators represent 300 ng, 200 ng, 150 ng, 100 ng and 50 ng of DNA respectively. The Calibration Control should compare to the 100 ng standard. Significant variation of the Calibration Control should be noted and investigated. Call Lifecodes to verify that there are no problems with the control, and/or run a new yield gel to confirm the results.

If samples contain significantly less than 50 ng of DNA, an additional aliquot of sample should be processed and combined. Reanalyze on another yield gel.

For samples containing significantly greater than 300 ng of DNA, dilute with 250 µl of Protein Lysis Buffer. Vortex to mix. Reanalyze sample on another yield gel.

2. High molecular weight DNA should appear as a tight band slightly above the uppermost band of the Visual Marker. If the sample shows “trailing”, use only the region above the upper band of the Visual Marker for quantifying high molecular weight DNA. If the DNA shows significant levels of degradation, a fresh sample may be required.
3. Calculate the concentration of DNA for each sample by dividing the quantity of DNA (ng) by the volume (µl) of the sample aliquot used in the yield gel. For example:

Sample compares to 100 ng calibrator (10 ng/µl)
 $100 \text{ ng DNA} / 2 \text{ µl sample} = 50 \text{ ng/µl}$

The LIFEPRINT Control should have a concentration of approximately 50 ng/µl. Significant variation may indicate a procedural error. A yellow discoloration of the original pellet may indicate age and/or improper storage. Call Lifecodes to verify that there are no problems with the control, and/or run a new yield gel to confirm results.

The concentration of DNA in each sample is required for subsequent restriction digestion. Record results and reagents on Yield Gel Worksheet.

APPENDIX C

APPENDIX C

RESTRICTION DIGESTION OF ISOLATED DNA

1. Label 3 sets of microcentrifuge tubes. Label one set with sample number and the date. Label the two sets with sample numbers only.
2. Using the table below, mix together in a labeled set of microcentrifuge tubes, the appropriate amount of sample and distilled H₂O. Refrigerate genomic DNA samples after using.

Table 6-Preparation for restriction digestion

Concentration of Samples	Amount of distilled H ₂ O	Amount of Sample DNA
250 ng/ul	180 ul	20 ul
150 ng/ul	166 ul	34 ul
100 ng/ul	150 ul	50 ul
75 ng/ul	133 ul	67 ul
50 ng/ul	100 ul	100 ul
25 ng/ul	-----	200 ul

3. Prepare a master mix of 154 ul Nuclease-free H₂O, 40 ul Digestion Buffer, 4 ul Spermidine, and 2 ul Pst I Enzyme for each sample, PLUS one extra aliquot to compensate for pipetting tolerances. Mix well by inversion. KEEP MASTER MIX ON ICE.

NOTE: Remove Pst I Enzyme from freezer immediately prior to use and place on ice. Prepare master mix immediately before use and place on ice. Return unused enzyme to freezer immediately.

4. Add 200 ul of master mix to each tube. Gently flick tube to mix.
5. Incubate at 37° C for 2 hours. After 30 minutes has elapsed, gently flick each tube. Microcentrifuge briefly at end of incubation.

NOTE: The procedure may be stopped at this point and samples stored at -20° C. Rewarm samples for 5 minutes at 65° C

6. Add 200 ul of lithium chloride Conditioning Salt. Vortex 5 seconds. Place on ice for 13 minutes.
7. Microcentrifuge for 10 minutes. Transfer supernatants to a fully labeled set of microcentrifuge tubes. Avoid transferring any of the precipitated protein and other debris. The pellet is often difficult to visualize.
8. Add 1.0 ml room temperature 95% Ethanol. Invert to mix. Incubate at room temperature for 20-30 minutes.
9. Microcentrifuge for 30 minutes to pellet DNA. Decant supernatant. Blot.
10. Add 1.0 ml room temperature 70% Ethanol. Vortex well for 3 minutes.
11. Microcentrifuge for 30 minutes to pellet DNA. Carefully decant supernatant. (Cell pellet does not adhere tightly to walls of tube). Blot.
12. Dry pellet with Speed-Vac for 15-30 minutes. (This may be shortened to 10 minutes if the heat is turned on; however, excessive drying will make the pellet hared to resuspend). The procedure may be stopped at this point and samples stored at -20° C.
13. Add 51 ul of Nuclease-free H₂O. Incubate at 65° C for 3-5 minutes. Vortex. Re-incubate at 65° C for 5 minutes.
14. Vortex to completely resuspend. Microcentrifuge briefly to bring contents to bottom of the tube.

NOTE: The procedure may be stopped at this point and samples stored at -20° C. (If stored:) Reheat samples for 5 minutes at 65° C.

APPENDIX D

APPENDIX D

DETERMINATION OF THE COMPLETENESS OF DIGESTION FROM TEST GEL

1. Refer to Appendix F, "Procedure for Preparation of Agarose Gels," for the preparation of the test gel(s).
2. If the restricted DNA has been stored at -20° C, heat samples for 5 minutes at 65° C, vortex, and microcentrifuge briefly. All 6 ul of restricted DNA samples and controls to 6 ul of Loading Buffer in the last set of labeled microcentrifuge tubes.

NOTE: Store the restricted DNA samples at -20° C immediately after use.

3. Incubate DNA/Loading Buffer mixtures, Visual Marker and Pst Test Gel Standard for 5 minutes at 65° C.
4. Vortex. Microcentrifuge all tubes briefly to bring contents to bottoms of the tubes.
5. Load first row of Test Gel in the following manner:

1.	Visual Marker	(10 ul)
2.	Pst Test Gel Standard	(10 ul)
3.	LIFEPRINT Control	(10 ul)
4.	GENOMIC DIGESTION Control	(10 ul)
5.	Sample	(10 ul)
6.	Sample	(10 ul)
.	.	(10 ul)
.	.	(10 ul)
(up to) 17.	Pst Test Gel Standard	(20 ul)

Subsequent row in the same gel may be run minus the standards.

6. Electrophoresis between 50-100 volts for one hour. Photograph under UV ("Low" setting).
7. Interpretation of test gel:
 - a. Determine for each sample if the Pst I digestion is complete by

comparing its pattern with the Pst Test Gel Standards. The digestion pattern should be a uniform smear with a well defined starting point slightly above the uppermost band of the Visual Marker.

- b. Compare the smear intensity of the samples to the standards to insure that the appropriate amount of DNA will be loaded onto the Analytical Gel. The fluorescent intensities of the Pst Test Gel Standard are equivalent to 500 ng and 1 ug of digested DNA. The 5 ul of sample used on the test gel should contain between 500 ng and 1 ug of DNA.
 - c. The DIGESTION Control should contain approximately 500 ng of Pst I digested DNA. This control should reproducibly cut to completion under the conditions of this protocol with Pst I Enzyme and is a control of enzyme reliability. If the digestion control has not completely restricted, the reactivity of the enzyme should be confirmed through Lifecodes. Redigestion of all samples may be necessary.
 - d. Significant variation in concentration must be corrected before samples are loaded onto the Analytical Gel. If fluorescence of any sample is significantly less than the 500 ng standard, an additional 5 ug of DNA must be digested. If the fluorescent intensity of a sample is significantly greater than the 1 ug standard, the sample should be diluted with appropriate amount of Loading Buffer.
8. If the Pst restriction digestion is complete, add 45 ul Loading Buffer to each sample. This amount may vary due to DNA concentration in the restriction. Proceed to Analytical Gel section.
 9. If the restriction is not complete, proceed as follows:
 - a. Remove sample to be "re-digested" from the freezer and add 5 ul Digestion Buffer and 2 ul Pst I Enzyme. Incubate 2 hours at 65°C. After 1 hour has elapsed, flick tubes to insure suspension of enzyme.
 - b. Add 2 ul Conditioning Salt and 100 ul 95% Ethanol. Invert tube to mix. Incubate 20-30 minutes at room temperature.
 - c. Microcentrifuge 20 minutes to pellet DNA. Decant supernatant. Blot. (Note: Pellet does not adhere tightly to wall of tube.)
 - d. Add 1.0 ml room temperature 70% Ethanol. Vortex well.
 - e. Microcentrifuge 10-20 minutes to pellet DNA. Carefully decant supernatant. Blot.

- f. Dry pellet with Speed-Vac for 5-10 minutes.
- g. Add 50 ul Nuclease-free H₂O.
- h. Repeat test gel procedure and interpretation. If restriction digestion is still incomplete, discard sample.

APPENDIX E

APPENDIX E

ANALYTICAL GEL ELECTROPHORESIS

1. Prepare as many Analytical Gels as needed to run completed samples and controls.

NOTE: Refer to Appendix F, "Procedure for Preparation of Agarose Gels", for the preparation and assembly of analytical gels.

2. Remove samples and controls from freezer and thaw.
3. Microcentrifuge briefly all samples , Allelic Control, Sizing standards, and other controls.
4. Incubate 5 minutes at 65° C. Vortex. Microcentrifuge for 5 minutes.
5. Load 10 ul of sizing standard and 20 ul of each sample into an analytical gel. The following pattern is recommended:

Lane	Sample
1.	Sizing Standard
2.	Sample 1
3.	Sample 2
4.	Sample 3
5.	Sample 4
6.	Sample 5
7.	Sample 6
8.	Sample 7
9.	Sample 8
10.	Sample 9
11.	Sample 10
12.	Sizing standard
13.	Allelic Control

NOTE: The Allelic Control **MUST** be run on every plate. The LIFEPRINT Control and /or Digestion Control must be run with each set of samples processed together.

7. Electrophorese at 14.5 volts across the top gel for 65 hours. If electrophoresis will take place for a longer or shorter period, adjust

voltage proportionately. After the first hour has elapsed, turn on the circulating pumps.

NOTE: If running more than one gel box, be certain to run the same number of gels in each box.

APPENDIX F

APPENDIX F

PROCEDURE FOR PREPARATION OF AGAROSE GELS

- Notes:
- a) Yield gels and test gels can be prepared in 12 x 15 cm or 12 x 27 cm gel trays. Analytical gels are always prepared in 12 x 27 cm gel trays. All the gels utilize 0.6% agarose.
 - b) The instructions below are for preparing one 12 x 15 cm (100 ml) or one 12 x 27 (200 ml) gel. The amount of agarose should be adjusted for additional gels.
 - c) The Gel Buffer concentrate must be diluted 1:40 with distilled water before use.

1. Determine the number of gels needed. Listed below are the amounts of agarose, Gel Buffer and ethidium bromide required per gel.

	Yield/Test Gels		Analytical Gels
	Small (100 ml)	Large (200 ml)	Large (200 ml)
Amount Agarose	0.6 g	1.2g	1.2g
Amount Gel Buffer	100ml	100 ml	200 ml
Ethidium Bromide	5ul	10 ul	-----

2. Prepare gel trays by taping the open ends.
3. Measure diluted Gel Buffer in the graduated cylinder and pour into an Erlenmeyer flask large enough to hold at least 2 times the volume of the buffer to be added.
4. Weigh agarose, add to flask, and swirl gently. Heat on a heated stir plate, until all agarose is dissolved. Make sure all crystals are dissolved before proceeding.
5. After heating, check volume of the solution in a graduated cylinder. Add distilled water to replace any volume lost in heating.
6. For yield and test gels, return solution to flask. Add 5 ul ethidium bromide per 100 ml of gel. Swirl contents to mix.

7. Pour molten agarose into center of gel tray. Be sure tray is on a level surface. Remove any bubbles with tip of transfer pipette. Place combs into position. One to four rows of wells may be used for yield gels and test gels to accommodate more samples.

APPENDIX G

APPENDIX G

PREPARATION OF TRANSFER WORKING SOLUTIONS

<u>DENATURING SOLUTION</u>	<u>10 L</u>
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NaCl	584 g
NaOH	200 g
Distilled H ₂ O	10 L

<u>NEUTRALIZATION SOLUTION</u>	<u>4 L</u>	<u>10 L</u>
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NaCl	584 g	1460 g
Tris	39 g	97 g
Tris HCL	661 g	264 g
Distilled H ₂ O	4 L	10 L

APPENDIX H

APPENDIX H

Southern Transfer of DNA onto nylon membrane

1. Stack gel trays on top of each other in a rectangular tray. Place an empty tray on top.
2. Carefully pour in enough Denaturing Solution to completely submerge gels (approximately 750 mls/gel). Let sit for 20-30 minutes.
3. Discard Denaturing Solution. Rinse gels carefully two times with distilled water to remove excess Denaturing solution.
4. Add enough Neutralization Solution to completely submerge gels (approximately 750 mls/gel.) Let set 20-30 minutes.
5. Prepare nylon membranes:
 - a. For each gel, label a membrane with a pencil in lower left corner with gel #, sample #'s and technologists' initials, and set up date.
 - b. Add 250 mls distilled water to a tray and carefully immerse membranes so that they wet evenly.
 - c. Replace water with 250 mls of Neutralization solution. Submerge membranes and let soak until ready for use.
6. Prepare sponges:
 - a. Rinse sponges thoroughly with tap water. Wring dry.
 - b. Rinse with distilled water. Wring dry.
 - c. Soak in Neutralization solution. Wring dry.
 - d. Saturate sponges with fresh Neutralization solution. Let stand until needed.
7. Onto Plexiglas bench shield, stack for each gel to be transferred, three saturated sponges and two sheets blotting paper pre-wet with neutralization solution. Remove air bubbles between the sponges by rolling gently with a disposable pipette.
8. Carefully remove gel trays from Neutralization solution and slide gel from tray onto the top of the blotting paper. Carefully smooth gels to remove excess buffer from tops. Remove air bubbles between gel and blotting paper.

9. Place pre-wet, labeled nylon membrane on top of the gel. The labeled side should be facing upwards, and the writing should be on the lower edge of the gel. Remove all air bubbles and ensure complete contact by gently rolling a disposable pipette over the membrane.
10. Pre-wet two sheets of blotting paper with neutralization solution and carefully place on top of the nylon membrane. Do not reposition blotting paper without checking for air bubbles that may have been introduced between the nylon membrane and the gel. Gently roll a pipette over the blotter paper to remove any bubbles.
11. Stack two inches of paper towels and a gel box lid or Plexiglas weight on top of the gel. Ensure uniform contact and even weight distribution. Make sure no paper towels overlap onto sponges or in buffer. They should only touch the blotter paper on top of the membranes.
12. Allow transfer to proceed for 3-4 hours. After one hour has elapsed, replace the wet papertowels with dry ones.
13. Disassemble transfers one at a time.
 - a. Carefully peel nylon membrane away from gel. Place membrane in approximately 500 ml of 1:5 dilution of neutralization solution and water.
 - b. With a gloved hand, gently rub the side of the membrane that was in contact with the gel to remove any residual agarose. Turn the membrane over and repeat on the other side.
 - c. Place membranes on a sheet of filter paper and allow to air dry until membranes are no longer glossy.
14. Repeat step 13 for each membrane. The same solution may be reused.
15. Sandwich the membranes between 12 X 28 cm sheets of filter paper in a stack. Do not stack more than 10 membranes. Bake at 65-80° C for one hour or until completely dry.
16. Place each membrane in a 302 nm UV transilluminator with DNA side facing the UV source (the writing should be facing upwards). Expose to UV for 90 seconds.
17. Place membranes into heat sealable bags until ready for hybridization. Membranes may be stored indefinitely.

APPENDIX I

APPENDIX I

HYBRIDIZATION OF NYLON MEMBRANES

Note: It is recommended that 1-5 membranes be processed together per vial of probe. Slightly more membranes may be processed with a small decrease of probe signal resulting. Membranes must be handled with gloves and should only be touched on the edges.

1. Prepare 1X Quick-Light Buffer, wash I and II as follows:

(prepare QUICK-LIGHT on day of use only)

1X QUICK-LIGHT Buffer	900 ml dH ₂ O 100 ml Quick-Light Buffer
Wash I	40 ml Wash Component A 250 ml Wash Component B 710 ml dH ₂ O
Wash II	4 ml Wash Component A 50 ml Wash Component B 946 ml dH ₂ O

2. To process 5 membranes, place Lumi-Phos 480 at room temperature, and incubate the following reagents at 55° C for 90 minutes prior to use:

75 ml Hybridization solution
2,000 ml Wash I
2,000 ml Wash II

Note: To hybridize fewer membranes, use 20-25 ml hybridization solution for one membrane (300 cm² membrane) and 10-15 ml for each additional membrane. Use up to 100 ml of all other solutions per membrane. It is important to add sufficient solution to have all the membranes completely immersed.

3. Trim membranes to approximately 25 cm in length, sort membranes, and place in appropriate sized clean container containing 500 ml of Wash I at 55° C. Using forceps, separate each membrane to allow complete wetting. Cover container with leakproof lid and shake at 55° C for at least 10 minutes.

4. During step 3, combine Probe Solution with 75 ml of Hybridization solution pre-heated to 55° C. Screw the cap on tightly, secure the cap with parafilm and incubate at 55° C for 10-20 minutes before use.
5. Pour out the Wash I solution and all 75 ml of the Probe Solution pre-heated to 55° C. Cover and shake at 55° C for 10-20 minutes.

Note: Incubations longer than 30 minutes may result in an increase in background.
6. Pour out the Probe Solution and add 1 liter of Wash I pre-heated to 55° C. Cover and shake at 55° C for at least 10 minutes.
7. Pour off the Wash Solution I and repeat step 6.
8. Pour off the second Wash Solution I and add 1 liter of Wash Solution II pre-heated to 55° C. Cover and shake at 55° C for at least 10 minutes.
9. Pour off the Wash Solution II and repeat step 8.
10. Pour off the second Wash Solution II.
11. Add 500 ml of 1X QUICK-LIGHT Buffer. Cover and shake the container vigorously. Using forceps, separate each membrane to allow complete wetting.
12. Pour off 1X QUICK-LIGHT Buffer and repeat step 11 three times.
13. Allow excess QUICK-LIGHT Buffer to drain. Position up to three rinsed membranes on an open development folder. Spray each membrane evenly with Lumi-Phos 480, using the spray applicator provided.
14. Close development folder over the sprayed membranes and remove air bubbles and excess Lumi-Phos 480 by firmly wiping the closed folder with a papertowel. Seal the folder with an impulse heat sealer. Inspect seals to ensure that the Lumi-Phos will not leak. (The folders and membrane should be trimmed so that they are approximately 25 cm in length, otherwise they will not fit into cassettes.)
15. Wipe the surface of the folder dry with a paper towel before inserting it into a X-ray cassette.

16. Expose film overnight at room temperature.
17. Develop film and review resulting Lumigraphs.

APPENDIX J

APPENDIX J

REAGENTS

- A. Reagents from Lifecodes, Inc.
1. Cell Lysis Buffer: Contains sucrose, magnesium chloride, and Triton X-100 in Tris Buffer. Store at 2-8° C.
 2. Protein Lysis Buffer: Contains sodium EDTA and sodium chloride in Tris buffer. Store at 2-8° C.
 3. Pro-K: Contains proteinase K in Tris buffer. Store at -20° C.
 4. Gel Buffer: Gel Buffer contains sodium EDTA in Tris-acetate buffer as a 40X concentration. Dilute 1:40 in nanopure water to prepare Working Gel buffer. Store at room temperature.
 5. Visual Marker: Visual Marker contains Hind III digested lambda DNA in loading buffer. Store at -20° C.
 6. Yield Calibrator Set: Yield Calibrator Set includes calibrators containing lambda DNA in loading buffer at concentration of 30 ng/ul, 20 ng/ul, 15 ng/ul, 10 ng/ul and 5 ng/ul.
 7. Calibration Control: Calibration Control contains 10 ng/ul high molecular weight human DNA in loading buffer. Store at 2-8° C.
 8. LIFEPRINT Control: LIFEPRINT Control contains 2.5 X 10 lyophilized K562 human cells per vial. Store at 2-8 ° C.
 9. Yield Gel Loading Buffer: Yield Gel Loading Buffer contains Ficoll 400, bromophenol blue, sodium EDTA, and SDS in Tris-acetate buffer as a 1.25X concentration. Store at room temperature.
 10. Ethidium Bromide: Ethidium Bromide contains 10 mg/ml ethidium bromide. Store at room temperature. **Mutagen**
 11. Conditioning Salt: Conditioning salt contains lithium chloride. Store at room temperature.

12. **Pst I Enzyme:** Pst I Enzyme contains 50 U/ul Pst I, Tris-hydrochloric acid, sodium chloride, EDTA, bovine serum albumin, and B-mercaptoethanol in Tris buffer. Store at -20° C.
13. **Digestion Buffer:** Digestion Buffer contains sodium chloride, magnesium chloride, bovine serum albumin and B-mercaptoethanol in Tris buffer. Store at -20° C.
14. **Spermidine:** Spermidine contains a diluted aqueous solution of spermidine. Store at -20° C.
15. **Nuclease-free H₂O:** Nuclease-free H₂O contains autoclaved, purified H₂O. Store at room temperature.
16. **Hybing Solution:** Hybing Solution contains polyethylene glycol, sodium chloride, sodium phosphate, EDTA, sodium dodecyl sulfate, sodium heparin and herring testes DNA. Store at 2-8° C.
17. **Wash Component A (SSPE):** Wash Component A contains sodium chloride, sodium phosphate and EDTA as a 25X concentration. Contains Sodium Azide. Store at room temperature.
18. **Wash component B (SDS):** Wash Component B contains sodium lauryl sulfate (SDS) as 20% solution. Contains Sodium Azide. Store at room temperature.
19. **Loading Buffer:** Loading Buffer contains Ficoll 400, Bromophenol blue, and sodium EDTA in Tris-acetate buffer. Store at room temperature.
20. **Digestion Control:** Digestion Control contains 100 ng/ul high molecular weight K562 human DNA and EDTA in Tris buffer. Store at 2-8° C.
21. **Pst Test Gel Standard:** Pst Test Gel Standard contains Hind III digested lambda DNA in loading buffer. Store at -20° C.
22. **Allelic Control:** Allelic Control contains 50 ng/ul of Pst I digested K562 human DNA in Loading Buffer. Store at -20° C.
23. **Sizing Standards:** Sizing Standards contains DNA fragments of various lengths originating from PhiX174, M13, Lambda and T7 phage in Loading Buffer. Store at -20° C.

24. **Combination Standards:** Combination Standards contains Adenovirus digested with Kpn I enzyme and the Sizing Standards in Loading Buffer. Store at -20° C.
 25. **Lumi-Phos 480:** Store at 2-8° C. **Avoid Aerosol**
 26. **I.D. Na Agarose:** Store in dry location at room temperature. Use for analytical gels.
 27. **QUICK-LIGHT Buffer:** Store at room temperature. Contains Sodium Azide.
 28. **Probe Solution I (D12S11/D17S79):** LIFEPRINT Probe Set I contains the enzyme labeled DNA probes D12S11 and D17S79 plus enzyme labeled phage DNA (PhiX174, M13, Lambda, T7) for the molecular weight markers. The chemiluminescent substrate (Lumi-Phos 480) is added to react to the hybridized probe. Store at 2-8° C.
- B. Reagent other than Lifecodes**
1. **Agarose, Seakem LE:**FMC Bioproducts, Cat. #50003. Store in dry location at room temperature. Use for yield and test gels.
 2. **Sodium chloride:** SP Cat #7581-12 NY. Store at room temperature.
 3. **Sodium hydroxide:** SP Cat #7708-2.5 NY. Store at room temperature.
 4. **Trizma base (Tris):** Sigma Cat. #T-1503. Store at room temperature.
 5. **Trizma-HCL (Tris-HCL):** Sigma Cat. #T-3253. Store at room temperature.
 6. **Ethanol, 95%:** AAPER Alcohol and Chemical, order through Sparrow Pharmacy. Flammable. Store in flame-proof chemical storage cabinet at room temperature.

APPENDIX K

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COMPUTER-ASSISTED LUMIGRAPH ANALYSIS SIZING PROGRAM

1. Call the DNA directory to your PC. After the C:\> prompt appears, type cd\DNAPHI91.
2. Begin the DNA sizing program. After the C:\DNAPHI91> type: D.
3. "LIFEPRINT (tm) SIZING PROGRAM FOR PATERNITY" will appear on the screen. Press the digitizer button once to insure proper hook-up. If a beeping sound is not heard, make sure connections are properly in place and start program again.
4. The computer will prompt you for optional information. Press "enter" to skip or type today's date, press "enter"; type date gel was run, press "enter"; type initials of person who ran the gel, press "enter." After pressing enter the last time, a question appears on the screen. Should you find that something was entered incorrectly, move the cursor to "2 no." Otherwise press "enter" to proceed.
5. The program requires you to input a gel number, which will be part of the name of the file under which the data are stored. Type gel number, without using a hyphen, and press "enter."
6. The other part of the file name is the initials of the person who is sizing the lumigraph. Type initials, press "enter."
7. The next screen will present the name of the file you are currently creating. If any lumigraphs have previously been sized under the same file name, you have a choice of whether you:
 - a. want the sizing you are currently working on to overwrite the previously named file or,
 - b. add what you are currently sizing to the existing file, or
 - c. rename the file that you are currently creating by reentering the gel number and your initials.

Choose the appropriate option, and press "enter." If there are no existing files with the same name, simply press "enter."

8. The next screen requests that you enter the number of paternity cases on the lumigraph. Generally, paternity cases are ordered so that standard size marker lanes flank each case. The program automatically takes this ordering system into account, and will, therefore, expect the number of standard size marker lanes that need to be sized, to be "one, plus the number of paternity cases on the lumigraph". Type the number of paternity cases, and press "enter."
9. For the first paternity case on the lumigraph (the left most), choose the option that accurately describes the paternity case. Move the cursor to the appropriate number and press "enter."

NOTE: If you choose "4. none of the above," you will be expected to first enter the number of lanes of the gel that the case occupies. This number should include mixture lanes, if any. Then, the case number should be specified. After typing the case number and pressing "enter," each lane is displayed on the monitor and it is necessary for you to type the suffix for each lane, in order, as it appears on the lumigraph. Use -10 for the mothers, -20 for children, -30 for alleged fathers, -X1 for first mix, -X2 for second mix, etc. After typing the suffix for the first sample, press "enter" and repeat for each subsequent sample lane for the case.

10. After each paternity case is described, the race of the mother and alleged father(s) may be entered, or for choice number "4", you are given the option to enter race for each sample lane. Move the cursor to the appropriate choice, and press "enter." (Note: enter "5-unknown" for each.)
11. The next screen gives you the option to edit information regarding case sample numbers and race. In addition, if you incorrectly entered too many samples, such that greater than 17 lanes including the control lane are recorded, a statement will appear on the top of the screen to inform you, and you must reenter the number of cases.

After reviewing the editing screen, and having determined that all sample and race information is accurate, you can proceed by answering "yes" to the question "has you sample number and race information been entered correctly?"

12. You must now make a choice of the probe, whose alleles (represented by bands on the lumigraph) for each sample lane will be what you are going to size. Move the cursor to the appropriate choice, and press "enter."

You can recheck that you have entered the probe information accurately. Move the cursor to “yes” when asked the question “Is the probe entered correctly?”, and press “enter.”

13. On the next screen are numbers corresponding to the size standards. To determine what standards you need to include, first look for the highest allele and lowest allele of the probe currently being sized (in all the sample lanes). Then choose a standard band, which is higher than the highest allele, as the standard with the greatest size. Choose a range that includes at least seven size standard bands, but no greater than 25 bands, to get an accurate determination of goodness of fit.

Type the number of the highest size standard band after the statement “enter the number of the standard with largest size [?].”

Next, choose a size standard band lower than the lowest allele. Type the number corresponding to the lowest size standard band after the statement “enter the number of standard with lowest size [?].”

If you entered either or both of these numbers incorrectly, you may reenter them. Choose the appropriate answer to the question “Are the standards correct?”, and press “enter.”

C. Sizing the standards:

The proper order for sizing these bands is to start with the band of greatest size and proceed, sizing each band in numeric order until you reach the one with the smallest size.

1. When “ENTER STANDARD #____” appears on the screen, center the cross-hairs of the mouse on the appropriate band (the largest standard you have chosen to size) in the standard lane, and press the button on the mouse.
2. Complete the sizing of all the bands in the lane. The screen will then provide information in columns, under the heading “LANE NO 1.”

Below is a list of the column descriptions:

- a. The number-from highest to lowest- associated with the respective size standard band.
- b. The Hi-pad’s corresponding number related to the point at which mouse button was pressed.

- c. Expected size of the standard band.
 - d. Observed size of the standard band.
 - e. Error associated with each observed sizing.
3. The goodness of fit is displayed below the numbers in the numbers in the columns. The goodness of fit must be below 2 in order to proceed. If the goodness of fit is greater than 2, after the question "Go on?", move cursor to "2 no", and press "enter". You can then resize the same standard lane.
 4. Once you get a goodness of fit less than 2, move cursor to "1 yes," and press "enter". Repeat the sizing procedure for the second standard lane. Depending on how many cases are on the lumigraph, you may then have to size standard lane #3, standard #4, etc. Once you have been successful in obtaining a goodness of fit below 2 for each standard lane, you may proceed to sizing of the sample lanes.

D. Sizing the Alleles in the Sample Lanes:

1. For each sample lane, the screen displays a table with the following headline: "Lane No_____ DIGITIZE sample # _____". The lane number corresponds to one of seventeen lanes (starting with the left most lane of the lumigraph = Lane No 1, and the rightmost lane = Lane No 17) in which the sample resides. The sample number plus the suffix indicating whether it is the mother, child, alleged father, or mixture lane.
2. Size the sample lane:
 - a. If the sample lane contains only one band (homozygous) for the probe system, center the mouse on the band, and hit the button on the mouse one time.
 - b. If the sample lane contains two bands (heterozygous) or more than two bands (in the case of a mixture lane), size each band once, starting with the greatest size, and proceeding in order of the lowest band. Center the cross-hairs of the mouse on the band, and press the button on the mouse.
3. Once you finish sizing the lowest band, you must place the cross-hairs of the mouse within 1 inch of the left hand side of the digitizer and press the button. This will indicate that sizing of the lane is complete, and allows you to proceed to the next

sample lane. Record results on worksheet before proceeding to next allele.

4. Repeat the same procedure for all sample lanes until you have completed the last sample on the lumigraph, which should always be the control lane.
5. The sizes of the alleles that are determined for the control lane must be within 2% variation compared to the expected sizes, otherwise repeat the sizing, since this may be an indication that sizing of standard bands was done inaccurately.

If the control can not be sized within the expected range, the plate must be re-run. No sizings are to be used from the plate where the standards may be incorrect.

6. After you have finished sizing each lane, the sizing information for that lane appears on the screen. The following is a description of the information contained within the columns on this table:
 1. sample number.
 2. allele number for each respective sample.
 3. race of sample.
 4. goodness of fit of left flanking size standard lane.
 5. goodness of fit of right flanking size standard lane.
 6. size of the allele (represented by band that was sized).

When asked the question "Are you finished?", move the cursor to the appropriate choice, and press "enter."

After completing the sizing of all samples, if your lumigraph contains more than one probe system, you may size the alleles of the second probe system. By answering "no" to the question "Are you finished", you will be brought back to the same screen that you have seen before regarding the information about probe systems, and asked to enter the second probe system. You then choose the number that corresponds to the second probe system. Repeat all necessary steps in order to do the sizing of all lanes for the second probe. If there is not another probe system for data analysis, answer "yes" to the above question.

APPENDIX L

APPENDIX L

Function of select reagents and controls

Agarose	Medium used to separate DNA fragments according to size.
Alcohol (70%)	Washes salts and buffers away; returns layer of hydration to the DNA.
Alcohol (100%)	Precipitates the DNA and removes protein and salt. Removes the layer of hydration of DNA.
Allelic Control	Human genomic control that must be run on every analytical gel. The allelic control is sized by the sizing program and must fall within a 2% size range of the expected kilobase size stated by Lifecodes.
Bovine Serum Albumin (BSA)	Enzyme stabilizer.
Calibration Control	Run, at least, in one lane on every yield gel. The Calibration Control compares to the 100 ng standard. Failure of this control may indicate a need to rerun yield gel(s).
Ethylenediamine Tetraacetic Acid (EDTA)	Chelates Mg so the nucleases cannot chew apart DNA.
Ethidium Bromide (EtBr)	Used as a fluorescent tracking dye and for quantitation of DNA. Interchelates between stacked bases of DNA. Excited at 300

	nm (UV light) quantitatively absorbed by DNA.
Genomic Digestion Control	Contains approximately 500 ng of Pst I digested DNA. This control is a check on enzyme reliability. This control is processed with every batch of restriction samples.
Herring Sperm	Blocks unrelated repetitive DNA probe and target; also used in labeling as a seed to precipitate DNA.
Hybridization Solution	PEG, SDS, SSPE, H ₂ O
Lifeprint Control	Should have a concentration of approximately 50 ng/ul. This control is started at the extraction and run through the yield gel, test gel and analytical gel.
Proteinase K	Proteolytic enzyme that degrades DNase and other proteins into their constituent amino acids.
Spermine	Binds unbound dNTP's in probe makeing; can be added at RE time to tie up negatively charged contaminants.
Sodium Chloride	Keeps DNA rigid so that proteinase K can react on it.
SDS	Sodium Dodecyl Sulfate; Protein denaturant; lyses cell wall; denatures enzymes; dissociate nucleic acid from proteins-DNA complex;

solubilizes membranes of nuclei.

SSPE

Sodium Chloride (NaCl) & Sodium Dihydrogen Phosphate (NaH₂PO₄) & Sodium EDTA Buffer used in hybridization and blot stripping.

Tris

Buffer used to maintain pH.

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