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1970

Cooksley, Joseph David, The Development and Evaluation of an Oligonucleotide Probe for Use in Quantifying Human Mitochondrial DNA Using the Quantiblot® Human DNA Identification Kit. Master of Science (Forensic Genetics), August 2003, 93pp., 3 tables, 14 figures, 1 appendix, references, 17 titles.

Nucleotide sequences from coding regions in the mitochondrial DNA genome were evaluated for use in designing an oligonucleotide probe specific to humans that could be used with the Quantiblot® Human DNA Identification kit from Applied Biosystems. Two probes were designed and evaluated using the conditions, reagents, and protocols recommended in the kits instructions, as well as with less stringent conditions than those recommended in the kit instructions. Probe 1 bound to amplified mtDNA at low stringency conditions, with greater probe amount, with heat denaturation of the probe, and more HRP-SA enzyme conjugate added than recommended in the protocol. Probe 1 did not hybridize to mtDNA extracted from a buccal swab, bloodstain cards, or a whole blood sample. Probe 2 did not show any hybridization to mtDNA when evaluated.

THE DEVELOPMENT AND EVALUATION OF AN OLIGONUCLEOTIDE PROBE FOR USE IN QUANTIFYING HUMAN MITOCHONDRIAL DNA USING THE QUANTIBLOT® HUMAN DNA IDENTIFICATION KIT

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July 2003

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the Graduate School of Biomedical Sciences University of North Texas Health Science Center at Fort Worth in Partial Fulfillment of the Requirements for the Degree of MASTER OF SCIENCE

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CHAPTER 1

INTRODUCTION AND BACKGROUND

A method for identifying and quantifying human mitochondrial DNA may be of use in the field of forensic DNA analysis. There is currently a kit available that will allow for the quantification of human nuclear DNA based on a slot blot hybridization procedure called Quantiblot® manufactured by Applied Biosystems. In this particular kit there is an oligonucleotide probe that will specifically bind to human nuclear DNA at a particular sequence on chromosome 17. A signal produced in subsequent reactions can be used to quantify DNA of unknown amount based on comparison to a set of known standard amounts. The main goal of this research project is to identify a sequence for an oligonucleotide probe that will specifically bind to human mitochondrial DNA for purposes of quantification. The probe will be designed based on sequence searches from DNA databases and will be evaluated using the procedures and equipment outlined in the Quantiblot® kit instructions. If necessary the parameters in the protocol will be adjusted to obtain proper probe binding and usable signals.

Mitochondria and the Mitochondrial DNA Genome

In addition to the nuclear DNA genome there is a mitochondrial DNA genome found in subcellular organelles called mitochondria. Mitochondria, located in the cytoplasm of cells are cylindrical in appearance and can range in size from 0.5um to 1.0um in length (1). Mitochondria have a double membrane structure consisting of an outer membrane and an inner membrane. The outer membrane consists of phospholipids normally associated with a phospholipid bilayer membrane and a protein known as porin that forms channels in the membrane allowing larger sized molecules into the mitochondrial inner membrane space, the space located between the outer and inner membranes (1). The inner membrane's lipid bilayer contains the phospholipid cardiolipin, which has four fatty acid tails instead of two. This membrane is impermeable to ions but contains proteins necessary in allowing the transport of small molecules into the matrix of the mitochondria (1). The inner membrane forms a number of folds called cristae that increase the overall surface are of the membrane. The process of oxidative phosphorylation occurs on the inner membrane where there are electron transport enzymes that transport electrons for use in generating ATP. In cells where a greater ATP production is required there will be larger surface area from a larger number of cristae (1). The matrix is a large internal space that contains the enzymes required for the citric acid cycle as well as the mitochondrial DNA genome, mitochondrial ribosomes, tRNAs and other enzymes required for the expression of mitochondrial genes, or coding regions (1).

The proteins subunits required for the biosynthetic processes in the mitochondria are encoded in the nuclear genome as well as the mitochondrial genome. The proteins subunits that are encoded in the nuclear genome are synthesized in the cytosol and inserted into the inner mitochondrial membrane whereas the other proteins and components required for mitochondrial synthesis functions are encoded in the mitochondrial genome and synthesized on ribosomes located within the mitochondrial matrix (1). The mitochondrial genome is a double-stranded, closed circular DNA molecule that in humans contains approximately 16, 569 base pairs (6). The components that are encoded by the mitochondrial genome consist of 2 ribosomal RNAs, 22 transfer RNAs, and 13 proteins (6). There is also a region known as the control region of approximately 1,100 base pairs that is non-coding (6). The control region contains the displacement loop, which serves as the origin of replication for the mtDNA genome. This region contains two hypervariable regions known as hypervariable region 1 (HV1) and hypervariable region 2 (HV2) that are valuable in forensic analysis because they show a high degree of polymorphism, or variability between individuals (7). The double-stranded mtDNA molecule has a heavy strand, which is purine rich that contains a higher percentage of the bases adenine and guanine. The light strand is pyrimidine rich having a higher percentage of cytidine and thymine (6).

Another feature of the human mitochondrial genome that makes it unique from nuclear DNA is that it has very dense gene packing with the vast majority of the nucleotides found in coding sequences for proteins, tRNAs, or rRNAs (1). Mitochondrial DNA has a relaxed coding scheme as compared to nuclear DNA. Whereas 30 tRNAs are

used to specify amino acids for protein synthesis in nuclear DNA only 22 tRNAs are utilized by the mtDNA genome. Many of the tRNA molecules will recognize any nucleotide in the 3rd codon position, "wobble" position, so that one tRNA molecule is capable of binding to four different codons allowing for a conservation of tRNAs (1). Also, the mtDNA genetic code, the sequence arrangement specifying the correspondence between nucleotide triplets or codons in DNA and RNA and amino acids from proteins, varies slightly from that of the nuclear DNA code. It has been found that in human mtDNA there are four codons that code for amino acids different from those of the human nuclear genome and other species genomes (1).

Mitochondrial DNA has other features that are of considerable importance when used for forensic analysis. One feature that makes it desirable for use in forensic casework analysis is its high copy number (6). Each mitochondria can have multiple copies of the mtDNA genome and there are multiple numbers of mitochondria in individual cells because mitochondrial reproduction, or division into two mitochondria from one, is independent of the cell cycle and can occur at higher rates depending on whether they are located in tissues with high energy needs or not (1). In cells with high energy needs, such as cardiac muscle tissue there can be thousands of mitochondria, whereas one nucleus is present in cells containing one copy of nuclear DNA genomes, bi-parentally inherited from the mother the father. High copy number of mtDNA makes it attractive to forensic DNA analysis when there are samples with very small amounts of nuclear DNA or very badly degraded DNA in which standard nuclear DNA typing procedures would not prove useful (6).

DNA from mitochondria is amplified with mtDNA specific primers bound to the mtDNA present in a genomic extract containing nuclear DNA and mtDNA. When cells are subjected to DNA extraction procedures, the conditions and reagents utilized serve to lyse, or break open the cellular, nuclear, and mitochondrial membranes. The most common method is the addition of stain extraction buffer (SEB) and Proteinase K to the intact cellular material and incubation at moderate heat. Stain extraction buffer contains a detergent, normally SDS (sodium dodecyl sulfate) that acts to individually surround the lipid components of the phospholipid bilayer, that membranes are composed of, separating them from each other and breaking the membrane open. The SEB also contains the salt NaCl, which also helps in disrupting the membranes, and EDTA, which binds to and sequesters cations required as cofactors for nucleases (6). Proteinase K is an enzyme that will digest proteins contained in the lysed cellular material such as membrane proteins and histone proteins utilized in DNA coiling. The heated water bath serves to denature the proteins in order to facilitate digestion by the Proteinase K. Once these membranes are lysed the nucleic acids or DNA are released into solution and can be separated from the organic cellular components such as proteins and lipids by the addition of organic solvents in the case where an organic extraction method is implemented (6). The most common solvent combination used is phenol:chloroform:isoamyl alcohol. The phenol serves as the solvent that will separate protein residues, which are soluble in phenol from the nucleic acids, the chloroform will separate lipid from the nucleic acids and act to clear up residual phenol, and the isoamyl alcohol helps to prevent foaming by reducing the vapor pressure produced by chloroform out-gassing. The nucleic acids are

then concentrated by precipitation in ethanol. Nucleic acids are not soluble in ethanol and will precipitate, thus allowing for purification and concentration of DNA (6).

Since mitochondria are composed of a double membrane system, the mtDNA within the mitochondria is more protected than nuclear DNA from the conventional cell lysis procedure. This is one reason that mtDNA is evaluated when dealing with badly degraded or low copy number nuclear DNA samples. Red blood cells present in whole blood samples do not contain nuclei, but do contain mitochondria. A saline sodium citrate with NaCl (SSC) is used during DNA extractions from whole blood to disrupt the red blood cell membranes leaving the white blood cell membranes intact (6). For this process the intent is to lyse the red blood cells to release hemoglobin-containing heme, a downstream PCR inhibitor, and to pellet intact white blood cells for further lysis (6). If whole blood is subjected to a procedure in which the red blood cells are lysed followed by the further lysis from the conventional lysis procedure, a greater amount of mtDNA may be released from the more resilient mitochondrial membranes.

When using DNA as a means for human identification the regions of DNA with high levels of polymorphism or variability between individuals are of the most interest. The mtDNA molecule does not undergo recombination, as is the case for nuclear DNA (6). Recombination is an occurrence where the linear arrangement of nucleic acid sequences in a chromosome is altered by cleaving and rejoining (13). This process is in part responsible for the variability of an individual's genetic makeup. The mtDNA genome however does have regions of high variability. One reason for this variability is that the mtDNA genome has a much higher mutation rate that that of the nuclear DNA

genome in part due to the low fidelity of mtDNA polymerase and the apparent lack of mtDNA repair mechanisms (6). It is estimated that there are on average eight nucleotide differences between individuals for unrelated Caucasians and fifteen for unrelated individuals of African descent (6). The regions of most interest in forensic casework are in the control region. This is a region of approximately 1,100 base pairs that contains two segments known as HV1 (hypervariable region 1) and HV2 (hypervariable region 2) extending from the 16024bp to 16365bp and 73bp to 340bp locations, respectively. The numbering of the base pairs is based on the Anderson or Cambridge reference sequence (2). These regions have been shown to vary between individuals at a range from 1 to 3% (7). The mtDNA genome sequence is considered to be a haplotype because it is haploid meaning the DNA is inherited through one individual lineage. Nuclear DNA is considered to be diploid because the DNA is contributed from two individuals having differing alleles, and the genome's genetic makeup is referred to as a genotype. In the case of mtDNA in mammals the mother of the offspring is the contributor. The mother contributes the mtDNA to the offspring because the ovum contributes the vast majority of cytoplasm to the zygote, thus contributing all of the mitochondria and the mtDNA genome (7). For forensic purposes, the mtDNA is sequenced for the HV1 and HV2 regions, and the sequence is referred to as an individual's haplotype. The haplotype of an individual can be compared to haplotypes located in mtDNA databases of unrelated individuals for the purpose of determining a relative frequency of the haplotype (7).

Another occurrence of note in mtDNA is the phenomena of heteroplasmy in which more than one mtDNA type is present in an individual (7). There are two types of heteroplasmy. One is sequence heteroplasmy where more than one base is visualized at a particular site in the sequence. An example of sequence heteroplasmy would be if a cytidine and thymine were present at the same location. The second type is length heteroplasmy, where there are differing lengths of a repeat sequence at a particular position.

Quantification

Quantifying the amount of DNA for use in forensics is important for determining the appropriate amount of DNA to input into the polymerase chain reaction (PCR) required for DNA amplification. PCR is a procedure for DNA amplification in which an oligonucleotide primer, complimentary to a sequence in the template DNA's region of interest, deoxynucleotide triphosphates containing the four bases required in DNA sequences, buffers, and a Taq DNA polymerase are utilized in creating copies of particular DNA sequences (8). In PCR, it has been demonstrated that there is an optimal amount of template DNA necessary to produce sufficient amplification product, which produces the fewest artifacts or stochastic effects (7). These effects are phenomena in which too little or too much DNA can influence how the template is amplified. An example of these affects includes high background in which there is non-specific binding of the primers to the DNA usually caused by too high input DNA. For PCR using nuclear

DNA as a template for amplification, the recommended amount of input DNA, or template is, typically about 1ng of DNA (6). It is recommended that a minimum of 100pg of extracted DNA, which is normally a mixture of nuclear and mitochondrial DNA, is input into the PCR when mtDNA is used as the template for amplification (6). Several methods for quantifying DNA have been developed and implemented since its discovery. Among these methods is ethidium bromide fluorescent visualization for double stranded DNA (6). For this method a series of dilutions are prepared from a known concentration of DNA. A 2% agarose gel is then typically prepared with ethidium bromide added and allowed to polymerize. A DNA ladder that contains fragments of differing sizes, the series of dilutions and the unknown DNA sample are loaded into wells located in the gel and electrophoresed. This method works by combining the separation of the DNA by electrophoresis and the addition of the fluorescent ethidium bromide to determine relative fragment size, relative quantity from comparison of signal intensity with that of the known standards, and relative quality of DNA in an unknown sample. These gels are commonly referred to as yield gels. For this method, the ethidium bromide is a fluorescent molecule that intercalates, or inserts between stacked aromatic and planar rings, located between the stacked DNA base pairs. When the intercalated DNA is exposed to ultra violet light the ethidium bromide fluoresces and that fluorescence can be used as a means of DNA quantity estimation because the amount of fluorescence is proportional to the total mass of DNA present, if a quantitative mass ladder is used. When electrophoresed the DNA molecules present in the ladder, the standard dilutions, and the unknown samples are separated based on charge and size. DNA is a negatively

charged molecule especially in higher pH buffers because the phosphate backbone of the DNA readily gives up hydrogen ions. When exposed to an electric current and in the presence of buffer containing sufficient ions for conduction, the DNA will migrate from a negatively-charged electrode to a positively-charged electrode. As the DNA passes through the agarose medium, the smaller fragments pass through the sieve faster than longer DNA fragments. For most yield gels the buffer used is Tris Acetate EDTA (TAE) at a 1X concentration because it provides better resolution for longer sized DNA fragments. A common ladder used is the lambda DNA marker with fragments of sizes ranging from 23,130 base pairs down to 125 base pairs. Normally the standards and the unknown samples will produce a band at the highest molecular weight marker. This indicates higher quality DNA that is not severely degraded or fragmented. The intensity of the band can be compared to those of the standards to generate an estimate of the unknown DNA concentration. This method is often used for determining extracted DNA concentration so that the ideal amount of DNA can be used for the PCR reaction. This method, however, is not species specific and DNA of different types such as nuclear and mitochondrial DNA can not be distinguished.

For DNA casework where nuclear DNA is typed for individualization, it is mandated in the National Standards that there be an attempt at quantifying the amount of human DNA in a sample (12). Yield gels are not sufficient. For this reason, methods have been developed for quantifying human nuclear DNA by hybridizing human-specific oligonucleotide probes to the extracts of DNA. One such method is called slot blot hybridization in which a probe is hybridized to a complimentary sequence at a particular

locus, such as the primate-specific D17Z1 locus on chromosome 17 (16), on the DNA after the DNA has been denatured and blotted onto a nylon membrane. There is currently a kit, the Quantiblot® Human DNA Identification Kit from Applied Biosystems that utilizes this process (3). In the Quantiblot® procedure, standard DNA samples of varying concentrations and DNA of unknown amounts are blotted onto a nylon membrane and immobilized. A biotinylated probe provided in the kit binds to complementary DNA sequence on the D17Z1 alpha satellite locus on chromosome 17 (16). This locus is specific to higher primates. An enzyme conjugate, horseradish peroxidase-streptavidin, is bound to the biotin on the probe following hybridization. Depending on which scheme is used, colorimetric, chemifluorescent, or chemiluminescent, a reaction is produced that generates a means of visualization. In the case of chemiluminescent detection, a luminol-based reagent will be oxidized in a reaction catalyzed by the horseradish peroxidase. This oxidation results in the emission of photons that can be detected on autoradiography film. The quantity of the sample DNA is determined by comparing the intensity of the resulting signal with those of the standard DNA dilutions.

Probe Development and DNA Hybridization

Certain aspects of probe development and DNA hybridization must be taken into consideration for a probe to be developed that, in theory, has the capability of being incorporated into the Quantiblot® kit procedure and used to quantitate human mitochondrial DNA. The probe included in the Quantiblot® kit is a biotinylated

oligonucleotide probe that is 40 nucleotides in length and will bind to single stranded DNA immobilized on a nylon membrane (3). This sequence is higher primate specific, which means that there is a particular alpha satellite sequence on chromosome 17 to which the probe will bind that is present in the nuclear DNA genomes of higher primates, such as chimpanzees, gorillas, and orangutans (16). The probe has a GC content of 35 % indicating that of all nucleotides present in the 40-nucleotide sequence 35% of them are guanidine and cytosine (3). Normally DNA sequences with higher GC contents will bind to complementary DNA strands more tightly than DNA sequences of lower GC contents because the G-C base pairing requires three hydrogen bonds making for stronger binding (17). The lower GC content of this probe indicates that it may not bind very tightly to target DNA so that enough DNA can be bound to produce a visual signal. The melting temperature of the D17Z1 probe is 79°C in a solution containing 1M Na+ (3). The melting temperature is the temperature at which 50% of the hybrids are dissociated (17). Normally when designing an experiment using probe hybridization it is recommended that the hybridization reaction temperature be 15-20 degrees Celsius lower than the Tm of the probe (17). The hybridization temperature and wash temperature for the D17Z1 probe in the Quantiblot protocol is 50°C (3).

In order to design a probe that can be used under similar conditions and with the same equipment, currently in place for Quantiblot® the probe must be designed to be chemically similar to the probe already included. This means that the probe must have a melting temperature very similar to that of the D17Z1 probe, as well as a similar GC

content. Different probes can be used in hybridization procedures such as RNA probes, long single-stranded DNA probes, double stranded DNA probes, and oligonucleotide probes. Oligonucleotide probes, usually between 20 and 40 bases in length can be readily synthesized from specific probe sequences designed from published sequences (17). They are usually labeled by incorporating modified nucleotides during chemical synthesis or by adding a tail of labeled nucleotides (17). A disadvantage of oligonucleotides is that they are less sensitive than longer nucleic acid probes.

Hybridization and washing conditions must also be considered when designing a probe. One consideration is that there are many factors that can influence the melting temperature of a probe. The nature of the probe and target sequences can influence the melting temperature. RNA:RNA hybrids are the most stable followed by RNA:DNA hybrids and finally DNA:DNA hybrids (17). The length of the probe should also be considered. Longer probes will form more stable hybrids (17). As mentioned above the GC content must also be evaluated. A greater GC content will increase the melting temperature of the probe (17). The homology between the probe sequence and its target sequence is also important. More homology in the sequences will allow for more stable hybrids (17). The hybridization and washing solutions must also be considered. Higher concentrations of monovalent cations, normally sodium ions will increase the stability of hybrids (17). If formamide is included in the solutions it will decrease the Tm allowing for lower temperatures to be implemented in achieving specificity of binding (17).

The kinetics of the hybridization also affect how the probe will bind to target sequence. If the concentration of probe is increased there will be more binding to the

target producing greater signals, however if the addition of probe is excessive the target may be saturated and the signal will contain background or non-specific binding (17). The stringency of hybridization is another parameter that will affect the binding. Stringency can be increased or decreased by adjusting the temperature during hybridization. Also fluctuations in salt concentration will affect the stringency of binding. There are also certain chemicals that will enhance binding stringency or decrease it. When the probe and target undergo washing, the non-specific binding of the probe, in which the probe binds to sequences to which it is not specifically intended, such as partial sequences, will be washed away. The probe is usually washed at moderate or high stringency to remove most of the non-specific binding. Probes that are high in A-T content will give poor signals if they are washed at too high a stringency (17).

A probe for use with the Quantiblot® kit would need to be labeled with biotin at the 5' end, as is the case with the D17Z1 probe. Biotin is a molecule that can be used as a tag for oligonucleotide or DNA probes. Biotin is a stable molecule and has a high affinity for streptavidin, a molecule that can be conjugated to horseradish peroxidase to produce reactions allowing for signal detection (9). In the Quantiblot® procedure, the streptavidin of the HRP-streptavidin complex binds to the biotin label on the probe. The horseradish peroxidase will make use of hydrogen peroxide to oxidize certain chemicals that will either produce a colored precipitate as is the case with chromagen:TMB used for colorimetric detection or in the emission of photons as is the case when a luminol-based reagent is oxidized (3).

If the probe designed for use in detecting and quantifying human mitochondrial DNA is similar in melting temperature, GC content, size to the D17Z1 probe and is labeled with biotin, theoretically it should be capable of being used with the Quantiblot® kit under similar conditions.

Species-specific Regions in Mitochondrial DNA

In addition to considering the similarity of the probe with that of the D17Z1 probe and the conditions for hybridization, it is of most importance to attempt to identify a probe sequence that will be specific to human mitochondrial DNA. There are currently methods involving the evaluation of mtDNA regions for species identification. One region that is commonly evaluated is the cytochrome b gene. Species determination on the basis of the phylogenetically variable cytochrome b gene is possible by sequencing of the cytochrome b DNA fragment (4). Species identification methods currently in use require amplification of mtDNA along with sequencing of the regions of interest to determine species affinity. In addition to cytochrome b, other regions in the mitochondrial genome have been evaluated. The D loop region of the mtDNA genome, normally outside of the hypervariable regions has been used as a target sequence for primers used to amplify the hypervariable regions for forensic identification purposes. These primers are considered human specific in that they will bind to human mitochondrial DNA for amplification.

For a probe to be developed that would bind specifically to human mitochondrial DNA for quantitation purposes, the sequence of the probe would have to be complementary to a sequence in the mitochondrial genome that is not highly conserved between humans and other species, but that is highly conserved within the human population. An ideal, conserved sequence would be one that has withstood considerable evolutionary changes over time and that is present in all humans. The hypervariable regions located in the control region would not be ideal candidates because these sequences are highly variable from one maternal lineage to another. A possible reason for this variability is that the region is non-coding and not under any selective pressure. As mentioned earlier, the mtDNA genome has a much higher mutation rate than that of the nuclear DNA genome. The entire mtDNA genome is subject to these mutational changes. Because only about 16,500 DNA nucleotides need to be replicated and expressed as RNAs and proteins in animal cell mitochondria, the error rate per nucleotide copied by DNA replication, transcribed by RNA polymerase, or translated into protein by mitochondrial ribosomes can be relatively high without damaging one of the relatively few gene products (1). It would be more tolerable in the genome if a mutation were to occur in a non-coding region than in one of the coding regions because the genes in the mtDNA are required for energy production, which is vital for survival. For this reason, the coding regions of the mtDNA genome would be of the most interest in developing a human specific mtDNA probe. It may be difficult to identify sequences in the human mtDNA genome that are both highly conserved in humans but not conserved between humans and other species. Researchers have found that there are differences in human

mtDNA coding sequences in which natural selection may have played a role in shaping regional mtDNA variation (10). It has been discovered that certain coding regions that are normally highly conserved when compared with sequences from distant species can have amino acid sequence variation when compared with other human mitochondrial genomes. In a study where climate was predicted as a factor in natural selection in gene coding regions of the mtDNA genome, it was found that the ATP6 gene was highly variable in mtDNA sequences from the Arctic zone, the cytochrome b gene was particularly variable in the temperate zone, and cytochrome oxidase I was variable in the tropics (10). These amino acid sequence changes stemming from mtDNA sequence variation also appeared to be functionally significant. This demonstrates that even though the genes coded for by the mtDNA genome are necessary for survival and will usually be conserved there are factors that can serve to alter their function thus making their sequences variable.

CHAPTER 2

MATERIALS AND METHODS

BLAST® Searches

BLAST® (Basic local Alignment Search Tool) searches can be used to search a particular DNA sequence against databases (usually Genbank) of published DNA sequences. The published DNA sequences in these databases are from mtDNA and nuclear DNA that have been sequenced from a variety of different species. The BLAST® search engine from the National Center for Biotechnology Information (NCBI) website, www.ncbi.nih.gov was used. NCBI was established in 1988 as a national resource for molecular biology information that creates public databases, conducts research in computational biology, develops software tools for analyzing genome data, and disseminates biomedical information (11).

The NCBI website's homepage has a search engine containing a pull-down menu with a list of databases that can be used for searches. In order to locate human mitochondrial DNA sequences of interest, the phrase 'human mitochondrial DNA sequences' was input into the search window. From the database pull-down menu, the *Nucleotide* database was selected. In some instances phrases including a certain mtDNA-coding region was input into the search window, such as 'human mitochondrial DNA

cytochrome b sequence'. Once the input phrase was searched a list of published sequences were displayed. The sequences used were listed as JO1415 human mitochondrion, complete genome, and AF234896 Homo Sapiens cytochrome b gene, complete cds; mitochondrial gene for mitochondrial product. The AF234896 contained the complete coding sequence for the cytochrome b sequence. The JO1415 sequence contained the entire human mitochondrial genome divided into the coding regions for the proteins, tRNAs, and rRNAs. The cytochrome b sequences listed in the two sequence printouts were identical.

For the BLAST® searches, the sequences of coding regions for the proteins encoded for by the mtDNA were highlighted from the JO1415 sequence. The highlighted sequences were then copied into a search window located at the BLAST® site on the NCBI website. In order to get to the BLAST® search engine from the homepage, *BLAST* was selected from the menu bar at the top left of the screen. At the BLAST® home page there are options for nucleotide and protein BLASTS. Under the nucleotide BLAST® search option '*standard nucleotide-nucleotide BLAST*' was selected. Once this option was selected, the highlighted sequence was pasted into a search window located at the top of the screen. Under the search window there is an option to where a subsequence of the entire sequence pasted into the search window can be searched against databases for a more refined search of the region of interest. The starting nucleotide position of the subsequence and the ending nucleotide of the subsequence must be input into the

appropriate windows. Directly below the subsequence search option is a pull-down menu with a list of databases that the BLAST® search can be performed in.

The sequences of all of the coding regions located within the mtDNA genome were searched against three of the databases present in the pull-down menu. The three databases were *est_others*, *est_mouse*, and *est_human*. The sequence of one coding region was copied and pasted into the BLAST® search window, the *est_others database* was selected and a BLAST® search was performed by selecting the *BLAST* button. A list of sequences and species possessing the sequence were displayed for regions in the sequence that aligned, or matched the input sequence. If there were any regions in the sequence that showed no alignment or little alignment then the subsequence search option was used to search the database in a narrower range of sequence that included the region with little alignment. The subsequence was then searched again against the *est_others database*. If a particular sequence was found that had little to no alignment with sequences from this database the same sequence was searched against the *est_mouse database*. If needed the subsequence search option was used for a more refined search. If it was found that a particular sequence had few to no significant matches in the *est_others* or *est_mouse databases*, the sequence was searched against the *est_human database* in order to confirm that the sequence of interest had the potential for conservation within the human population. The search process was performed for each of the coding regions in the mtDNA genome including the coding regions for proteins, tRNAs, and rRNAs.

DNA Extractions

DNA extractions were performed on samples collected from a human male volunteer from different substrates using a phenol-chloroform DNA extraction protocol outlined below (14). DNA was extracted from a buccal cell swab containing epithelial cells from the cheeks and gums of the mouth, from bloodstains blotted onto Whatman filter paper blood cards, and from the red blood cell portion of whole blood.

For the DNA extracted from the blood cards and the buccal swab, pieces of the substrates were cut away using a sterile technique and placed into labeled tubes; designated JC, the number of the sample, and the date. For each extraction there were a JC1 and a JC2 containing pieces of the sample substrate, and a reagent blank in which all of the reagents required for the subsequent extraction were added without any source of DNA present, or no substrate to monitor for extraneous contamination. The sterile technique used for the buccal swab cuttings included rinsing scissors with 70% ethanol and distilled water, drying the scissors with a kimwipe, and cutting the substrate into each of the two sample tubes. For the samples isolated from the blood cards a standard one-hole punch was used. A sheet of FTA™ paper containing, strong buffers, protein denaturants, chelating agents, and a UV-absorbing, free radical trap, was punched twice with the hole puncher followed by the punching of a blood stained portion of the card into the individual sample tubes.

Once the sample substrates were placed into their respective tubes, the following protocol, provided by Orchid Cellmark-Nashville was followed to extract and purify the DNA present in the cells located on the sample substrates.

1. 500ul of Stain Extraction Buffer (Tris, NaCl, EDTA, 10% SDS, distilled water) and 25ul of Proteinase K (20mg/ml) were added to the tubes. The tubes were capped, briefly vortexed, and centrifuged for 5 sec at 14,000 RPM to force any substrate into the bottom of the tubes.
2. The samples were then incubated at 56° C for a period ranging from 2 hours to 24 hours.
3. The samples were then centrifuged to force any substrate and condensation down into the tube.
4. 500ul of phenol:chloroform:isoamyl alcohol at a ratio of 25:24:1 was added to each of the tubes. The tubes were vortexed until a milky emulsion was achieved, and centrifuged at 14,000 RPM for 5 minutes.
5. New tubes were labeled and the upper aqueous layer from the sample tubes that were centrifuged were transferred using a transfer pipette to the newly labeled tubes. The tubes containing the bottom organic layer were discarded.
6. 1.0 ml of cold 100% ethanol was added to the tubes, which were capped and mixed by inverting.
7. The tubes were placed in a freezer at –70 degrees Celsius for a minimum of 30 minutes.

8. The tubes were removed from the freezer and centrifuged at 14,000 RPM for 10 minutes.
9. The ethanol was decanted from the tubes by use of a transfer pipette without disturbing the pellet located at the bottom of the tube.
10. 1.0 ml of 70% ethanol at room temperature was added to each of the tubes, which were then centrifuged again at 14,000 RPM for 10 minutes.
11. The ethanol was again decanted.
12. The tubes were placed in a speed vac centrifuge for 30 minutes in order to dry the pellet
13. There were two extractions performed on blood from blood cards at separate times. For these extractions the DNA pellets were resuspended in 30ul of TE^{-4} (Tris-HCl, .1mM EDTA) and 200ul DNA, respectively. The extraction from the pellet extracted from the buccal swab was resuspended in 300ul of TE^{-4}. The resuspended samples were then incubated for 2 to 24 hours in a 56°C water bath.
14. The samples were removed, briefly centrifuged and stored at 20°C until further use.

The DNA from the whole blood was extracted by the following procedure provided by Orchid Cellmark-Nashville:

1. Blood was taken from the bottom layer of a vacuum tube containing blood and aliquoted into two micro-centrifuge sample tubes JC1 and JC2

2. The samples were centrifuged for 5 minutes at 14,000 RPM and the top, clear layer from each tube was removed and discarded.
3. Step 2 was repeated
4. 1.0 ml of SSC (Saline Sodium Citrate with 0.5mM EDTA) at a concentration of 1X was added to the sample tubes, which were capped and placed in a freezer at –70°C for 20 minutes.
5. The samples were centrifuged at 14,000 RPM for 5 minutes and the supernatant liquid was removed and discarded.
6. 1.0 ml of SSC was again added and the sample tubes were centrifuged at 14,000 RPM for 5 minutes.
7. The supernatant was once removed and discarded
8. Steps 1 through 14 from the above listed protocol were then performed

DNA Quantification

The DNA that was extracted was quantified using yield gels in order to estimate the amount of total DNA that was present in each of the extracts. The following protocol was followed for each yield gel.

1. Yield gel tubes were labeled for each sample including the reagent blank.
2. Four micro-liters of loading buffer (0.1% bromophenol blue, 0.1M EDTA in TE^{-4} and 50% glycerol) was pipetted into each tube along with 8ul of the DNA

sample suspended in TE. Pipetting up and down mixed the loading buffer and sample.

3. A solution of dissolved 2% agarose, 1X TAE (0.8M Tris, 0.4M Acetic acid, 0.5M EDTA) and ethidium bromide stored in a 56 degrees Celsius water bath was poured into a gel cast tray with edges taped. A well comb was inserted into the solution inside of the tray. The solution was allowed to polymerize.
4. The gel cast tray, with gel, was inserted into an electrophoresis chamber following the removal of the tape on the edges of the tray. The electrophoresis chamber contained 1X TAE buffer that covered the entire gel.
5. A set of prepared DNA dilution standards of human DNA at concentrations of 20ng/ul, 15ng/ul, 10ng/ul, 5ng/ul, 2.5ng/ul and 1ng/ul and a Hind III restricted Lambda DNA fragment ladder were added to the appropriate wells in the gel along with the extracted DNA at a volume of 5ul.
6. A plastic covering was placed over the chamber and the positive and negative nodes from the chamber were plugged into the corresponding ports on the power output device.
7. The power supply was turned on and a voltage of 200 volts was applied to the gel for approximately 15 minutes, unless more separation was desired in which case extra time was allowed.
8. The current in mili-amps was recorded for each gel

9. After fifteen minutes the power supply was turned off and the gel was removed from the chamber to be exposed to UV light from a UV trans-illumiminator.
10. Once the gel was exposed to UV light a photograph of the gel was taken and given approximately a minute to develop.
11. The intensity of the bands located at the highest molecular weight marker from the DNA extractions were compared with those of the standard DNA dilution concentration in order to estimate the concentration of DNA present.

The protocol described in the Quantiblot® kit instruction manual (3) was followed for quantifying the nuclear DNA present in the DNA extracts in order to evaluate the reliability of the estimate from the yield gels and to serve as a control for the procedure making sure that it was functioning properly. The same protocol was followed using the possible human-specific mitochondrial DNA probes instead of the nuclear DNA probe included in the kit in order to identify mtDNA present in the sample extracts and to possibly quantify it. Certain parameters of the protocol were altered when using the mitochondrial probes in further experiments. The following is the manufacturer's recommended protocol for the Quantiblot® kit that was used.

1. All reagents required for the procedure were prepared as recommended in the Quantiblot® instruction manual (3) if they were not already prepared.
2. Seven 1.5-ml microcentrifuge tubes were labeled A through F (A-2.0ng/ul, B-1.0ng/ul, C-0.5ng/ul, D-0.25ng/ul, E-0.125ng/ul, F-0.0625ng/ul) for the

standard dilutions with the corresponding concentration of human genomic DNA.

3. The DNA standard A provided in the kit was vortexed and 120ul of it was added to the tube labeled A.
4. Sixty microliters of TE^{-4} buffer was added to the remaining tubes.
5. Sixty micro-liters of Standard A was added to the tube labeled B. Sixty micro-liters of Standard B was added to the tube labeled C. This process was repeated down to tube F with a concentration of .0625ng/ul of DNA.
6. The number of tubes required for the samples, standards, and calibrators (known DNA concentrations falling between standard amounts prepared at concentrations of 0.7ng/ul, and 0.15625ng/ul) were determined, labeled, and 150ul of spotting solution was added to each tube.
7. The standards, calibrators, and samples were vortexed and 5ul of each was added to the tubes containing the 150ul of spotting solution.
8. A Biodyne positively charged membrane was placed in a hybridization tray containing 50ul of pre-wetting solution for anywhere from 1 to 30 minutes.
9. Using forceps sterilized with 70% ethanol, the nylon membrane was removed from the hybridization tray and placed onto the gasket of the slot blot apparatus. The top of the apparatus was placed over the membrane. The vacuum source attached to the apparatus was turned on. The sample vacuum was checked to make sure it was in the off position. The clamp vacuum was turned on to form a tight seal over the membrane.

10. With a new pipette tip for each sample the standards, calibrators, and DNA samples all in spotting solution were pipetted into their appropriate slots on the membrane. The full 155ul was added to each well.
11. The sample vacuum was turned on for approximately 30 seconds until a uniform blue band was observed in each well.
12. The sample vacuum was turned off and the membrane was removed from the slot blot apparatus and placed into the hybridization tray containing100ul of pre-warmed (56°C) hybridization solution
13. 5 ml of 30% hydrogen peroxide were added to the hybridization solution and the tray was placed in a rotating 50°C water back for 15 minutes.
14. The solution was poured out of the tray
15. Thirty mililiters of hybridization solution (5X SSPE, 0.5% w/v SDS) was added to the tray containing the membrane.
16. Twenty micro-liters of probe (1pmole/ul) was added to the tray. When three or two separate membranes were being run for the different probes three or two different trays were used. Each probe, D17Z1, P1 (Probe 1 ordered from Invitrogen™ life technologies), and P2 (Probe 2 ordered from Invitrogen™ life technologies) were all at concentrations of 1pmole/ul.
17. The tray was rotated in a 50°C water bath for 20 minutes.

18. The solution was poured off and the membrane was washed briefly in 100ml of pre-warmed 56°C wash solution (1.5X SSPE, 0.5% SDS) and the wash solution was poured off.
19. A strict wash/conjugation was performed in which 30ml of the pre-warmed wash solution was added to the tray along with 90ul of the horseradish peroxidase-streptavidin (HRP-SA) conjugate for the chemiluminescent detection.
20. The tray was rotated in a 50°C water bath for 10 minutes.
21. The solution was poured off and rinsed for 1 minute in 100ml of pre-warmed wash solution. The solution was poured of and the membrane was rinsed again for 1 minute in 100 ml of wash solution.
22. The solution was poured off and 100ml of pre-warmed wash solution was added to the tray. The tray was placed on an orbital shaker and allowed to shake for 15 minutes at room temperature.
23. The solution was poured off and 100ml of citrate buffer (0.1M Sodium Citrate, pH 5.0) was added to the tray and rocked briefly.
24. For the chemiluminescent detection procedure 5ml of ECL reagent 1 and 5ml of ECL reagent 2 were mixed thoroughly and added to the membrane after the citrate buffer was poured off. The membrane was exposed to the ECL reagent for two minutes then the ECL reagent was poured off.
25. The membrane or membranes were sealed in plastic laminate and exposed to X-ray film in a dark room for approximately 1-hour at room temperature.

26. After 1 hour of exposure the film was placed in a developer following two test pieces of film.

27. Once the film was developed it was cut to the proper size and stored.

The probes P1 and P2 listed above are oligonucleotide probes designed to be complimentary to specific sequences in the human mtDNA genome. Both of the probes were ordered from Invitrogen™ life technologies in order to evaluate their effectiveness in hybridizing to and quantifying human mtDNA.

The Quantiblot® protocol above was used for the following membranes to test the following alterations in protocol parameters.

Membrane 1:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	B1	C1	D1	E1	F1	C1	C2	SSB			
B	A3	B3	C3	D3	E3	F3						
C	A2	B2	C2	D2	E2	F2	G2	H2	I2			
D												
E												
F												
G												
H												

Figure 1: Membrane 1. The letters A1-F2 in row A represent the standard dilutions from the standard A provided in the kit (2.0ng/ul, 1.0ng/ul, 0.5ng/ul, 0.25ng/ul, 0.125ng/ul, and 0.0625ng/ul, respectively). C1 and C2 stand for calibrator 1 (0.7ng/ul) and 2 (0.15625ng/ul) (between B1 and C1, and between E1 and F1, respectively). SSB stands for spotting solution blank. A3 through F3 are dilutions of JC1 buccal swab DNA matching those of A1-F1 based on the concentration estimate of the yield gel. A2 through I2 are serial dilutions of the JC1 buccal swab (12ng/ul, 6ng/ul, 3ng/ul, 1.5ng/ul, .75ng/ul, .375ng/ul, .1875ng/ul, .09375ng/ul, and .046875ng/ul, respectively).

Parameters: The protocol listed above was followed without any deviations

Membrane 2:

D17Z1 P1 P2

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	C1			A1	C1			A1	C1		
B	B1	C2			B1	C2			B1	C2		
C	C1	SSB			C1	SSB			C1	SSB		
D	D1	JC1			D1	JC1			D1	JC1		
E	E1	mtDNA			E1	mtDNA			E1	mtDNA		
F	F1				F1				F1			
G												
H												

Figure 2: Membrane 2. For descriptions refer to figure 1. JC1 represents the DNA extracted from the buccal swab. The mtDNA represents amplified mtDNA containing approximately 16.3kb of the human mtDNA genome obtained from Dr. James Sligh at Vanderbilt University. Five micro-liters of JC1 and the mtDNA at concentrations of 1ng/ul were spotted onto the membrane.

Parameters: The protocol outlined above was followed with the following exceptions.

1. After the 15 minute pre-hybridization step in which the membrane was in a rotating 50°C water bath in 100ml of pre-warmed hybridization solution, the membrane was cut with scissors sterilized in 70% ethanol into thirds.
2. Each of the membrane sections was placed in its respective hybridization tray labeled D17Z1, P1, and P2.
3. 20ul of each probe (1pmole/ul) was added to the respective trays and the remaining steps were performed for each membrane section.
4. All three sections were laminated together for exposure to film and development.
5. After the exposure to X-ray film and film development, the membrane sections that were subjected to the P1 and P2 probes were cut away from the laminate and the steps following the membrane spotting were followed with the exception of 200ul of P1 and P2 being added to their respective trays.

Membrane 3:

A nylon membrane was pre-wetted in 50ml of pre-wetting solution. The membrane was then removed from the hybridization tray containing the pre-wetting solution and placed on sheet of plastic sterilized with 70% ethanol. On the left side of the membrane probe 1 (P1) was spotted in volumes of 5ul, 10ul, 15ul, and 20ul and left to soak into the membrane. The same amounts of probe 2 (P2) were spotted onto the right side of the membrane and left to soak into the membrane. The membrane then underwent all of the steps in the Quantiblot protocol after the hybridization step, with the times of the wash steps reduced as to allow the probe to stay bound to the membrane. This process was performed in order to test whether the biotin that the probes were labeled with was reacting with the HRP-SA conjugate.

Membrane 4:

P1 P2

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	C1							A1	C1		
B	B1	C2							B1	C2		
C	C1	SSB							C1	SSB		
D	D1	JC1							D1	JC1		
E	E1	mtDNA							E1	mtDNA		
F	F1								F1			
G												
H												

Figure 3: Membrane 4. For descriptions refer to figure 1. Five nanograms of JC1 (1ng/ul) buccal swab DNA was spotted. Five nanograms of mtDNA (1ng/ul) were spotted.

Parameters: The Quantiblot® protocol was followed with the following exceptions.

1. After the pre hybridization step the membrane was cut into two sections.
2. Each section was placed in its respectively labeled hybridization tray where the remaining steps were followed with probe 1 (1pmole/ul) and probe 2 (1pmole/ul) added to the appropriate trays.

3. The rotating water bath was set to 40 degrees Celsius for the hybridization and wash steps, and 150ul of P1 and P2 were added to the corresponding hybridization trays.

Membrane 5:

P1 P2

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	C1							A1	C1		
B	B1	C2							B1	C2		
C	C1	SSB							C1	SSB		
D	D1	JC1							D1	JC1		
E	E1								E1			
F	F1								F1			
G												
H												

Figure 4: Membrane 5. For descriptions refer to figure 1. Ten nanograms of JC1 buccal swab DNA (2ng/ul) was spotted .

Parameters: The protocol listed in the Quantiblot® instructions was followed with the following exceptions.

1. After the pre hybridization step the membrane was cut into two sections and each section was placed into the appropriate hybridization tray labeled P1 and P2.
2. The rotating water bath was set at 45° C.
3. Forty milliliters of hybridization solution was added to the tray during the hybridization step.
4. The probes were reconstituted in a TE buffer with a pH of 7.75. The TE buffer that the probes were suspended in previously at a concentration of 1pmole/ul had a pH of 5.0.
5. 200ul of each probe at a concentration of 1pmole/ul was added to the appropriate hybridization tray.
6. Each of the probes were placed in a 56° C water bath for 4 minutes and were snap cooled for 2 minutes before being added to the 40ml of hybridization solution.

The mtDNA was excluded from this membrane because there was a small amount left. The mtDNA was intended for use again if there was any binding detected in the mitochondrial DNA portion of the JC1 extract with lower stringency conditions.

Membrane 6:

P1

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	C1										
B	B1	C2										
C	C1	SSB										
D	D1	JC1										
E	E1	mtDNA										
F	F1											
G												
H												

Figure 5: Membrane 6. For descriptions refer to figure 1. Ten nanograms of JC1 buccal swab DNA was spotted. Seven nanograms of mtDNA (2ng/ul) were spotted.

Parameters: The protocol listed in the Quantiblot® instructions was followed with the following exceptions.

1. The rotating water bath was set at 45° C.
2. Forty milliliters of hybridization solution was added during the hybridization step.

3. Two hundred and fifty micro liters of Probe 1 (1pmole/ul) was added to the tray during the hybridization step.
4. Probe 1 was heated at approximately 85° C for approximately 3 minutes and snap cooled for 3 minutes.
5. One hundred and twenty micro liters of HRP-SA was added to the wash solution during the stringent wash/conjugation step.
6. The membrane was exposed to X-ray film for approximately 1 hour and 20 minutes.

Probe 1 was the only probe evaluated on this membrane in order to focus more efforts on the probe that showed more promise, to conserve time so that more membranes could be run, and to conserve reagents.

Membrane 7:

P1

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	C1										
B	B1	C2										
C	C1	C3										
D	D1	SSB										
E	E1	mtDNA										
F	F1											
G												
H												

Figure 6: Membrane 7. A1 through F1 in row 1 represent a series of dilutions from the JC1 buccal swab DNA extraction of the following concentrations (12ng/ul, 6ng/ul, 3ng/ul, 1.5ng/ul, .75ng/ul, .375ng/ul). C1 represents calibrator 1 prepared from JC1 at a concentration of 10ng/ul, C2 at 5ng/ul, and C3 at 1ng/ul. SSB represents the spotting solution blank. Five nanograms of mtDNA (5ng/ul) were spotted. Only 1ul remained for this membrane blotting.

Parameters: The protocol listed in the Quantiblot® kit was followed with the following exceptions.

1. Dilutions were prepared from the DNA extracted from JC1 of the buccal swab at the above concentrations.
2. The rotating water bath was set at 45°C.
3. Forty milliliters of hybridization solution was used in the hybridization step
4. Probe 1 was heated for 4 minutes at approximately 90 degrees Celsius and snap cooled for 3 minutes before being added to the hybridization tray.
5. Three hundred micro-liters of probe 1 were added during the hybridization step.
6. One hundred and fifty micro-liters of HRP-SA were added during the stringent wash/conjugation step.

Membrane 8:

D17Z1 P1

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	60ng				A1	A2	60ng			
B	B1	B2	45ng				B1	B2	45ng			
C	C1	C2	30ng				C1	C2	30ng			
D	D1	D2	15ng				D1	D2	15ng			
E	E1	E2					E1	E2				
F	F1	F2					F1	F2				
G	C1	C3					C1	C3				
H	C2						C2					

Figure 7: Membrane 8. For descriptions refer to figure 1. A2 through F2 in columns 2 and 8 represent dilutions of JC1 extracted from bloodstains matching the concentrations of the standards as estimated from the yield gel. C3 represents JC1 at a concentration of 3ng/ul (15ng added). The amounts in columns 3 and 9 represent amounts of JC1 spotted onto the membrane.

Parameters: The protocol listed in the Quantiblot® instructions was followed with the following exceptions.

1. After the pre-hybridization step the membrane was cut into two sections, one to be probed with D17Z1 and the other to be probed with probe 1.
2. Probe 1 was placed in a 56°C water bath for 2 hours and cooled prior to addition during the hybridization step.

Membrane 9:

D17Z1 P1

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1						A1					
B	B1						B1					
C	C1						C1					
D	D1						D1					
E	E1						E1					
F	F1						F1					
G												
H												

Figure 8: Membrane 9. A1 through F1 in column 1 represent amounts of JC1 extracted from whole blood. The amounts were as follows: 60ng, 30ng, 15ng, 7.5ng, 3.75ng, and 1.875ng.

Parameters: The protocol listed in the Quantiblot® instructions was followed with the following exceptions.

1. No standards were blotted to the membrane.
2. Thirty micro-liters of P1 and D17Z1 were added during the hybridization steps.
3. The rotating water bath was set at 45°C.

CHAPTER 3

RESULTS

BLAST® Searches

After performing the BLAST® searches for each of the coding regions in the mtDNA genome and refining the search when necessary three sequences were discovered that could be used as human mtDNA specific probes. One of the sequences was found in the ATP Synthase 6 gene, one of the sequences was located in the NADH Dehydrogenase 5 gene, and the other was a sequence outside of hypervariable region 1 in the D loop, which part is used for primers in mtDNA amplification reactions. The possible probe sequence identified in the ND5 coding region was noted for possible use in later studies. Biotin labeled oligonucleotide probes selected from the ATP 6 and D loop regions were obtained for evaluation. BLAST® results performed on the two probes as well as the D17Z1 probe are outlined.

Probe	Putative Probe Sequence location (BP)	Tm	Sequence	Length	%GC	nmoles	Nmoles/ OD
1 (ATP 6)	9038 – 9075	82	ATG CAC CTA ATT GGA AGC GCC ACC CTA GCA ATA TCA A	37	46	138.7	2.40
(ND5)	12407 - 12447	80	TTA ACC CTA ACA AAA AAA ACT CAT ACC CCC ATT ATG TAA A				
2 (D loop)	15953 - 15992	80	CAA ATC AGA GAA AAA GTC TTT AAC TCC ACC ATT AGC ACC	39	39	167.0	2.23

Table 1. Sequences and sequence locations of the three possible human mtDNA specific oligonucleotide probes and properties of Probes 1 and 2 supplied by Invitrogen™ life technologies. The base pair locations are derived from the numbering system of the published human mtDNA sequence JO1415. The Tm was calculated for a 1M salt solution.

Probe 1: 5' Biotin – ATG CAC CTA ATT GGA AGC GCC ACC CTA GCA ATA TCA A 3'

The sequence for Probe 1 produced no significant similarities when searched against the *est_others database* and produced one BLAST® hit, which refers to a sequence alignment when searched against the *est_mouse database*. The hit was a sequence of 16 nucleotides long. When the Probe 1 sequence was BLAST® searched in the *est_human database* there were one hundred hits which is what the search filter was set for.

Probe 2: 5' Biotin- CAA ATC AGA GAA AAA GTC TTT AAC TCC ACC ATT AGC ACC 3'

The sequence for probe 2 produced 7 hits in the *est_others database*, which were all 17 nucleotides long of differing sequence. When searched in the *est_mouse database*, there were13 hits of 16 and 17 nucleotides in length. When searched in the *est_human database* there were 100 hits at which the search filter was set.

D17Z1 probe: 5' Biotin – TAG AAG CAT TCT CAG AAA CTA CTT TGT GAT GAT TGC ATT C 3'

There were six hits when the D17Z1 probe sequence was searched against the *est_others database* with lengths of 28, 19, and 17 nucleotides. When searched in the *est_mouse database* there were 16 blast hits with lengths of 16 and 18 nucleotides.

Probe	*Est_others*	*Est_mouse*	*Est_human*	Sequence Lengths
P1	No significant alignments	1	100	16 nucleotides
P2	7	13	100	17 nucleotides (*est_others*), 16 and 17 nucleotides (*est_mouse*)
D17Z1	6	16	100	28, 19, 17 nucleotides (*est_others*), 16 and 18 nucleotides (*est_mouse*)

Table 2. Results of BLAST® searches for probe 1, probe 2, and D17Z1. The filter for the *est_human database* was set at 100 alignments. For alignment sequences with species, refer to appendix 1.

Quantification

The sample DNA JC1 extracted from a buccal cell swab was estimated to contain 12ng/ul of total DNA based on the visual comparison of the band intensity produced by the sample with the intensities of the DNA standards. JC1 extracted from bloodstains on Whatman bloodstain cards produced no visible result when electrophoresed on a yield gel. DNA was extracted from bloodstains on a different Whatman bloodstain card at a separate time producing an estimated total DNA yield of 3ng/ul based on the visual comparison of the band intensity produced by the sample with those produced by the

DNA standards. JC1 extracted from the red blood cell portion of whole blood was estimated to contain approximately 150ng/ul of DNA, presumably mtDNA, based on the visual comparison of the band intensity produced by the sample with those produced by the DNA standard after a 10-fold dilution.

Genomic DNA extract	Concentration (ng/ul)
JC1 (buccal cell)	12ng/ul
JC1 (bloodstain)	3ng/ul
JC1 (whole blood)	150ng/ul

Table 3. Relative genomic DNA concentrations estimated from yield gels. Signal intensity comparisons to standard of known concentrations (20ng/ul, 15ng/ul, 10ng/ul, 5ng/ul, 2.5ng/ul, and 1ng/ul) were used.

Quantiblot® Results:

Membrane 1: The standard DNA dilution series prepared from DNA standard A, appeared to be linear from the highest DNA concentration to the lowest (Figure 9). Calibrator 1 has an intensity between standards B and C at 0.7ng/ul. On this membrane it appeared to be between standards A and B (Figure 9). A possible reason for this listed in the Quantiblot® kit trouble-shooting guide is that the standards were not prepared with the

proper concentrations. The row containing the dilutions of the JC1 buccal extract matching the concentrations of the standards appeared to be linear with intensities highly comparable to the ones produced by the standards (Figure 9). The serial dilutions from 12ng/ul of JC1 appeared to be linear after the first two dilutions.

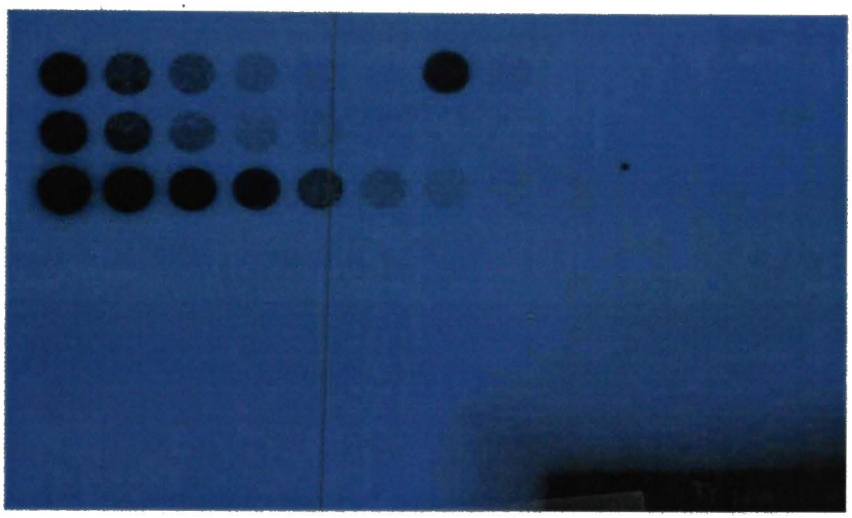

Figure 9: X-ray film exposed to membrane 1. Row 1 contains the genomic DNA standards A through F provided in the kit. Row 2 contains the dilutions matching the standards from the JC1 buccal swab. Row three contains the serial dilutions from 12ng/ul of the JC1 buccal swab DNA. For descriptions and concentrations refer to figure 1.

Membrane 2: For the membrane section that was exposed to the D17Z1 probe during hybridization, the standard dilution series prepared from DNA standard A, appeared to be linear and the calibrators appeared to have intensities that fell between the correct DNA standards (Figure 10). JC1 appeared to contain 5ng of DNA at a concentration of 1ng/ul.

The amplified mtDNA did not display a blot on the membrane (Figure 10). This indicates that the reaction parameters and the D17Z1 probe were working properly. The correct amount of JC1 was estimated from the standards and there was no cross reactivity with the mtDNA. The membrane sections that were exposed to P1 and P2 produced no visible results for any of the DNA spotted onto the respective membrane sections (Figure 10). There were also no visible results after the two sections were subjected to the protocol again with 200ul of probe added.

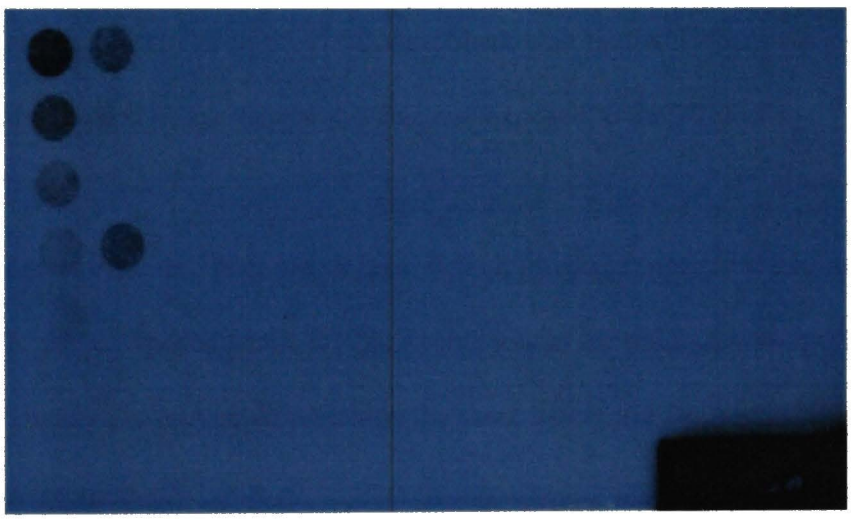

Figure 10: X-ray film exposed to membrane 2. Column 1 shows standards A through F probed with D17Z1. Column 2 shows C1, C2, and JC1 buccal swab DNA probed with D17Z1. For descriptions and concentrations refer to figures 1 and 2.

Membrane 3: The entire membrane appeared to be very dark, but when held up to the light very dark spots where the different amounts of probe were spotted appeared at intensities darker than the background of the membrane.

Membrane 4: There were no visible results for either of the membrane sections exposed to P1 and P2.

Membrane 5: For the membrane section that was exposed to P1, rings of seemingly equal intensities were present in each of the spots where DNA was spotted as well as the region where the spotting solution blank was spotted. There were no visible results for the section of the membrane that was exposed to the P2 probe.

Membrane 6: There was a spot with an intensity approximately matching DNA standard F, when exposed to the D17Z1 probe, where the mtDNA was spotted onto the membrane. There were no visible results in the areas where the DNA standards, calibrators, or JC1 were spotted.

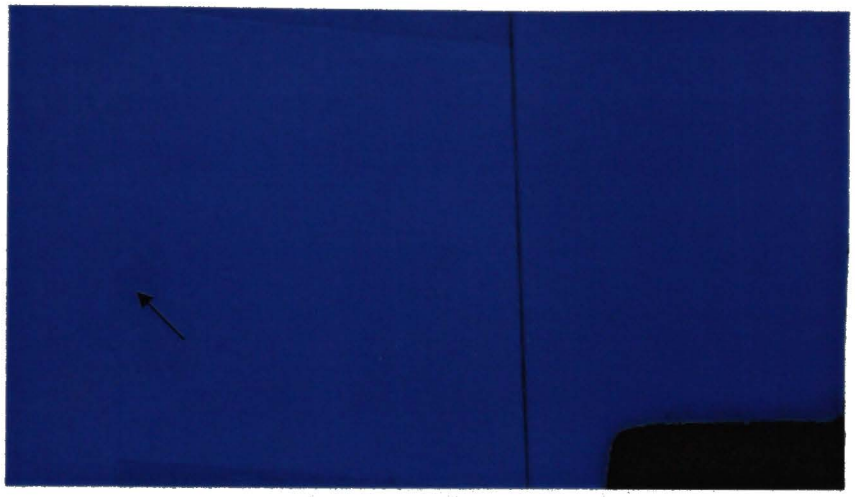

Figure 11: X-ray film exposed to membrane 6. The arrow points to the faint signal produced where the mtDNA was spotted and probed with P1. For descriptions and concentrations refer to figure 5.

Membrane 7: There was a spot with an intensity approximating DNA standard F, when exposed to the D17Z1 probe, where the mtDNA was spotted onto the membrane. There were no visible results in the areas where the DNA standards prepared from the extracted JC1, or calibrators prepared from JC1 were spotted.

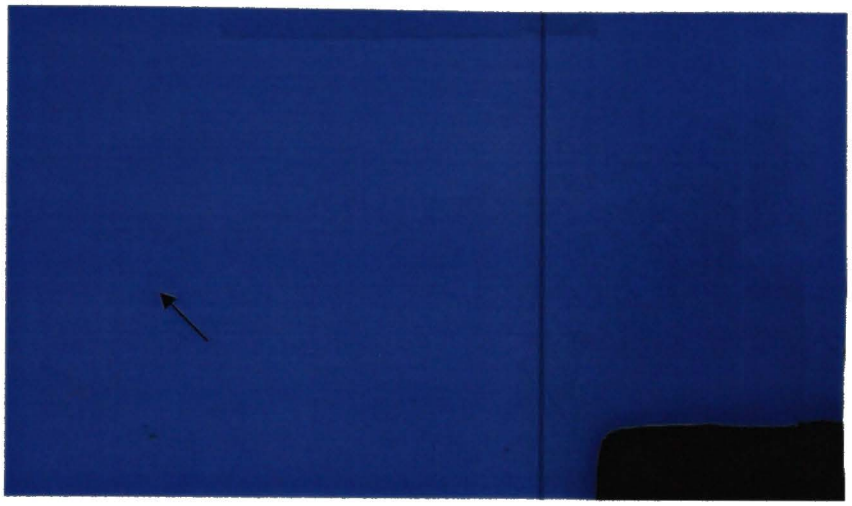

Figure 12: X-ray film exposed to membrane 7. The arrow points to the faint signal produced where the mtDNA was spotted and probed with P1. For descriptions and concentrations refer to figure 6.

Membrane 8: For the section of the membrane exposed to the D17Z1 probe the standard DNA dilutions prepared from the DNA standard A were linear in intensity and the calibrator's intensities fell within their appropriate ranges. The dilutions prepared from the JC1 bloodstain DNA extract with intended matching concentrations to those of the standards appeared linear in intensities, but with slightly darker intensities than those of the corresponding standards. The differing amounts of JC1 all above the detection limit of the Quantiblot standards were intensely dark uniform spots.

For the section of the membrane exposed to P1 there were no visible results in the areas where the DNA was spotted, but there was background present near the interface of the two membrane sections and on the top right corner of the membrane.

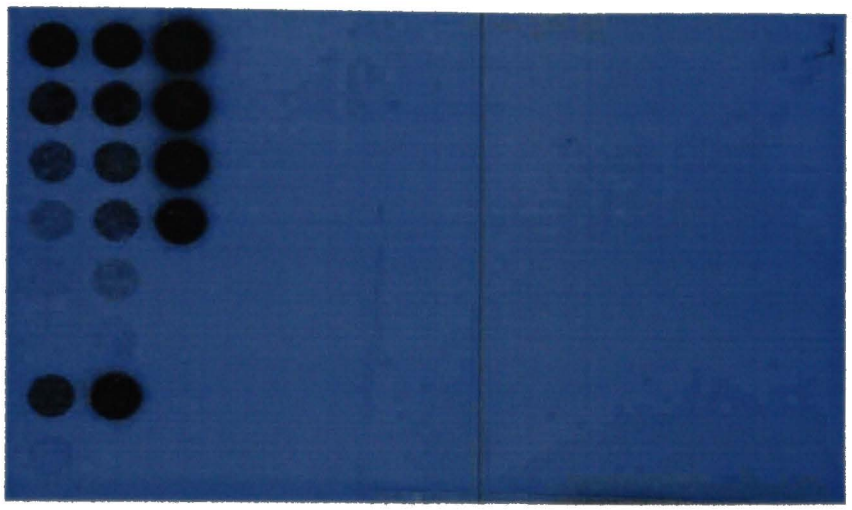

Figure 13: X-ray film exposed to membrane 8. Row 1 shows the standards with calibrators probed with D17Z1. Row 2 shows dilutions matching the standard concentrations of JC1 extracted from bloodstains with calibrators probed with D17Z1. Row 3 shows a serial dilution of JC1 bloodstain extract DNA probed with D17Z1. For descriptions and concentrations refer to figures 1 and 7.

Membrane 9: For the section of the membrane exposed to D17Z1 there were faint spots that appeared in non-linear intensities for the 60ng, 30ng, 15ng, and 7.5ng amounts of DNA diluted from 150ng of the genomic DNA extracted from whole blood. The blot at where the 30ng was spotted appeared the most intense.

For the section of the membrane exposed to P1 there were no visible results present.

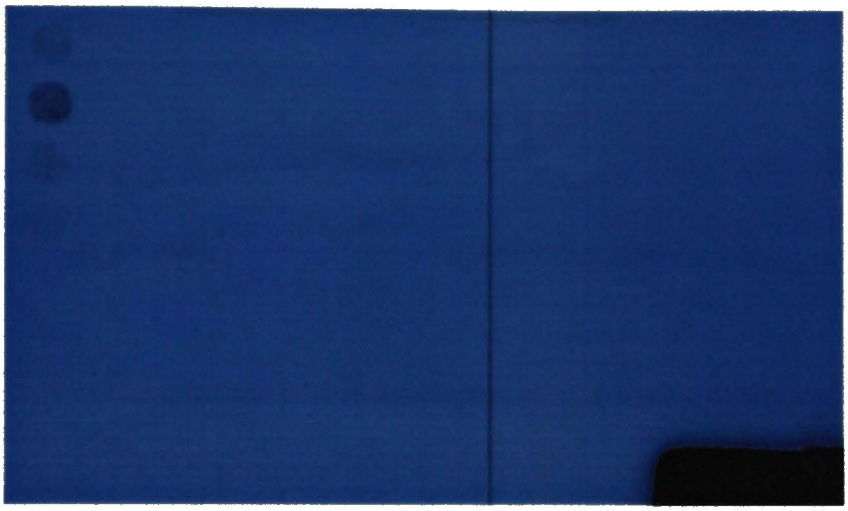

Figure 14: X-ray film exposed to membrane 9. Row 1 shows serial dilutions of JC1 extracted from whole blood and probed with D17Z1. For descriptions and amounts refer to figure 8.

CHAPTER 4

DISCUSSION

The principle goal in developing an oligonucleotide probe for use in identifying a particular DNA sequence specific to a given species is to find a sequence that is well conserved within the species of interest, but that is not well conserved when compared to other species. The BLAST® searches utilizing the Genbank database of non-human and human DNA sequences can serve as a valuable tool for evaluating sequences in order to accomplish this. The sequence used for Probe 1 when searched against non-human databases showed few sequence alignments under the BLAST® search parameters that were used and many alignments when searched against the human database. This indicates that the sequence is potentially specific to human mtDNA. The sequence used for Probe 2, based upon a sequence already used as a primer for human mtDNA amplification, showed more alignments with sequences from non-human databases. However, the sequences were of short length when compared with the overall length of the probe's sequence. The sequences of Probe 1 and Probe 2 were shortened in order to achieve a melting temperature similar to that of the D17Z1 probe, 37 and 39 nucleotides respectively. If the probes were to have worked as efficiently as the D17Z1 probe and have been human-specific, they would not have bound to any foreign DNA because the binding would not have been strong enough for them to stay bound during the stringent

wash steps of the Quantiblot procedure. The smaller lengths of the sequence alignments show promise that the sequence may specifically bind to human mtDNA. The D17Z1 probe sequence used in the ® kit to detect human nuclear DNA had an alignment that matched 28 of its 40 nucleotides demonstrating that even this probe is not entirely conserved among humans when compared against other species. Some of the signal detected when DNA is probed with D17Z1 may show cross-reaction with a nucleotide sequence from another species raising the question of how human-specific a probe can really be designed to be.

During the experiments evaluating the probes' performances when subjected to the reagents, conditions, and protocol used in the Quantiblot® kit there were varying results. When the Quantiblot® kit's manufacturers recommended instructions were implemented using the D17Z1 probe as a reaction control, the membrane sections exposed to the D17Z1 probe presented expected results. The membrane sections exposed to P1 and P2 showed no binding to little binding. This indicates that the reaction conditions are optimal for using the D17Z1 probe to identify and quantitate human nuclear DNA. These results also demonstrate that among other factors, the conditions may not be optimal for binding of the P1 and P2 probes to human mitochondrial DNA. From the different experiments performed, it is evident that Probe 1 will bind to human mtDNA without binding to human nuclear DNA. In the experiments with membranes 6 and 7, binding of P1 to the amplified target mtDNA was apparent at target amounts of 7ng and 5ng, respectively. The intensity of the blot was very faint. Also, the parameters for these two membranes were the least stringent of all the membranes run through the

Quantiblot® procedure. This suggests that either the probe wasn't binding to the mtDNA efficiently, there was not enough probe being added, or there was not enough target mtDNA present to produce more distinguishing signals. In DNA extracts from cellular material, nuclear DNA and mitochondrial DNA is present. In theory, there should be enough target mtDNA for the probe to bind to since mtDNA is released from cells with a far higher copy number than nuclear DNA. Also, it seems that the 20pmoles of probe required for the Quantiblot procedure should be adequate for binding the target mtDNA that should be present. The probes were designed with melting temperatures similar to the D17Z1 probe, therefore the 50°C hybridization temperatures should have been sufficient for the binding.

There are many possible reasons for the inefficient binding of the probes to the mtDNA. There was slight binding of P1 to the amplified mtDNA in two separate instances, but no binding was observed for any of the genomic DNA extracts. One possible reason for this occurrence is that a high majority of the mitochondria present in the cellular material remained intact thus sequestering the mtDNA. On membrane 9 the blots appearing on the section of the DNA exposed to the D17Z1 probe containing far more total DNA than the standards were faint as compared to the standard dilutions from Standard A demonstrating that in the 150ng/ul of DNA estimated from the yield gel, it may be possible that a small percentage was nuclear DNA and the remaining DNA was mitochondrial. The spotting of the JC1 extract on the section of the membrane exposed to P1 still showed no result.

The non-binding or faint binding of P1 and P2 to known mitochondrial DNA suggest that either the probes were not efficiently binding to the target DNA or that there was not sufficient target DNA for binding. In the experiments for membranes 6 and 7, where binding to 7ng and 5ng of mtDNA was observed, a much greater amount of P1 (250ul and 300ul, respectively) was added than was added to the membrane containing the JC1 whole blood extracted DNA. Also, the parameters were much less stringent than for the JC1 buccal cell extract that was subjected to P1 and the probe was heat denatured before the hybridization step. Overall, more probe, less stringent hybridization and wash conditions, greater amount of HRP-SA conjugate, and heat denaturation of the probe before use were required to see faint binding of Probe 1 to target amounts of mtDNA, which were much greater than those of the detection range for the D17Z1 probe. Membrane 3 was blotted with straight probe and exposed to the ECL reagents in order to determine if the biotin was functioning properly and reacting with the HRP-SA conjugate. The dark blots on the membrane where differing amounts of the probe were added indicate that the biotin on the labeled probe was effective. Either the probe was incapable of binding to the target DNA because of the conditions it was exposed to or the probe was comprised of a sequence that was incapable of binding to the target DNA effectively. The melting temperatures of probes P1 and P2 were very similar to that of the D17Z1 probe, but the GC contents of both probes were higher which may have affected binding. Normally higher GC contents are desired for tighter, more specific binding because there are 3 hydrogen bonds between the two bases as opposed to the 2 hydrogen bonds in A-T binding. However, the high GC content may have made the probes too specific and

affected their binding ability. It has also been discovered that certain coding regions of the human mtDNA genome can have differing sequences based on how natural selection may affect inhabitants of different regions, including the ATP6 region where the sequence for probe 1 is located. The JC1 mtDNA may have had a sequence divergent enough from the probe that binding was ineffective. A future consideration would be to amplify and sequence the mtDNA from the genomic extract and look for alignment of the sequence with that of the probe. Another possible suggestion as to why the probes didn't effectively bind to the target DNA is that the pH of the TE buffer that the probes were originally suspended in was discovered to be at 5 which is much more acidic than normally required for probes to be stored at. At low pH where there are more free hydrogen ions in solution there is an increased chance that the probes would have dimerized or bound to one another at complimentary nucleotides or formed hairpin loops by binding of complimentary sequences in the same oligonucleotide. Also, the probes may have been partially degraded in the low pH environment. In future experiments if there is a low degree of hybridization detected a probe with, a complimentary sequence, could be added to test and see whether it is intact and hybridizing. The probes were later resuspeded in TE^{-4} buffer with a higher pH of 7.75 to help keep them denatured, but it may have not been high enough for effective denaturation. The probes were further denatured by heat and alkali conditions.

It also may have just been that the conditions were still too stringent for the probes even after less stringent conditions were attempted. For future evaluation of these probes, it may be beneficial to attempt the procedure with extremely low stringency conditions

such as a lower temperature, much higher salt concentration, more probe, and more enzyme conjugate to achieve a desired amount of binding. The reaction parameters could then be augmented in order to optimize the reaction. Also, the probe must be suspended in a buffer with a desirable pH so that there are no problems with dimerization.

APPENDIX

BLAST® SEARCH RESULTS

P1 (*est_others*):

BLASTN 2.2.6 [Apr-09-2003]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1054688558-017756-30022

Query=
(37 letters)

Database: GenBank non-mouse and non-human EST entries 7,926,587 sequences; 4,093,482,730 total letters

If you have any problems or questions with the results of this search please refer to the BLAST FAQs
No significant similarity found. For reasons why, click here.

Database: GenBank non-mouse and non-human EST entries
Posted date: Jun 2, 2003 7:57 PM
Number of letters in database: 2,069,430,259
Number of sequences in database: 4,284,121

Lambda	*K*	H
1.37	0.711	1.31

Gapped

Lambda	*K*	H
1.37	0.711	1.31

Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 31,411
Number of Sequences: 10227022
Number of extensions: 31411
Number of successful extensions: 31339
Number of sequences better than 10.0: 0
Number of HSP's better than 10.0 without gapping: 0
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 5999
Number of HSP's gapped (non-prelim): 0
length of query: 37
length of database: 4,093,482,730
effective HSP length: 18
effective length of query: 19
effective length of database: 3,950,804,164
effective search space: 75065279116
effective search space used: 75065279116
T: 0
A: 0
X1: 6 (11.9 bits)
X2: 15 (29.7 bits)
S1: *12* (24.3 bits)
S2: 17 (34.2 bits)

P1(*est_mouse*):

BLASTN 2.2.6 [Apr-09-20031

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schdffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), 'Gapped BLAST and PSI-BIAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1054689044-022777-25135

Query=
(37 letters)

Database: GenBank Mouse EST entries

3,733,855 sequences; 1,675,975,278 total letters
If you have any problems or questions with the results of this search please refer to the BLAST FAQs
Taxonomy reports

Distribution of 1 Blast Hits on the Query Sequence

Sequences producing significant alignments:	Score (bits)	E Value
gi/ 29491027/ gb/ CB571497.1/ CB571497 AGENCOURT-12975535 NIH-M...		32

Alignments

gil29491027igbiCB571497.11CB571497 AGENCOURT-12975535 NIH-MGC-165 Mus
musculus CDNA clone

IMAGE:30279547 5'.
Length = 976

Score = 32.2 bits (16), Expect = 6.5
Identities = 16/16 (100%)
Strand = Plus / Minus

Query: 11 ttggaagcgccaccct 26
Sbjct: 592 ttggaagcgccaccct 577

Database: GenBank Mouse EST entries
Posted date: Jun 2, 2003 7:57 PM
Number of letters in database: 1,088,892,180
Number of sequences in database: 2,548,704

Lambda	*K*	H
1.37	0.711	1.31

Gapped Lambda	*K*	H
1.37	0.711	1.31

Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 10,198
Number of Sequences: 8491605
Number of extensions: 10198
Number of successful extensions: 10148
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 2831
Number of HSP's gapped (non-prelim): 1
length of query: 37
length of database: 1,675,975,278

effective HSP length: 17
effective length of query: 20
effective length of database: 1,612,499,743
effective search space: 32249994860
effective search space used: 32249994860
T: 0
A: 0
Xl: 6 (11.9 bits)
X2: 15 (29.7 bits)
S1: 12 (24.3 bits)
S2: 16 (32.2 bits)

P2 (*est_others*):

BLASTN 2.2.6 [Apr-09-2003]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
'Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

RID: 1054687938-012207-29070

Query=
(39 letters)

Database: GenBank non-mouse and non-human EST entries 7,926,587 sequences;
4,093,482,730 total letters

If you have any problems or questions with the results of this search please refer
to the BLAST FAQs
Taxonomy reports

Distribution of 7 Blast Hits on the Query Sequence

Sequences producing significant alignments:	Score (bits)	E Value
gil297519551gbICB694808.11CB694808 AMGNNUC:SRPB2-00091-DlO-34	4.2	
gil278928341gbICB079397.11CB079397 hp7Ofll.bl Hedyotis term	34	4.2
gil2l882234lembiA-L840296.11AL840296 AL840296 FOOOC Takifugu	34	4.2
gil2l3717321gblBQ512863.11BQ512863 EST620278 Generation of	34	4.2
gil9862987]gbIBE605718.11BE605718 fll7dO7.xl Zebrafish Rese	34	4.2
gil871279OIgbIBE200621.11BE200621 fk88cO7.xl Zebrafish Rese	34	4.2
gil3343194ldbjlAU006735.11AU006735 AU006735 Schizosaccharom	34	4.2

Alignments

gil297519551gbICB694808.11CB694808 AMGNNUC:SRPB2-00091-DlO-A srpb2 (10220)
Rattus norvegicus CDNA

clone srpb2-00091-dlO 51.
Length = 426

Score = 34.2 bits (17), Expect = 4.2
Identities = 17/17 (100%)
Strand = Plus / Plus

Query: 5 tcagagaaaaagtcttt 21
Sbjct: 247 tcagagaaaaagtcttt 263

gil27892834igbICB079397.11CB079397 hp7Ofll.bl Hedyotis terminalis flower Stage 2 (NYBG) Hedyotis terminalis CDNA clone hp7Ofll.
Length = 514

Score = 34.2 bits (17), Expect = 4.2
Identities = 17/17 (100%)
Strand = Plus / Plus

Query: 17 tctttaactccaccatt 33
Sbjct: 240 tctttaactccaccatt 256

gii2l882234lembIAL840296.11AL840296 AL840296 FOOOC Takifugu rubripes CDNA clone FOOOClOaClO.

Length = 423

Score = 34.2 bits (17), Expect = 4.2
Identities = 20/21 (95%)
Strand = Plus / Plus

Query: 4 atcagagaaaaagtctttaac 24
Sbjct: 248 atcagagagaaagtctttaac 268

gil2l3717321gblBQ512863.11BQ512863 EST620278 Generation of a set of potato CDNA clones for microarray

analyses mixed potato tissues Solanum tuberosum CDNA clone STMIB11 31 end.
Length = 479

Score = 34.2 bits (17), Expect = 4.2
Identities = 20/21 (95%)
Strand = Plus / Plus

Query: 5 tcagagaaaaagtctttaact 25
Sbjct: 181 tcagagaaaaaatctttaact 201

gil9862987igblBE605718.11BE605718 fll7dO7.xl Zebrafish Research Genetics C32 fin Danio rerio CDNA 31

similar to SW:SFR5 HUMAN Q13243 SPLICING FACTOR, ARGININE/SERINE-RICH 5
Length = 685

Score = 34.2 bits (17), Expect = 4.2
Identities @ 17/17 (100%)
Strand = Plus / Plus

Query: 20 ttaactccaccattagc 36
Sbjct: 124 ttaactccaccattagc 140

gii871279OIgbIBE200621.11BE200621 fk88cO7.xl Zebrafish Research Genetics C32
fin Danio rerio CDNA

31 similar to SW:SFR5 RAT Q09167 SPLICING FACTOR, ARGININE/SERINE-RICH
Length = 616

Score = 34.2 bits (17), Expect 4.2
Identities = 17/17 (100%)
Strand = Plus / Plus

Query: 20 ttaactccaccattagc 36
Sbjct: 50 ttaactccaccattagc 66

gil3343194idbjlAU006735.11AU006735 AU006735 Schizosaccharomyces pombe late
log phase CDNA

Schizosaccharomyces pombe CDNA clone spcOO362.
Length = 461

Score = 34.2 bits (17), Expect = 4.2
Identities = 17/17 (100%)
Strand = Plus / Minus

Query: 7 agagaaaaagtctttaa 23
Sbjct: 189 agagaaaaagtctttaa 173

Database: GenBank non-mouse and non-human EST entries
Posted date: Jun 2, 2003 7:57 PM
Number of letters in database: 2,069,430,259
Number of sequences in database: 4,284,121

Lambda	*K*	H
1.37	0.711	1.31

Gapped Lambda	*K*	H
1.37	0.711	1.31

Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 82,446
Number of Sequences: 10227022
Number of extensions: 82446
Number of successful extensions: 82148
Number of sequences better than 10.0: 7

Number of HSP's better than 10.0 without gapping: 10
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 21829
Number of HSP's gapped (non-prelim): 10
length of query: 39
length of database: 4,093,482,730
effective HSP length: 18
effective length of query: 21
effective length of database: 3,950,804,164
effective search space: 82966887444
effective search space used: 82966887444
T: 0
A: 0

X1: 6 (11.9 bits)
X2: 15 (29.7 bits)
S1: 12 (24.3 bits)
S2: 17 (34.2 bits)

P2 (*est_mouse*):

BLASTN 2.2.6 [Apr-09-2003]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search

programs", Nucleic Acids Res. 25:3389-3402.

RID: 1054689250-024612-18486

Query=
(39 letters)

Database: GenBank Mouse EST entries
3,733,855 sequences; 1,675,975,278 total letters

If you have any problems or questions with the results of this search please refer to the BLAST FAQs
Taxonomy reports

Distribution of 13 Blast Hits on the Query Sequence

Sequences producing significant alignments:	Score (bits)	E Value
gil273220321gbICA870483.11CA870483 K0902EO2-5N NIA Mouse Ne	34	1.8
gil22351577igblBQ936194.11BQ936194 AGENCOURT - 8863311 NCI-CG	34	1.8
gil8776020ldbjlBB123452.11BB123452 BB123452 RIKEN full-leng	34	1.8
gil22842451gblAA538252.11AA538252 vi98dl2.rl Barstead mouse	34	1.8
gil27196968ldbjlBY761366.11BY761366 BY761366 RIKEN full-len	32	7.1
gil26817121ldbjlBY482742.11BY482742 BY482742 RIKEN full-len	32	7.1
gil241284831gbIBU939664.11BU939664 AGENCOURT 10503333 NIH M	32	7.1
gil236367631gbiBU706365.11BU706365 UI-M-FOO-caf-a-14-0-UI.r	32	7.1
gil232575151gbIBU583550.11BU583550 maiO7cO7.yl McCarrey Edd	32	7.1
gil224987181gbIBU058429.11BU058429 UI-M-FOO-caf-b-13-0-UI.r	32	7.1
giil6482027ldbjlBB647749.11BB647749 BB647749 RIKEN full-len	32	7.1
gi]14296695igb[BG916219.11BG916219 602814937Fl NCI - CGAP-Mam	32	7.1
gil65157861gbIAW209846.11AW209846 ul47gl2.yl Rashbass mouse	32	7.1

Alignments

gil27322032igbICA870483.11CA870483 K0902EO2-5N NIA Mouse Neural Stem Cell
(Undifferentiated) CDNA
Library (Long) Mus musculus CDNA clone NIA:KO902EO2
IMAGE:30084433 5'.
Length = 503

Score = 34.2 bits (17), Expect = 1.8
REFERERENCES
20/21 (95%)
Strand = Plus / Minus

Query: 2 aaatcagagaaaaagtcttta 22
Sbjct: 202 aaatcagagaaaaagacttta 182

gil22351577igblBQ936194.11BQ936194 AGENCOURT-8863311 NCI-CGAP-Mam2 Mus musculus CDNA clone

IMAGE:6440166 5'.
Length = 1154

Score = 34.2 bits (17), Expect = 1.8
Identities = 17/17 (100%)
Strand = Plus / Minus

Query: 6 cagagaaaaagtcttta 22
Sbjct: 815 cagagaaaaagtcttta 799

gil8776020ldbjlBB123452.11BB123452 BB123452 RIKEN full-length enriched, adult male urinary bladder Mus

musculus CDNA clone 9530092J20 31 similar to AF049879 Mus musculus TGFB inducible early protein MRNA. Length = 292

Score = 34.2 bits (17), Expect = 1.8
Identities = 17/17 (100%)

Strand = Plus / Minus

Query: 6 cagagaaaaagtcttta 22
Sbjct: 288 cagagaaaaagtcttta 272

gil22842451gblAA538252.11AA538252 vi98dl2.rl Barstead mouse pooled organs MPLRB4 Mus musculus CDNA

clone IMAGE:920279 51 similar to gb:Ul5647-cdsl Mus rnusculus (MOUSE);.
Length = 339

Score = 34.2 bits (17), Expect = 1.8
Identities = 17/17 (100%)
Strand = Plus / Minus

Query: 11 aaaaagtctttaactcc 27
Sbjct: 259 aaaaagtctttaactcc 243

gil27196968idbjlBY761366.11BY761366 BY761366 RIKEN full-length enriched, bone
marrow macrophage Mus
musculus CDNA clone G530011CO9 3'.
Length = 694

Score = 32.2 bits (16), Expect = 7.1
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 4 atcagagaaaaagtct 19
Sbjct: 623 atcagagaaaaagtct 638

gil26817121|dbj|BY482742.1|BY482742 BY482742 RIKEN full-length enriched, bone
marrow macrophage Mus

musculus CDNA clone G530014C24 31.
Length = 439

Score = 32.2 bits (16), Expect = 7.1
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 4 atcagagaaaaagtct 19
Sbjct: 373 atcagagaaaaagtct 388

gil241284831|gb|BU939664.1|BU939664 AGENCOURT-10503333 NIH-MGC-169 Mus
musculus CDNA clone

IMAGE:6708004 51.
Length = 726

Score = 32.2 bits (16), Expect = 7.1
Identities = 16/16 (100%)
Strand = Plus / minus

Query: 8 gagaaaaagtctttaa 23
Sbjct: 567 gagaaaaagtctttaa 552

gi[236367631|gb|BU706365.1|BU706365 UI-M-FOO-caf-a-14-0-UI.rl NIH-BMAP-FOO Mus
musculus CDNA clone

IMAGE: 6410389 51.
Length = 707

Score = 32.2 bits (16), Expect = 7.1

Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 4 atcagagaaaaagtct 19
Sbjct: 342 atcagagaaaaagtct 357

gil23257515igbIBU583550.11BU583550 maiO7cO7.yl McCarrey Eddy 18 20 day sertoli cell mus musculus CDNAclone
IMAGE:6369396 5'.
Length = 578

Score = 32.2 bits (16), Expect = 7.1
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 16 gtctttaactccacca 31
Sbjct: 562 gtctttaactccacca 577

gil224987181gbIBU058429.11BU058429 UI-M-FOO-caf-b-13-0-UI.rl NIH-BMAP-FOO Mus
musculus CDNA clone

IMAGE:6410412 5'.
Length = 787

Score = 32.2 bits (16), Expect = 7.1
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 4 atcagagaaaaagtct 19
Sbjct: 341 atcagagaaaaagtct 356

gil16482027ldbjlBB647749.11BB647749 BB647749 RIKEN full-length enriched,

adult male corpus striatum Mus musculus CDNA clone C030026A16 51.
Length = 683

Score = 32.2 bits (16), Expect = 7.1
Identities = 16/16 (100%)
Strand = Plus / Minus

Query: 22 aactccaccattagca 37
Sbjct: 673 aactccaccattagca 658

gil42966951gblBG916219.11BG916219 602814937F1 NCI-CGAP-Mam4 Mus musculus
CDNA clone
IMAGE:493719351.
Length = 891

Score = 32.2 bits (16), Expect = 7.1
Identities = 16/16 (100%)
Strand = Plus / Minus

Query: 4 atcagagaaaaagtct 19
Sbjct: 94 atcagagaaaaagtct 79

gil6515786igbIAW209846.11AW209846 ul47gl2.yl Rashbass mouse MOV 9 5 optic
vesicle Mus musculus CDNA clone
IMAGE:2101510 5' similar to gb:X57377 Mouse dilute myosin heavy chain gene for novel heavy chain with (MOUSE);.
Length = 740

Score = 32.2 bits (16), Expect = 7.1
Identities = 16/16 (100%)
Strand = Plus / Plus

Query:4 atcagagaaaaagtct 19
Sbjct: 4atcagagaaaaagtct 19

Database: GenBank Mouse EST entries
Posted date: Jun 2, 2003 7:57 PM
Number of letters in database: 1,088,892,180
Number of sequences in database: 2,548,704

Lambda	K	H
1.37	0.711	1.31

Gapped Lambda	*K*	H
1.37	0.711	1.31

Matrix: blastn matrix:1 -3
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 40,086
Number of Sequences: 8491605
Number of extensions: 40086
Number of successful extensions: 39916
Number of sequences better than 10.0: 13
Number of HSP's better than 10.0 without gapping: 16
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 11488
Number of HSP's gapped (non-prelim): 16
length of query: 39
length of database: 1,675,975,278

effective HSP length: 17
effective length of query: 22
effective length of database: 1,612,499,743
effective search space: 35474994346
effective search space used: 35474994346
T: 0
A: 0
Xl: 6 (11.9 bits)
X2: 15 (29.7 bits)
Sl: 12 (24.3 bits)

S2: 16 (32.2 bits)

D17Z1 (*est_others*):

BLASTN 2.2.6 [Apr-09-20031

Reference:
Altschul, Stephen F., Thomas L. madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BIAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1054686092-025573-19799

Query =

(40 letters)

Database: GenBank non-mouse and non-human EST entries

7,926,587 sequences; 4,093,482,730 total letters

If you have any problems or questions with the results of this search please refer to the BLAST FAQs

Taxonomy reports

	Score	
Sequences producing significant alignments:	(bits)	V
IAU291313.11AU291313 AU291313 zinnia culture	56	le
82.11BF066382 stlOhO2.yl Gm-clO65 Glyc	38	0.
75.11CB212675 OML02955 Oryza minuta Hy	34	4.
SJEDED12 SJE Schistosoma	34	4.

BPO13256 Nori Satoh unp..... 34 4.
347214 MARC 1PIG Sus scr..... 34 4.

Alignments

F->gil2425182|dbj|AU291313.1|AU291313 AU291313 zinnia cultured mesophyll cell eqt elegans CDNA clone Z5931.
Length = 264

Score = 56.0 bits (28), Expect = 1e-06
Identities = 34/36 (94%)
Strand = Plus / Minus

Query: 5 agcattctcagaaactactttgtgatgattgcattc 40
Sbjct: 133 agcattctcagaaacttctttgtgatgtttgcattc 98

r->gi|10843053|gb|BF066382.1|BF066382 stlOhO2.yl Gm-clO65 Glycine max CDNA clone
ID: Gm-clO65-940 51 similar to TR:Q9$KY5 Q9SKY5
PUTATIVE HYDROLASE.
Length = 425

Score = 38.2 bits (19), Expect = 0.28
Identities = 22/23 (95%)
Strand = Plus / Plus

Query: 5 agcattctcagaaactactttgt 27
Sbjct: 49 agcattctcagaaacgactttgt 71

r->gi|28258766|gb|CB212675.1|CB212675 OML02955 Oryza minuta HybriZAP-2.1 XR librai
Length = 554

Score = 34.2 bits (17), Expect = 4.4

Identities = 17/17 (100%)

Strand = Plus / Plus

Query: 12 tcagaaactactttgtg 28
Sbjct: 234 tcagaaactactttgtg 250

r->gil283442611gbIBU776945.11BU776945 SJEDED12 SJE Schistosoma japonicum CDNA.
Length = 519

Score = 34.2 bits (17), Expect = 4.4

Identities = 17/17 (100%)

Strand = Plus / Plus

Query: 18 actactttgtgatgatt 34
Sbjct: 449 actactttgtgatgatt 465

r->gill9504733ldbjlBPO13256.11BPO13256 BPO13256 Nori Satoh unpublished CDNA librai intestinalis CDNA clone ciad56b2l 51.

Length = 534

Score = 34.2 bits (17), Expect = 4.4

Identities = 20/21 (95%)

Strand = Plus / Plus

Query: 13 cagaaactactttgtgatgat 33
Sbjct: 280 cagaaacgactttgtgatgat 300

r'>gill4Ol96381qblBG733354.11BG733354 347214 MARC 1PIG Sus scrofa CDNA 51.
Length = 558

Score = 34.2 bits (17), Expect = 4.4

Identities = 17/17 (100%)

Strand = Plus / Minus

Query: 4 aagcattctcagaaact 20
Sbjct: 98 aagcattctcagaaact 82

Database: GenBank non-mouse and non-human EST entries
Posted date: Jun 2, 2003 7:57 PM
Number of letters in database: 2,069,430,259
Number of sequences in database: 4,284,121

Lambda	K	H
1.37	0.711	1.31

Gapped Lambda	K	H
1.37	0.711	1.31

Matrix: blastn matrix:1 -3

Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 74,183
Number of Sequences: 10227022
Number of extensions: 74183
Number of successful extensions: 73887
Number of sequences better than 10.0: 6
Number of HSP's better than 10.0 without gapping: 7

Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 18533
Number of HSP's gapped (non-prelim): 7
length of query: 40
length of database: 4,093,482,730
effective HSP length: 18
effective length of query: 22
effective length of database: 3,950,804,164
effective search space: 86917691608
effective search space used: 86917691608

T: 0
A: 0

Xl: 6 (11.9 bits)
X2: 15 (29.7 bits)
Sl: 12 (24.3 bits)
S2: 17 (34.2 bits)

D17Z1 (*est_mouse*):

BLASTN 2.2.6 [Apr-09-20031

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs". Nucleic Acids Res. 25:3389-3402.

RID: 1054686593-029400-19957

Query=

(40 letters)

Database: GenBank Mouse EST entries

3,733,855 sequences; 1,675,975,278 total letters

If you have any problems or questions with the results of this search please refer to the **BIAST IFAQs**

Taxonomy reports

Sequences producing significant alignments:	Score (bits)	Value
gil5374114ldbjlAV167677.11AV167677	AV167677 Mus musculus he	36
gil293537291qblCB520374.11CB520374	UI-M-GIO-cei-1-18-0-UI.r	32

	qil288453781gbICB321143.11CB321143	AGENCOURT 12236800 NIH
M	32	7.5
	gil2732596SIgbICA874419.11CA874419	K0932CO3-5N NIA Mouse
Ne	32	7.5
	qil251212851qblCA576583.11CA576583	K0646HlO-5N NIA Mouse
He	32	7.5
	gil251203951qblCA575694.11CA575694	K0634DO8-5N NIA Mouse
He	32	7.5
	gil251203821qblCA575681.11CA575681	K0634CO4-5N NIA Mouse
He	32	7.5
	qil250671111gbICA534612.1]CA534612	C0207EO1-5N NIA Mouse 7.....32
	qil25067072igbICA534602.11CA534602	C0207COl-5N NIA Mouse 7 32
	qil228126061qblBU506373.11BU506373	AGENCOURT 10015485 NIH
m	32	7.5
	qil2l46380OIgblBQ562913.11BQ562913	H4078FlO-5 NIA Mouse 7.4.....32

gill94020551qbiBM942192.11BM942192 UI-M-CGOp-bfo-a-10-0-UI	32
gill25483701qblBG065807.11BG065807 H3035EO4-3 NIA Mouse 15K	32
gil6261900ldbjlAV273863.11AV273863 AV273863 RIKEN full-leng...	32
qil2517555ldbjIC77225.lIC77225 C77225 Mouse 3.5-dpc blastoc	32
gil2517315ldbjIC76985.lIC76985 C76985 Mouse 3.5-dpc blastoc	32

Alignments

F->gil5374114ldbjlAV167677.11AV167677 **M** AV167677 Mus musculus head C57BL/6j 13-da-1,
CDNA clone 3110056HO6.

Length = 303

Score = 36.2 bits (18), Expect = 0.48
Identities = 18/18 (100%)

Strand = Plus / Minus

Query :9 ttctcagaaactactttg 26
Sbjct: 59 ttctcagaaactactttg 42

F->gil293537291gbICB520374.11CB520374 UI-M-GIO-cei-1-18-0-UI.rl NIH-BMAP-GIO Mus r
IMAGE: 6840067 5'.

Length = 840

Score = 32.2 bits (16), Expect = 7.5

Identities = 19/20 (95%)

Strand = Plus / Minus

Query: 20 tactttgtgatgattgcatt 39
Sbjct: 392 tactttgagatgattgcatt 373

r->gil288453781gblCB321143.11CB321143 AGENCOURT-12236800 NIH-MGC-136 Mus musculus
IMAGE:30288592 5'.

Length = 930

Score = 32.2 bits (16), Expect = 7.5

Identities = 16/16 (100%)

Strand = Plus / Plus

Query: 4 aagcattctcagaaac 19
Sbjct: 58 aagcattctcagaaac 73

F->gil273259681gblCA874419.11CA874419 K0932CO3-5N NIA Mouse Neural Stem Cell (Undi
Library (Long) Mus musculus CDNA clone NIA:KO932CO3
IMAGE:30087290 51.
Length = 567

Score = 32.2 bits (16), Expect = 7.5

Identities = 19/20 (95%)

Strand = Plus / Minus

Query: 20 tactttgtgatgattgcatt 39
Sbjct: 258 tactttgagatgattgcatt 239

F->gil251212851gbiCA576583.11CA576583 K0646HlO-5N NIA Mouse Hematopoietic Stem Cel
CDNA Library (Long) Mus musculus CDNA clone NIA:KO646HlO
IMAGE:30073245 5'.

Length = 509

Score = 32.2 bits (16), Expect = 7.5

Identities = 16/16 (100%)

Strand = Plus / Plus

Query: 3gaagcattctcagaaa 18

Sbjct: 306 gaagcattctcagaaa 321

F->gil251203951qblCA575694.11CA575694 K0634DO8-5N NIA Mouse Hematopoietic Stem Cel
CDNA Library (Long) Mus musculus CDNA clone NIA:KO634DO8 IMAGE:30072043 51.

Length = 522

Score = 32.2 bits (16), Expect = 7.5

Identities = 16/16 (100%)

Strand = Plus / Plus

Query: 3gaagcattctcagaaa 18
Sbjct: 306 gaagcattctcagaaa 321

F->gil251203821qblCA575681.11CA575681 K0634CO4-5N NIA Mouse Hematopoietic Stem Cel
CDNA Library (Long) Mus musculus CDMA clone NIA:KO634CO4 IMAGE:30072027 5'.

Length = 440

Score = 32.2 bits (16), Expect = 7.5

Identities = 16/16 (100%)

Strand = Plus / Plus

Query: 3 gaagcattctcagaaa 18
Sbjct: 260 gaagcattctcagaaa 275

F->gil250671111qblCA534612.11CA534612 C0207EO1-5N NIA Mouse 7.5-dpc Whole Embryo
musculus CDNA clone NIA:CO207EO1 IMAGE:30011280 51.
Length = 319

Score = 32.2 bits (16), Expect = 7.5
Identities = 16/16 (100%)
Strand = Plus / Minus

Query: 2 agaagcattctcagaa 17
Sbjct: 102 agaagcattctcagaa 87

r->gil250670721,bICA534602.11CA534602 C0207COl-5N NIA Mouse 7.5-dpc Whole Embryo
musculus CDNA clone NIA:CO207COl IMAGE:30011256 51.
Length = 107

Score = 32.2 bits (16), Expect = 7.5
Identities = 16/16 (100%)
Strand = Plus / Minus

Query: 2 agaagcattctcagaa 17
Sbjct: 102 agaagcattctcagaa 87

r->gil228126061qblBU506373.11BU506373 AGENCOURT-10015485 NIH-MGC-94
Mus musculus c 51.
Length = 964

Score = 32.2 bits (16), Expect = 7.5
Identities = 19/20 (95%)
Strand = Plus / Minus

Query: 20 tactttgtgatgattgcatt 39
Sbjct: 547 tactttgagatgattgcatt 528

>gil214638001qblBQ562913.11BQ562913 H4078FlO-5 NIA Mouse 7.4K CDNA Clone Set Mu!
H4078FlO 51.
Length = 564

Score = 32.2 bits (16), Expect = 7.5
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 3 gaagcattctcagaaa 18
sbjct: 306 gaagcattctcagaaa 321

r->gill94020551gblBM942192.11BM942192 11 UI-M-CGOp-bfo-a-10-0-UI.rl NIH-BMAP-Ret4-E clone UI-M-CGOp-bfo-a-10-0-UI 51.
Length = 659

Score = 32.2 bits (16), Expect = 7.5
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 3 gaagcattctcagaaa 18
Sbjct: 66 gaagcattctcagaaa 81

r->gill25483701qblBG065807.11BG065807 H3035EO4-3 NIA Mouse 15K CDNA Clone Set Ml H3035EO4 31.
Length = 870

Score = 32.2 bits (16), Expect = 7.5
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 2 agaagcattctcagaa 17
Sbjct: 336 agaagcattctcagaa 351

F->gil6261900ldbjlAV273863.11AV273863 AV273863 RIKEN full-length enriched, adult Mus musculus CDNA clone 4932414JO4 31.
Length = 238

Score = 32.2 bits (16), Expect = 7.5
Identities = 19/20 (95%)
Strand = Plus / Plus

Query: 10 tctcagaaactactttgtga 29
Sbjct: 50 tctcagaaactactgtgtga 69

r->qil2517555ldbjIC77225.lIC77225 M C77225 Mouse 3.5-dpc blastocyst CDNA Mus musci J0027FO8 31.
Length = 569

Score = 32.2 bits (16), Expect = 7.5
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 2 agaagcattctcagaa 17
Sbjct: 333 agaagcattctcagaa 348

r->gil2517315ldbjIC76985.lIC76985 MM C76985 Mouse 3.5-dpc blastocyst CDNA Mus r
J0023GO9 31.
Length = 603

Score = 32.2 bits (16), Expect = 7.5
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 2 agaagcattctcagaa 17
Sbjct: 333 agaagcattctcagaa 348

Database: GenBank Mouse EST entries
Posted date: Jun 2, 2003 7:57 PM
Number of letters in database: 1,088,892,180
Number of sequences in database: 2,548,704

Lambda	K	H
1.37	0.711	1.31

Gapped Lambda	K	H
1.37	0.711	1.31

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