THE DEVELOPMENT OF A COSMID MAP OF CHROMOSOME 12P13

STEPHANIE HEATHER BELK

SENIOR HONORS THESIS

MAY 5, 1998

Acknowledgements

I would like to thank the following people for their help with my work on this Honors Thesis. Dr. Ann Fabirkiewicz, Professor of Chemistry at Randolph Macon Woman's College, and Dr. Jill Granger, Professor of Chemistry at Sweet Briar College, for their time as readers for my defense. I thank Dr. Robin Davies, Professor of Biology at Sweet Briar College, for support and suggestions to guide my project and Cassie Thomas, Sweet Briar College Class of 1997, for her time spent in cosmid and primer preparation and her aid in the exploration of the project's techniques. Mr. and Mrs. Belk, Mr. Thomas Loter, and my fellow undergraduates were also essential for the successful completion of this thesis because of their unwavering support. This project was funded by support from the Jeffress Memorial Trust, grant number J-353.

Table of Contents

ABSTRACT

The Human Genome Project is a cooperative global effort, implemented in 1990, with the ultimate goal of determining the genetic sequence of the entire human genome. Also included are plans to study the structure of the genome and advance the current genetic technologies for use with other model organisms. The production of a high-resolution map of Chromosome 12 and the subsequent determination of the nucleotide sequence for the same will aid in the completion of the Human Genome Project. High-resolution maps pinpoint the location of a particular gene to a small region of the chromosome, within 100,000 base pairs.[12] The goal of this project was to develop high-resolution cosmid contigs for the p13 region of Chromosome 12. The specific goals were to use the polymerase chain reaction and hybridization techniques to map cosmids to their respective Sequence-Tagged Site (STS) markers, located within this region. Through these techniques a small cosmid contig was developed for one STS marker within the 12p13 region of the genome.

I. Introduction

I. A. *Human Genome Project History.*

Congress implemented the Human Genome Project in October 1990 after the National Academy of Sciences conducted several years of research in collaboration with the Department of Energy. The original goals were to locate the "building blocks necessary to develop an unprecedented understanding of the basic biochemical processes of living organisms," to work toward molecular medicine based on early detection of disease and preventative medicine.[2] As a result of the technologies developed in the project, there are also hopes of advances in agriculture, environmental science, and industrial processes. The ultimate goal is to improve existing human genetic maps in order to discover the location and sequence of all 80,000 human genes, as well as the sequence of the entire genome, and to render them accessible for further study. Parallel studies are being carried out in several model organisms to gain greater understanding of their genes in disease processes and to facilitate interpretation of human gene function.

The structure of the human genome and of eukaryotic chromosomes is complex. The genome is a term used to describe the entire set of genes within a haploid set of

chromosomes. The chromosomes, though, are made up of a complex of DNA, RNA, and proteins known as chromatin. There are 46 chromosomes in a normal human cell and they each contain between 48 and 240 million base pairs apiece. The base sequences of the chromosomes are the specific ordering of the four nucleotide triphosphates, dATP, dTTP, dGTP, and dCTP. Within the base sequence of a chromosome there are gene coding sequences, promotor regions, random repeated sections, and the telomeres and centromeres. The genes also contain introns, or non-coding regions. The complexity of the chromosomes adds to the difficulty of sequencing the entire human genome.

Parallel studies being carried out in model organisms help in fragmenting the complex sequencing into more manageable segments. Many of the genes of model organisms are very similar to human genes. The use of model organisms such as bacteria, yeast, crop plants, farm and laboratory animals is a cost effective way to follow the inheritance of genes through many generations in a relatively short time-frame. They are also of use in comparative genetics to examine evolution more closely. The current phylogenetic tree is based on the ribosomal RNA sequences. A phylogenetic tree based on the DNA sequences of genes will provide more comprehensive information about evolutionary relationships.[2, 28]

Adding to the complexity of the genome project is the cost of sequencing. Current technology is thought to be too expensive and time consuming to attain the long-term objective of mapping the DNA sequence of the entire human genome. Current technology estimates $2 to $5 per base pair. Goals were to reach $0.50 cost per base or better for large-scale sequencing to be cost effective. In some laboratories, one individual can generate about 2000 base pairs of finished DNA sequence per day per automated sequencing machine.[2] Thus the primary goal of the first ten years of the Human Genome Project was to optimize the existing methods and to develop new technologies to increase efficiency in DNA mapping and sequencing greater than ten-fold. Studies of average sized chromosomes, such as Chromosome 12, are important in optimizing mapping and sequencing methods. Methods that work efficiently for this chromosome are likely to be equally effective in mapping larger chromosomes.

I.A.1. *GENETIC VS. PHYSICAL MAPS.*

Genetic and physical maps of the genome are very different in the information they provide human genome project researchers. A genetic map describes the linear order of genes along a chromosome's length. Homologous chromosomes are really two copies of the same chromosome that are found in each cell. They carry differing allele combinations, or two copies of a given gene, which are distributed to the gametes during meiosis. The different copies of the alleles lie at the same point on the homologous chromosomes. Studying recombination frequencies among differing genes on homologous chromosomes produces genetic maps; the more frequently recombination occurs between two genes, the farther apart they are positioned on the chromosome. The key is that crossing over frequency increases with distance. Two genes whose alleles are close together rarely separate by crossing over. Genes that are separated at a greater distance are separated more frequently (FIGURE 1).[15] A centimorgan, cM, is the term used to arbitrarily define the distance between two positions on a chromosome corresponding to a one-percent recombination frequency. This map unit designation is in honor of the geneticist Thomas Hunt Morgan.[19]

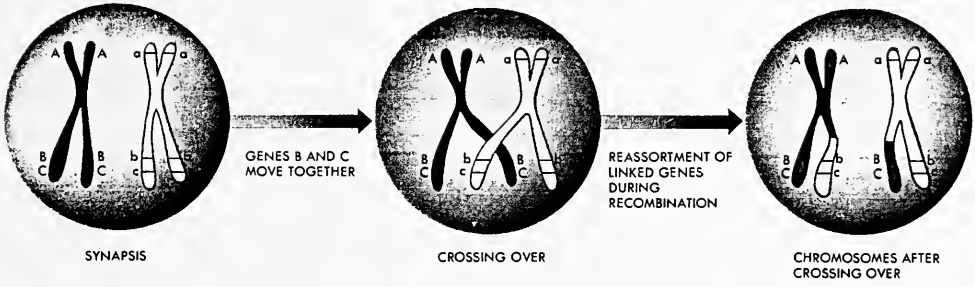


FIGURE 1. Crossing over between genes on homologous chromosomes.[31]

One of the five-year plan (1990-1995) goals for the genome project was that a full genetic map be completed with markers, identified by Sequence-Tagged Sites, spaced 2 to 5 cM apart. This goal was more than fulfilled with a map of 0.7 cM density.[24] Genetic maps were used to discover five separate regions that play a role in insulin-dependent diabetes and in 1995 genome developed technologies played a key role in the isolation of thirteen disease genes, including BRCA-1, the gene for hereditary breast cancer.[11]

Physical mapping is another method for studying the relationship between genes and other parts of the genome. A physical map of the genome is based on the actual distances between different nucleotide stretches as traversed by pieces of recombinant DNA in comparison with the less accurate genetic map. Physical maps of the genome are generally produced first with low resolution and then with higher resolution as the regions between the markers are explored. The goal for physical mapping, using overlapping sets of cloned DNA covering large portions of the genome, is to find markers spaced at 100,000 base pair intervals. The production of detailed physical maps is critical to understanding the basis of many diseases and complex disorders that are the result of interactive effects of multiple genes or the environment (FIGURE 2). The benefits of the Human Genome Project and its impacts are widespread also, reaching into fields other than those that are related to the health sciences. It has also developed the infrastructure for future research, providing technology for the emerging biotechnology industry.

Sequencing will provide information about the instructions encoded within a certain piece of DNA and is critical to understanding gene function and roles in disease processes. Along with large-scale sequencing come advances in the development of genetic tests to indicate a predisposition to disease and eventually the treatments for these diseases based on gene therapy will be developed.

But genetic testing, with all its benefits to preventative health care, can also be misused. Genetic testing could lead to denying of jobs and insurance on the basis of genetic predisposition. The Ethical, Legal, and Social Issues (ELSI) section of the Human Genome Project focuses its attention on protecting the public through rulings based on fairness in use of genetic information, privacy and confidentiality. They also study the psychological impact of testing, reproductive issues related to genetic testing, conceptual and philosophical implications, and genetic testing in general.[2,11] For example, they examine whether parents should be encouraged to have extensive prenatal testing for Down's Syndrome, Tay Sachs Disease, or other diseases. If parents are determined to be carriers, or the fetus expresses the disease gene, what should be done? ELSI also addresses the gene therapy and germ-line gene therapy debates.[19] Their goals are based on fostering greater acceptance of human genetic diversity and enhancing public and professional education on genetic testing policy.[17]

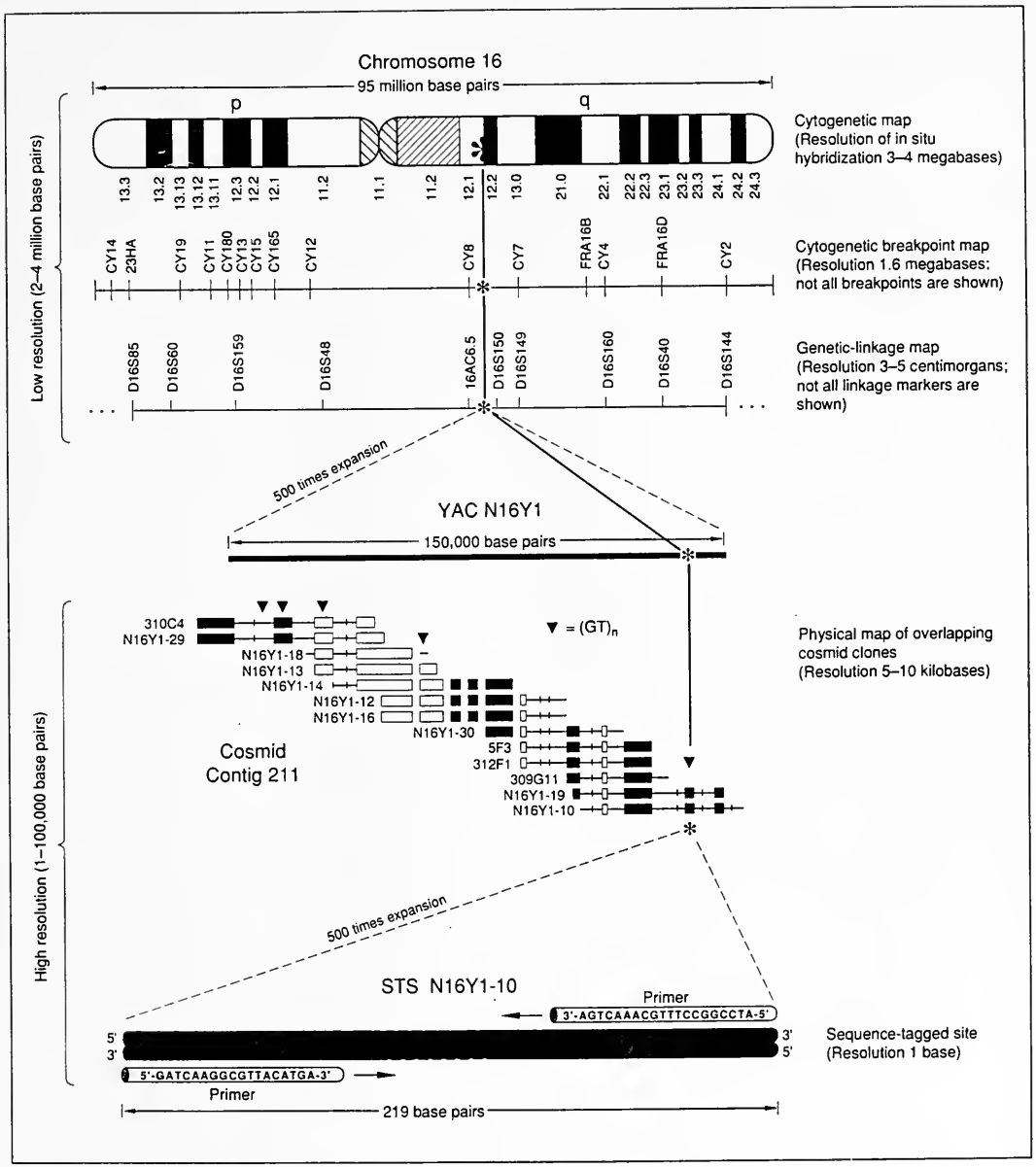


FIGURE 2. A Chromosome Map at Differing Levels of Resolution.[12]

I.A.2. *PROGRESS TOWARDS THE GOALS.*

In 1996, at the end of the first five years of the Human Genome Project, many of the goals had been met or exceeded. The genetic map was complete, the chromosome physical maps were very close to completion and pilot programs for sequencing were underway. The physical map goal was to place a marker every 100,000 bases, or about 30,000 total markers. The most current map has approximately 8,000 markers at twice the resolution of the previous map.[2]

I.A.3. *CHROMOSOME 12 AS A STANDARD FOR GENOME MAPPING.*

Chromosome 12 is an average sized chromosome within the human genome. Projects undertaken to map this chromosome with high efficiency are important because techniques that work with this chromosome are likely to also be effective when working with the larger or smaller chromosomes in the human genome. There are also many genes and cDNA markers located on Chromosome 12. As of March 12, 1998 the chromosome has 357 mapped genes and 8 pseudogenes, or inactive copies of other genes. Some of these genes code for Stickler syndrome, primary osteoarthritis, the Fragile-X mental retardation gene, autosomal dominant hypertension, HIV-1 expression, and phenylketonuria (PKU).[2] Between the telomere and STS marker D12S91, the region being studied in this particular paper, there are 24 cDNA markers. As part of the Human Genome Project, physical maps of Chromosome 12 were produced as a preliminary step to the sequencing procedures. The first generation YAC map of Chromosome 12 contained overlapping clones, or contigs, for 56 STS markers.[23] This was the foundation upon which a second generation high-resolution map was based (FIGURE 3). A contig map using YAC hybridization and Alu-PCR, (PCR for YACs based on repetitive Alu sequences), was made with 13 contigs and 13 gaps. Approximately 75% of Chromosome 12 is estimated to be spanned by these contigs and 412 markers are contained within these contigs.[4, 18]

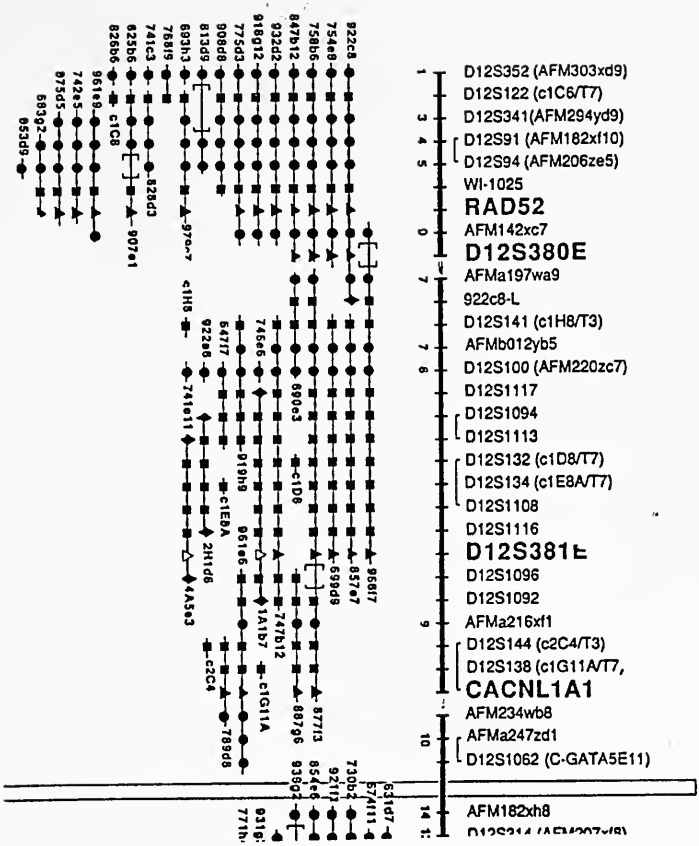


FIGURE 3. Second generation Yeast Artificial Chromosome Map for the p13 region of Chromosome 12.[7] This is an example of a physical map using Sequence-Tagged Sites and YACs. The STSs are positioned along the right side of the vertical cosmid line. The YACs are found on the left portion of the figure and are shown overlapping each other and the STS markers that they contain.

I.B. *RECOMBINANT DNA AS AN EXPLORATION VECTOR.*

I.B.1. *RECOMBINANT DNA.*

Recombinant DNA is formed when fragments of target DNA, broken apart by restriction enzymes or other methods, are inserted into a vector.The recombinant DNA is then replicated along with the vector inside a host cell. Common vectors include plasmids, bacteriophages, cosmids, and Yeast Artificial Chromosomes. Plasmids are one of the more commonly used vectors for recombinant DNA studies and can hold the smallest amount of recombinant material. Plasmids are circular, double-stranded, extrachromosomal DNA. They range from a few thousand base pairs to an excess of 100

kilobases and are self-replicating in the cell. Plasmids that occur naturally often provide selective advantages to their hosts. Many carry antibiotic resistance genes that protect the host and allow for easy identification of those hosts which have taken up a particular recombinant plasmid in the laboratory.[21] The DNA to be cloned is obtained through the use of restriction endonucleases to produce restriction fragments. The restriction fragment may be inserted into the cloning vector (plasmid) by using a restriction endonuclease with the same recognition site on the vector to open it. The complementary ends of the two DNA fragments associate and are spliced together with DNA ligase. An advantage to this method is that the DNA insert can be precisely excised from the cloned vector, if need be, by using the same restriction enzyme as before.[29]

Bacteriophages are the next smallest vectors in the amount of inserted material that they can hold. Up to twenty-five kilobases of foreign DNA can be inserted in the bacteriophage vector. The phage genome contains certain genes that are not needed for the lytic growth pathway and once these are removed, the foreign DNA can be inserted. The recombinant DNA is packaged to form the active bacteriophage vireons that are capable of both replicating and forming plaques on *E. coli* host cells. This process is approximately a thousand times more effective than the transformation found with plasmid vectors.[21]

Cosmid vectors are the particular recombinant DNA clones used in this project. Cosmids are recombinant plasmids that can hold up to 45kb of inserted DNA and are efficiently incorporated into host bacteria cells, usually *E. coli* (FIGURE 4). The vectors are produced by incorporating the COS site of a lambda bacteriophage into a plasmid vector of *E. coli*.[21] COS site incorporation was accomplished by the use of restriction endonucleases to cleave the plasmid vector and insert the COS site gene into the opening. The COS site is used in packaging the DNA to be cloned into the bacteriophage heads. Cosmids are more efficient than both lambda bacteriophage and plasmid vectors in cloning larger pieces of DNA but not cannot hold nearly the amount of cloned DNA as a Yeast Artificial Chromosome (YAC).[21] The 45kb insertion length increases the chances of obtaining clones with the entire sequence of a human gene, since many eukaryotic genes are 30-40kb in length, over the chances of obtaining the sequence when plasmids or bacteriophage vectors are used.

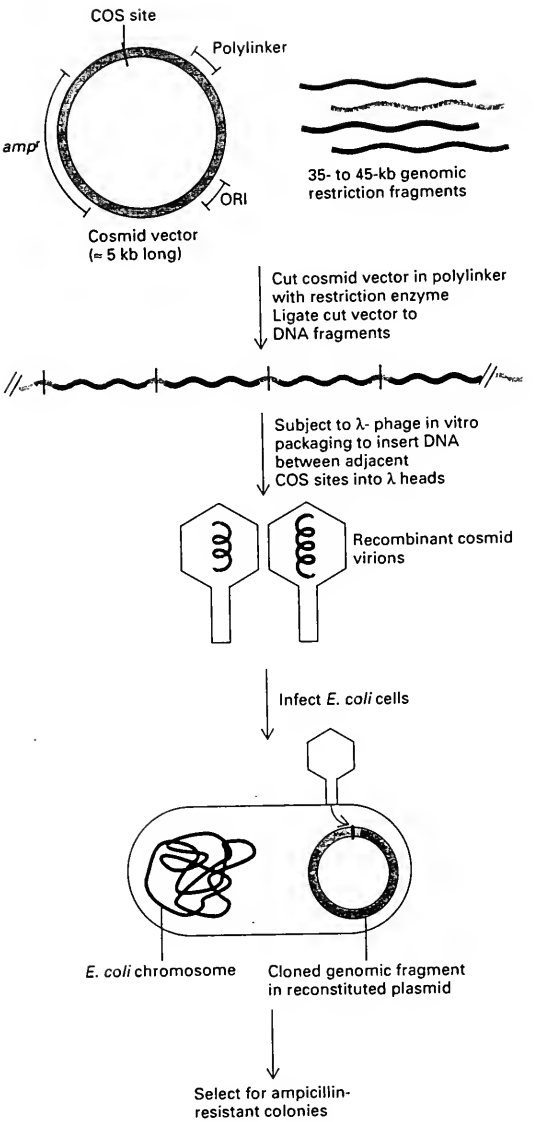


FIGURE 4. Cloning DNA fragments into Cosmid Vector.[21]

Yeast Artificial Chromosomes, YACs, are also important in constructing physical maps of the human genome. Human DNA up to 1000 kilobases in length can be inserted into a YAC. YACs replicate in yeast cells, along with the natural yeast chromosomes, and are taken up with very high efficiency.[21] The use of YACs in physical mapping of the human genome has made possible the isolation of YAC clones that overlap to cover nearly the entire genome. These yeast-based clones can be mapped to Sequence-Tagged Sites or other markers along the chromosomes. Yeast Artificial Chromosome maps are not useful for sequencing DNA, however, because they are too large to be sequenced using current methods. This is why cosmid vectors are valuable in the sequencing of the human genome; they are of manageable size for sequencing after they have been mapped to marker sites within the genome.

I.B.2. *Mapping Recombinant DNA to Locations in the Genome.*

In order to accurately map recombinant DNA we need to be able to clearly establish its position. One way of doing this is by assignment to a Sequence-Tagged Site marker, or STS. Sequence-Tagged Sites are unique, 200 to 300 base sequences located only once throughout the genome that are used as markers for physical mapping (FIGURE 5).[14] The STSs are generally portions of the cDNA sequence of gene transcripts whose physical location relative to one another were determined by the use of YAC human genome DNA libraries. Sequence-Tagged Sites are the markers used to produce ordered maps of contiguous overlapping recombinant DNA clones.

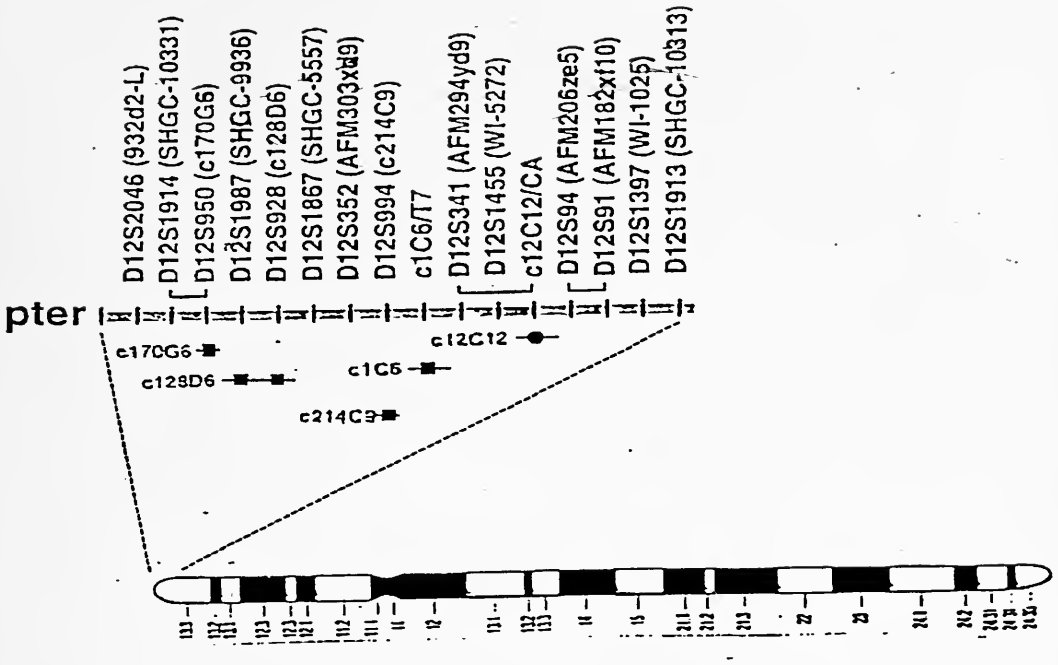


FIGURE 5. STS map of Chromosome 12p13. The sixteen markers, listed along the top of the figure, are being used in the current research. Five cosmids are shown mapped to their respective markers within the lower portion of the figure. (Davies, Jeffress Grant progress report 1996).

I.C. *PRODUCTION OF A PHYSICAL MAP.*

In order to produce a physical map there are several steps that must occur in a specific order (FIGURE 6). First the cosmids which contains the inserted DNA from chromosome 12 must be replicated to produce a large amount for use in the next procedures. Following cosmid replication there are two possible scenarios; the cosmid DNA can be used as a template in the polymerase chain reaction (PCR) in an attempt to replicate a particular Sequence-Tagged Site marker found within the DNA sequence or it can be aliquoted onto a nylon membrane for use in hybridization procedures. If the cosmid DNA was used as a template in PCR the product is electrophoresed on an agarose gel to identify a positive or negative replication of the marker being tested. Positive replication identifies that the cosmid contains the marker sequence and places the cosmid at a physical location within the genome. If the cosmids were placed on a membrane a particular marker was then replicated via PCR and was allowed to hybridize to cosmids that contain its sequence during hybridization and detection protocols. All cosmids that

were detected as positive for the marker, whether determined by PCR or hybridization, were next digested with restriction endonucleases and the resulting fragments electrophoresed on an agarose gel. The gel allows a determination of the overlap between cosmids for a particular marker and allows the production of a cosmid map for the marker. A Southern Blot is used to determine the exact fragment of the restriction endonuclease digestion containing the marker sequence.

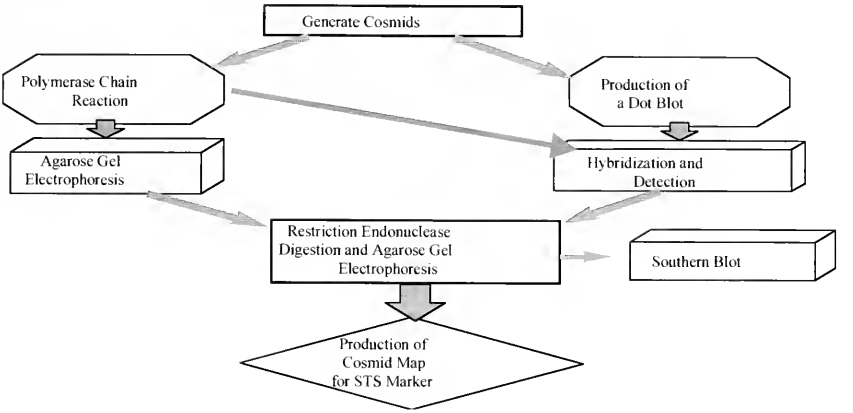


FIGURE 6. Flowchart of steps required to produce a physical map of a chromosome. This a a very brief overview. Refer to entirety of section I.C. for a more complete description of the overall procedures followed.

I.C.1. *POLYMERASE CHAIN REACTION.*

In order to map the human genome we need techniques that would show that a group of recombinant DNAs contained an STS marker sequence within its own DNA base sequence. Two broad techniques were explored in this project, polymerase chain reaction and hybridization.

The first method of mapping used here involves the polymerase chain reaction. The polymerase chain reaction (PCR) is used to amplify a section of DNA found between two primers for a known sequence. There are many of these known sequences, called Sequence-Tagged Site (STS) markers, along the chromosomes. Oligonucleotide primers flank the regions of STS markers and are complementary to the DNA. *Taq* DNA polymerase, a heat-stable enzyme, is used to catalyze the amplification reaction by replicating the region of DNA between the STS primers. The template DNA strand is

first denatured by heating to boiling and then allowed to cool for the oligonucleotide primers to anneal and begin building complementary strands to the DNA template. The process is performed with an excess of primers and the four deoxynucleotide triphosphates. A series of denaturing, annealing, and synthesis cycles are carried out with a two-fold amplification of the select sequence occurring with each cycle (FIGURE 7). The polymerase chain reaction is so specific that single copy genes may be routinely visualized as distinct bands on an agarose gel.[16] Applications for PCR amplification include the generation of cDNA libraries from small amounts of RNA and the generation of large amounts of DNA for sequencing. Rapid screening and/or sequencing of inserts directly from aliquots of bacterial colonies is also possible using PCR.[16]

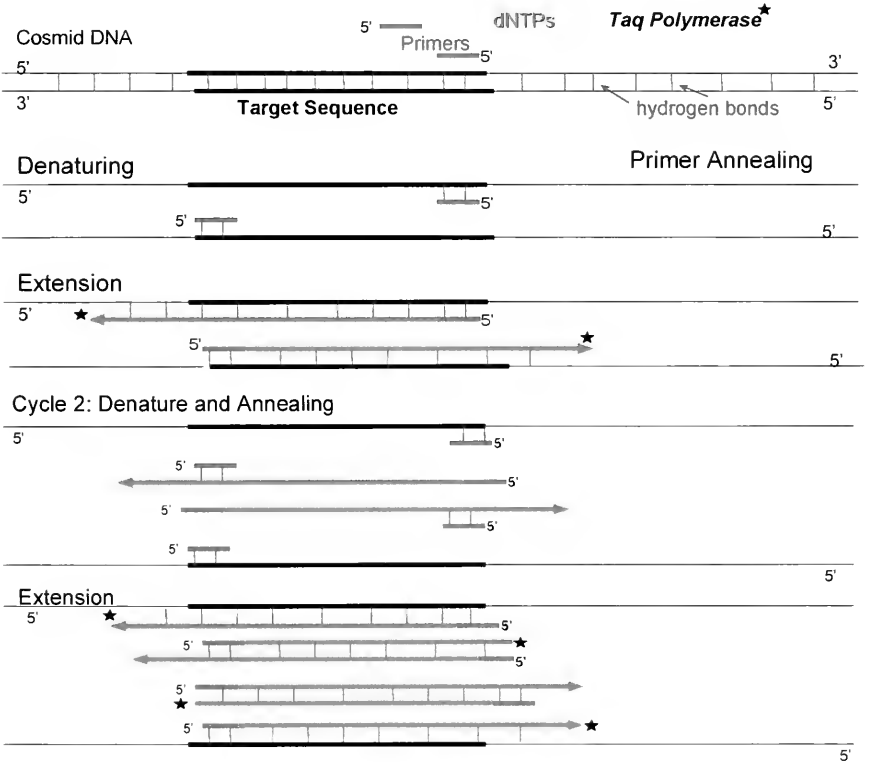


FIGURE 7. Polymerase Chain Reaction.

I.C.2. *Dot Blot.*

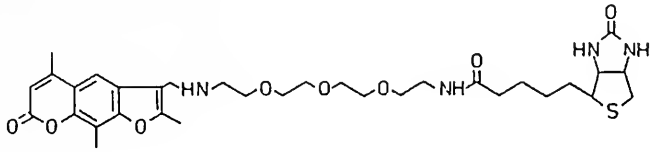
A dot blot is a nylon or nitrocellulose membrane to which single stranded DNA has been bound. Dot blots are extremely useful in probing for complementary sequences between recombinant DNA and particular markers because it allows testing of many pieces of DNA simultaneously. The template DNA from cosmids or other sources is bound to the membrane while the marker DNA sequence is used as a probe. The DNA to be bound is first boiled for ten minutes at 100°C then chilled quickly on ice before being aliquoted onto the membrane. The boiling denatures the DNA and allows single stranded DNA to be bound to membrane for hybridization procedures. The dot blots are then used in hybridization for the large-scale probing and detection of marker sequences within particular bound DNAs.

I.C.3. *Hybridization.*

The second method of determining clone matches to STS markers is hybridization. Hybridization is the binding of complementary base pairs of DNA together via hydrogen bonding. In hybridization, probes of a known sequence or location within the genome or chromosome are labeled with a radioactive label or with a non-radioactive label such as psoralen biotin or digoxigenin (DIG) so that positively hybridized sequences can be determined. Non-radioactive labeling is advantageous over radioactive labeling because it gives faster results with safer handling of both the probe and the labeled product.[5] Non-radioactive labeling can also provide sensitive results in hybridization assays. The non-radioactive DIG DNA labeling kit, from Boehringer Mannheim, detects down to 0.1 pg of homologous DNA. The first visible results can be detected within 20 minutes to three hours after x-ray film exposure.[5] In comparison, radioactive labeling has a high ionizing radiation and requires a lengthy incubation, sometimes weeks, depending upon the isotope, to expose x-ray film for detection. Radioactive probes also have a shorter shelf-life than non-radioactive probes. Under the best conditions, hybridization can detect as little as 0.1 pg DNA that is complementary to a ^{32}P radioactively labeled probe.[25]

One mode of non-radioactive labeling is the use of a psoralen biotin label. Psoralens are planar tricyclic compounds capable of being incorporated into double or

single stranded nucleic acids and being covalently bound to thymidines (FIGURE 8). Psoralen biotin derivatives are incorporated into oligonucleotide probes for use in hybridization. It is the psoralen tri-ring structure that covalently binds to the nucleic acid while the biotin unit, attached via a hydrophilic spacer, serves as the tag for chemiluminescent detection.



Psoralen Biotin

FIGURE 8. Psoralen Biotin Derivative

There are specific psoralen biotin derivatives that have a high affinity, via covalent attachment, for nucleic acids.[20] DNA can be labeled with such derivatives and used in hybridization. The DNA to be identified is placed on a dot blot and the labeled probes are allowed to hybridize to the DNA blots. Cosmids, gene sequences, and low copy repeat sequences are most often tested in this manner.[25] If the probe sequence is present in the dot blot DNA, the probe will remain bound to the membrane blot after washing and will be detected through the detection procedure. If a radioactive probe is used the detection procedure involves production of an image on a x-ray film sheet. If a psoralinated probe is used the detection may be by colormetric or chemiluminescent techniques. The results of colormetric detection are readily visible to the naked eye but chemiluminescent detection of hybridization results requires the use of X-ray film imaging. If detection and labeling are performed correctly it should be possible to detect as little as 50 pg of control DNA, provided in the kit, with chemiluminescent detection.[26]

Another method of producing a non-radioactive label for hybridization uses a steroid hapten known as digoxigenin (DIG) coupled to dUTP to label DNA, RNA, or oligonucleotides (FIGURE 9). First, the labeled STS marker is produced during PCR by incorporating DIG during the replication process.

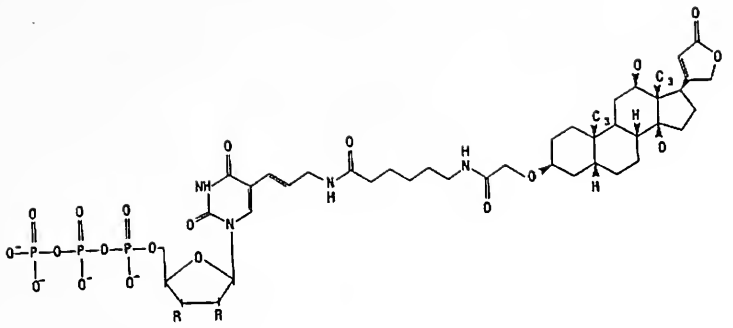


FIGURE 9. Digoxigenin-11-dUTP.

The labeled STS marker probes will then bind with great specificity to cosmids that contain their complementary sequence. This hybridization is easily visualized by immunochemical detection of the hybridized probes. Anti-digoxigenin fragments conjugated to alkaline phosphatases bind CSPD, a chemiluminescence substrate, to produce luminescence on the nylon membrane of the dot blot.[7,8] The luminescence can be recorded on film. It is possible to produce probes specific to a particular STS for use in hybridization to cosmid dot blots, by using primers specific for STS markers in the p13 region of Chromosome 12 (FIGURE 10).

I.C.4. *ADVANTAGES TO USING DIGOXIGENIN HYBRIDIZATION.*

The digoxigenin system has a much easier method of labeling, using PCR, compared to the long irradiation step and multiple extraction steps required in psoralen biotin labeling. Hybridization and chemiluminescent detection procedures are similar in both techniques. In the digoxigenin labeled control it is possible to detect a DNA concentration down to 0.03 pg of homologous DNA using chemiluminescent detection.[7] The first detectable results are visible after only fifteen to twenty minutes, depending on the strength of the signal, and are visible for up to two days, allowing for multiple exposures.[7] In contrast, chemiluminescent control labeling with psoralen biotin is less sensitive and will detect 50 pg of DNA within two or three hours of film exposure.[26]

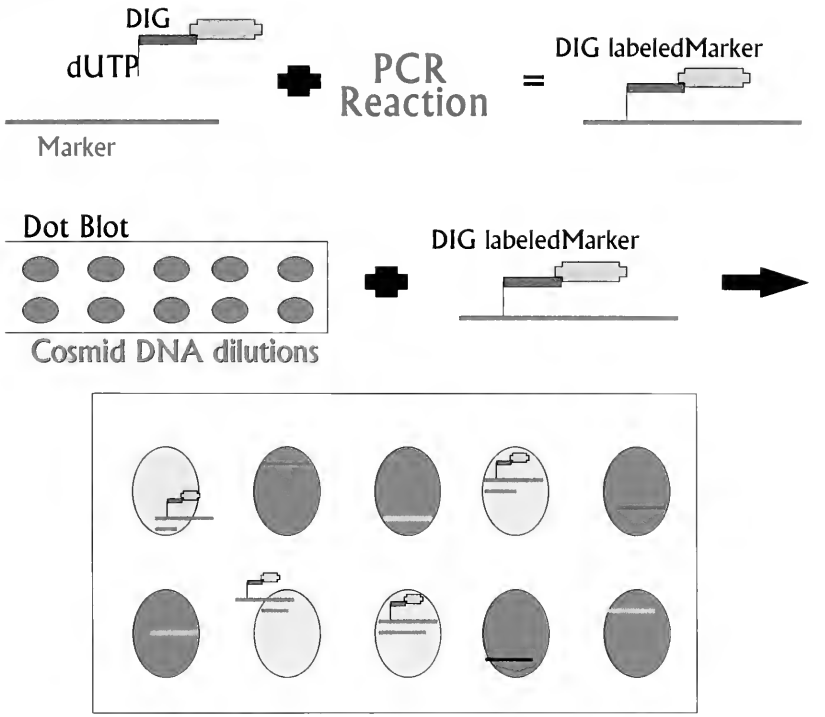


FIGURE 10. Digoxigenin Hybridization of Target STS marker to Cosmid DNA Dot Blot. The gray represents cosmid DNA which has bound the DIG labeled marker DNA.

I.C.5. *AGAROSE GEL ELECTROPHORESIS.*

Agarose gel electrophoresis was employed to determine the relationship among positive cosmids from hybridization and to analyze PCR results. Agarose gel electrophoresis is the standard method of separating DNA of lengths from 200 base pairs to 50 kilobases for identification and purification procedures.[25] A rectangular plate of agarose gel is subjected to a constant voltage and the DNA loaded into the gel, obtained by PCR or other means, migrates toward the positive electrode due to its negative charge (FIGURE 11.a). When a gel is run with a DNA ladder of known size it is easy to determine the sizes of the DNA that are separated across the gel in the procedure (FIGURE 11.b). By plotting the migration distances of known fragments as well as unknown fragments versus the sizes of the known fragments on a logarithmic plot one can

determine the size of the unknown fragments. In the analysis of a PCR reaction, the appearance of a band, other than one at the size consistent for primers, may be indicative of a positive match of the cosmid to the marker tested.

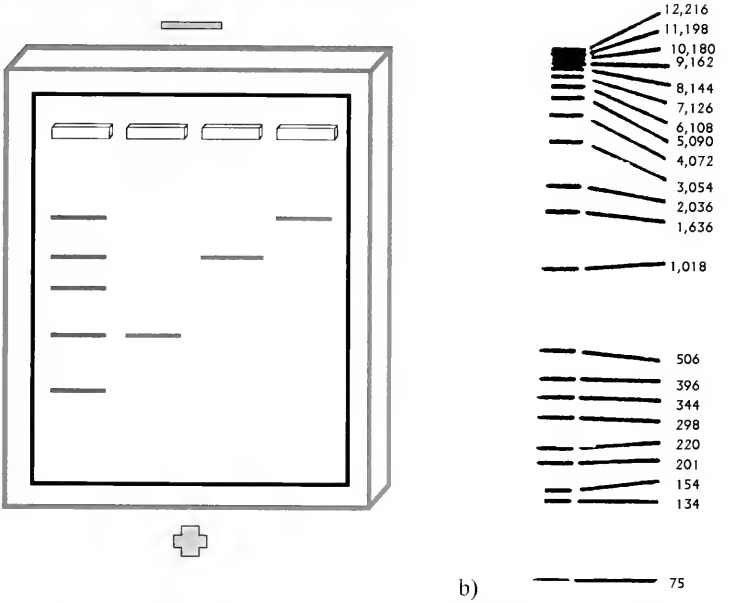


FIGURE 11. a) Electrophoresis of agarose gel. b) 1 KB ladder. This is the DNA of known fragment size that is run as a standard for size migration on all agarose gels that are electrophoresed.

Detection of the bands by ultraviolet light requires the gel to either be made with ethidium bromide (0.5 μg/ml) or to be stained with ethidium bromide (~200ng/ml) before photographing (FIGURE 12). As ethidium bromide is a carcinogen, its use requires extreme caution and gels and buffers containing the solution must be properly destained before disposal.[25] Another possible stain to use is SYBR Gold Nucleic Acid Stain, a proprietary unsymmetrical cyanine dye. This stain is one of the most sensitive available for detecting double or single-stranded DNA or RNA in electrophoretic gels. It is more sensitive than ethidium bromide in many applications and does not require destaining prior to visualization. In addition the dye can be readily removed from the nucleic acids leaving the templates pure for further study.[22]

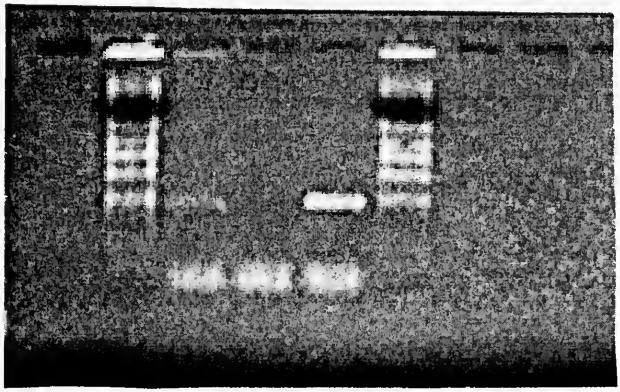


FIGURE 12. PCR of Cosmid 170G6 with Forward and Reverse 950 Primers. A positive band for STS marker D12S950 is observed. Lane 1 is empty, Lanes 2 and 6 contain 1 KB ladder, Lane 3 contains Total Human DNA (K562), Lane 4 contains Chromosome 12, and Lane 5 contains cosmid 170G6.

I.C.6. *RESTRICTION ENZYME DIGESTIONS.*

The use of restriction endonucleases is one way to show the relationships between different pieces of recombinant DNA. Restriction endonucleases recognize a specific four to eight base sequence within double-stranded DNA and cleave both strands, cutting approximately every 256 bases for a four base recognition site and every 4096 bases for a six base recognition site.[30] Of the three types of endonucleases the Type II restriction enzymes are the most useful for DNA manipulation because they cleave at sites within their recognition sequence whereas Types I and III cleave at sites distant from the recognition site. In nature, bacteria use restriction endonucleases for protection from viral infection. In the laboratory, restriction enzymes can be used to characterize DNA. Restriction maps show relative positions of restriction cleavage sites within a DNA molecule and can provide a framework for locating base sequences within a fragment of DNA. Treatment of several different DNAs with the same restriction enzymes produces a series of fragments of defined sizes. If these fragmented DNA molecules are run on gel electrophoresis the identically sized regions within the DNAs are easily identified. If two pieces of DNA share a portion of the same sequence they will be fragmented in equal sized pieces and these are visualized through electrophoresis (FIGURES 13, 14).

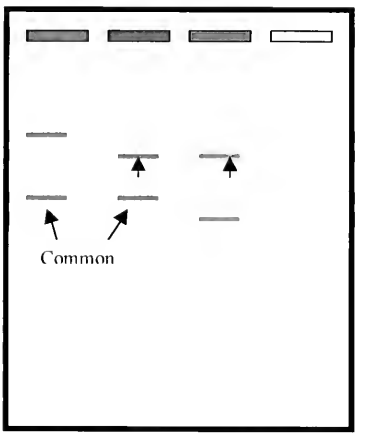


FIGURE 13. Restriction Endonuclease Digestion of three different DNA molecules as electrophoresed within an agarose gel. Common bands represent regions of the same molecular size thus representing a region of identical sequence between the DNA molecules.

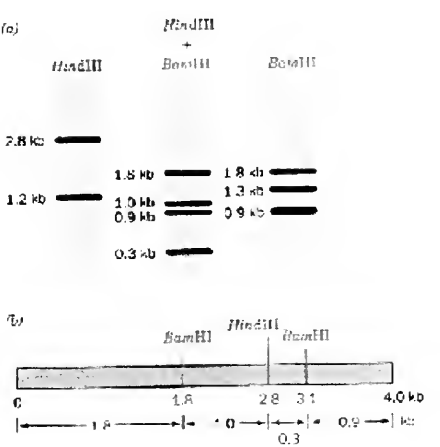


FIGURE 14. Sample restriction enzyme map. A piece of 4.0 kb DNA fragmented by two separate endonucleases and the two endonucleases together to produce a restriction enzyme map of the fragment. A) Agarose gel electrophoresis of the endonuclease results for the fragment. B) A restriction enzyme map showing the results of the enzyme digestions and the relative positions of the restriction enzyme recognition sites.[30]

Using hybridization, PCR, and gel electrophoresis techniques it should be possible to accurately identify the presence of particular primer sequences in a set of cosmids from the p13 region of chromosome 12. The matching of several cosmids to a particular STS marker within the region will allow for the production of a cosmid contig (FIGURE 15). Cosmid contigs, named for the pieces of DNA that constitute them, are overlapping maps formed when cosmids positive for a DNA sequence are ordered according to identical sequence regions. By using restriction enzyme digestion with multiple restriction enzymes it is possible to determine the amount of overlap between neighboring cosmids that share positive bands for a particular marker. If the cosmids share common bands when electrophoresed after being exposed to multiple restriction enzymes then they should contain identical sequences for that region. When cosmid contigs are produced for several markers along the region it may be possible to produce a genetic map of the region, thus aiding in the Human Genome Project goal of forming high-resolution genetic maps for the entire genome.

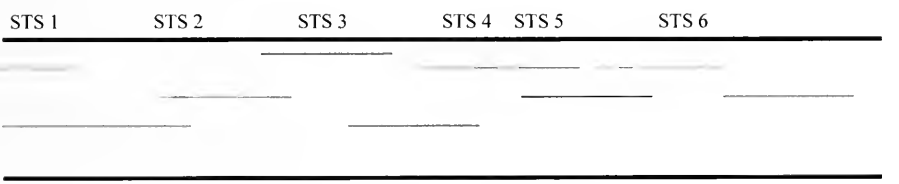


FIGURE 15. This is a small cosmid contig that could have been built by the techniques described for this project. The dark band at the top of the contig is representative of the portion of chromosomal DNA being mapped with STS markers shown. The colored bands represent individual cosmids.

I.C.7. *SOUTHERN BLOT.*

Southern blotting is an additional method of determining the relationships of cosmids to an STS. During Southern Blot in this project, agarose gels of restriction endonuclease digested cosmid DNA were exposed to a nylon membrane in the Southern Blot apparatus and the DNA fragments within the gel diffused onto the membrane. Following diffusion times of approximately 18-24 hours the membrane was removed and the DNA permanently attached via crosslinking with an ultraviolet light source. The membrane could then undergo hybridization procedures with a DIG labeled STS marker to identify the restriction fragment that contained the STS sequence specifically.

II. Materials and Methods

II.A. *Cosmid Preparation.*

Bacteria containing cosmids were first streaked onto 2x TY agar plates to which 20 µg/mL Kanamycin was added (Appendix A). The plates were incubated at 37°C to obtain single colonies. A single colony was transferred to sterile 125 mL flask that contained 30 mL 2x TY broth and 30 µLof Kanamycin. The cultures were incubated overnight at 37°C with vigorous aeration, at approximately 240 RPM, on the LabLine® Orbit Environ-Shaker. Flask cultures were centrifuged at 2000 RPM for thirty minutes in a tabletop Beckman TJ-6 Centrifuge with rotor TH-4 1-85. The supernatant was removed and the pellet re-suspended in 1.5 mL of Solution I (50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA). Three milliliters of freshly prepared Solution II (0.2 M NaOH, 1% SDS) were added and the solution inverted to mix. After several minutes 2.25 mL of Solution III (2.9 M KoAc, 1.99 M glacial acetic acid) were added and the solution was mixed by inversion. The resulting solution, which resembles egg albumin in viscosity, was transferred to 50 mL centrifuge tubes and pelleted in the Sorvall RC-5B Centrifuge with an SS-34 rotor at 4°C at 17000 RPM for fifteen minutes. The supernatant was then filtered through Kimwipes™ into 50 mL conical tubes to remove the cellular debris and high molecular weight bacterial DNA. Isopropanol at one-half volume was added and the solution was allowed to precipitate at room temperature for at least thirty minutes. The suspension was centrifuged in the Sorvall centrifuge for thirty minutes at 17000 RPM to recover the precipitate. The pellet was resuspended in 400 µl TE and transferred to 1.5 mL Eppendorf tubes. Two microliters of 10 mg/mL RNAase A and of 1000 U/mL RNAase T1 were added to the tubes to a final concentration of 0.05 mg/mL RNAase A and 5 U/mL RNAase T1. The tubes were then incubated in a water bath for thirty minutes at 37°C. Added to the tubes were 8.1 µL of 10 mM EDTA and 8.1 µL of 0.2% SDS before transfer to 1.5 mL PhaseLock™ gel tubes, manufactured by 5 Prime→3 Prime Inc. The Phase Lock™ tubes were previously layered with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). PhaseLock™ gel tubes recover nucleic acids from the denatured proteins at the aqueous and organic interface with relative ease following extraction. During centrifugation the Phase Lock Gel™ migrates to form a seal between organic and aqueous phases of the sample, trapping the organic material below the gel and allowing the aqueous phase containing the DNA to be recovered by

pipetting.[1] The PhaseLock™ tubes were spun at 14000 RPM for two minutes and the top layer was removed. To this layer were added 150 µL of 1M NaOAc to a final concentration of 0.3 M and 2.5 volumes of ethanol to precipitate the DNA. The mixture was placed in -80°C freezer for a minimum of two hours. The cosmid DNA preparations were removed from the -80°C freezer and centrifuged at 1400 RPM for ten minutes in the Eppendorf centrifuge model number 5402. The supernatant was discarded, the pellet was washed with 1 mL of 70% ethanol/30% TE, was allowed to air dry and was then resuspended in 90 µL of TE and stored at -20°C. The final step was to determine the OD260/280 ratio by reading the absorbance of the diluted sample on the Beckman DU-640 spectrophotometer. A ratio of 1.8 or better signified cosmid preparations free of protein and ready to use. If the ratio was lower than 1.8 the cosmid was subjected to additional rounds of RNAase treatments, phenol extraction, and ethanol precipitation until such time as the ratio was of an acceptable value.

II.B. *Polymerase Chain Reaction.*

II.B.1. *Target DNA Amplification.*

Oligonucleotide primers for STS markers on the P arm of Chromosome 12, obtained from Kate Montgomery at Albert Einstein College of Medicine, were adjusted to 100 ng/ µL concentrations. A Master Mix was prepared using calculations for n+1 tubes (Appendix B). The Master Mix contained ddH_2O, 1x PCR buffer, 0.2 mM of each dNTPs (dATP, dTTP, dGTP, dCTP), 0.064 Units/µl *Taq*, 1.5 mM $MgCl_2$, 5 ng/µL forward primer, and 5 ng/µL reverse primer per tube. The 10x PCR buffer is composed of 100 mM Tris HCl, and 500 mM KCl adjusted to pH 8.3. One half milliliter Eppendorf tubes were prepared with 19 µL Master Mix and 1 µL template, where the template was total human DNA, Chromosome 12, or an individual cosmid. Cosmid pools were made up of five separate cosmids in equal volumes. If cosmid pools were to be tested, 18 µL Master Mix and 2 µL cosmid pool were used. The Eppendorf tubes were placed in an Ericomp Easy Cycler Series thermal cycler and run on program 10. Program 10 consisted of one repetition of 94°C for two minutes, thirty repetitions of 94°C for thirty seconds, 58°C for thirty seconds, and 72°C for thirty seconds, one repetition of 72°C for fifteen minutes of final extension, and ending with a 4°C holding phase. After amplification, the tubes were

centrifuged on the short cycle of the Eppendorf Centrifuge 5402 for ten seconds to spin the product contents to the bottom of the tube.

II.B.2. *Agarose Gel Electrophoresis.* Running buffer to a final concentration of 0.04% bromophenol blue and 6.4% w/v sucrose were added to each PCR reaction tube and 20 µL of each were loaded into the wells of a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide (APPENDIX B). One-half of a microgram of 1KB ladder was loaded into one of the wells. The gel was electrophoresed for thirty to forty minutes at 180V in a buffer of 1x Tris Borate EDTA (0.45 M Tris Base, 0.44 M boric acid, 0.5 M EDTA). Gels were then photographed with Polaroid 667 film using the Fotodyne Ultraviolet transilluminator. An f-stop of 5.6 or 4.5 and an exposure length of 1/2 seconds were used for photographing the gels.

II.C. *COSMID HYBRIDIZATION USING PSORALEN BIOTIN.*

II.C.1. *Labeling.*

Using a Rad-Free Universal Oligo Labeling and Hybridization Kit from Schleicher and Schuell, a single primer of known sequence was bound with ultraviolet radiation to psoralen biotin and brought to a concentration of one microgram per milliliter (APPENDIX C). Under reduced light the psoralen biotin reagent and oligonucleotide solution were mixed in a ratio of 1:10. A Rad-Free UV lamp was positioned over the samples and irradiation carried out for one hour. TE was added to aid in sample removal to Eppendorf tubes. Two volumes of dH_2O-saturated n-butanol were then added to extract unincorporated psoralen biotin. The tubes were then vortexed and centrifuged at approximately 7000 RPM to separate the two phases of the sample. The n-butanol layer was then removed and the extraction repeated. The labeled oligonucleotides were then ready for hybridization or storage at -20°C.

II.C.2. *Preparation of Dot Blot.*

Previously prepared cosmid DNAs (average concentration of 1.993 µg/µL) were pipetted onto a Schleicher & Schuell Nytran™ nylon membrane filter, producing what is known as a dot blot. The cosmids were denatured by heating to 100°C for ten minutes, cooled rapidly on ice, and then aliquoted on the membrane. The cosmids were attached

to the damp membrane using a Stratagene UV Stratalinker 2400 (autocrosslink at 1200 μJ (x100) for 25-50 seconds).

II.C.3. *Hybridization.*

The labeled psoralen oligonucleotides were used with the prepared dot blots for hybridization procudures (Appendix C). Ten milliliters Southern pre-hybridization solution (0.75 M NaCl, 0.075 M NaCitrate $2H_2O$, 5% Rad-Free Blocking Powder™, 0.035 M SDS) per 100 cm^2 membrane was incubated at optimal probe annealing temperature for one hour. The pre-hybridization solution was replaced with an equal amount of the same solution plus labeled primer (25 ng/mL) and incubated at the same temperature for another hour with gentle agitation. The membrane dot blot was then washed twice in 500 mL Wash Solution I (0.3 M NaCl, 0.03 NaCitrate $2H_2O$, 3.4 mM SDS) at room temperature for five minutes with gentle agitation and twice in 500 mL pre-warmed Wash Solution II (0.05 M NaCl, 1.5 mM NaCitrate $2H_2O$, 3.4 mM SDS) in a 40°C water bath for five minutes.

II.C.4. *Detection.*

Detection of positive hybridization of the psoralen probe to the dot blot used the Schleicher and Schuell Rad-Free Universal Oligo Labeling and Hybridization Kit. Five milliliters detection blocking solution (0.05 M Tris Base, 0.15 M NaCl, 3% Rad-Free Blocking Powder™, 3.4 mM SDS) and fifty microliters 10% Sodium dodecyl sulfate (SDS) were placed with the dot blot in a hybridization bag and incubated for one to three hours at room temperature with gentle agitation. The blocking solution and 10% SDS were replaced with equal amounts of both. Three microliters (at concentration indicated by vial) streptavidin-alkaline phosphatase was added to the bag and then incubated for one hour at room temperature with gentle agitation. The blot was washed three times in 500 mL Wash Solution III (0.05 M Tris Base, 0.15 M NaCl, 3.4 mM SDS) for ten minutes at room temperature with gentle agitation, once in 1x TBS (0.5 M Tris Base, 1.5 M NaCl, pH 7.5) for ten minutes at room temperature with gentle agitation, and placed in an imaging cassette to expose a sheet of film to reveal positive hybridization results. A Konica SRX-101 Medical Film Processor was used to develop the single exposure x-ray film following exposure times of 30 minutes to 12 hours.

II.D. *COSMID HYBRIDIZATION USING DIGOXIGENIN.*

II.D.1. *Labeling.*

Using the polymerase chain reaction, digoxigenin labeled deoxyuracil, DIG-11-dUTP, was incorporated into the growing replicated strands of the STS markers (Appendix C). Evidence of positive incorporation was obtained by running 10 μL of the PCR reaction mixture on a 1.5% agarose gel in electrophoresis. The reagents and DIG-11-dUTP used in the PCR reaction were obtained from the Boehringer Mannheim PCR DIG Probe Synthesis Kit.

II.D.2. *Hybridization.*

Hybridization following the Boehringer Mannheim DIG Hybridization protocol was performed next (Appendix C). Prewarmed Standard Hybridization Solution (5x SSC, 0.1% (w/v) N-lauroylsacrosine, 0.02% (w/v) SDS, 1% blocking reagent) was added to the dot blot at 20 mL/100 cm^2 and incubated for thirty minutes at the temperature for hybridization appropriate to the labeled probe. The DIG labeled probe (5 μL/ mL), denatured by heating to 100°C for five minutes, was included with 2.5 mL/100 cm^2 Standard Hybridization Solution and the membrane was incubated in this mixture in a 68°C water bath overnight. The 68°C hybridization temperature is used for homologous probes when the standard hybridization buffer is used, otherwise the temperature is dtermined to be 20-25°C below the calculated T_m for the probe. Following the hybridization incubation, four post-hybridization washes were performed. Two five minute washes in 2x SSC and 0.1% SDS were carried out at room temperature and two fifteen minute washes in 0.1x SSC, 0.1% SDS were carried out with constant agitation on the Lab-Line® Orbit Environ-Shaker at 68°C.

II.D.3. *Detection.*

Detection of hybridization with the DIG labeled probe was performed using the DIG Luminescent Detection Kit from Boehringer Mannheim (Appendix C). The membrane was first rinsed for five minutes in Washing Buffer (maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), 0.3% (v/v) Tween 20) then incubated for thirty minutes at room temperature in 100mL/100cm^2 1x Blocking Solution (10% (w/v)

blocking reagent in maleic acid buffer). Anti-digoxigenin alkaline phosphatase conjugate was diluted to 75 mU/mL in 1x Blocking Solution and the membrane was incubated for thirty minutes in 20 mL/100cm^2 of the antibody solution. Two fifteen minute washes in 100 mL/100cm^2 Washing Buffer were followed by a five minute equilibration in 20 mL/100cm^2 Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). A dilution of chemiluminescence substrate CSPD® 1:100 in Detection Buffer was performed and the membrane incubated in a hybridization bag for five minutes in 2 mL/100cm^2 of the CSPD® solution (CSPD® is Disodium 3-(4-methoxyspiro{1,2-dioxetane-3, 2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate).[6] The membrane was blotted; DNA side up, on Whatman 3MM papers and then sealed in a hybridization bag while damp. Following a ten-minute incubation at 37°C the membrane exposed x-ray film for at least twenty minutes at room temperature. Luminescence of the membrane continues for at least twenty-four hours and the visible signal increases in intensity during the first few hours.[6]

II.E. *SOUTHERN BLOT.*

Southern blot techniques were used in attempts to determine the position of STS markers within the fragments of endonuclease digested cosmids. The cosmids were separated via agarose gel electrophoresis following endonuclease digestion. After electrophoresis the gel was subjected to two DNA denaturation washes in 1 M NaCl and 0.5 M NaOH with constant agitation on the Lab-Line® Orbit Environ-Shaker at room temperature. Next the gel was exposed to two DNA neutralization washes in 1.5 M NaCl and 0.5 M Tris, pH 7.4, at room temperature. The DNA within the gel was then transferred onto a Schleicher and Schuell Nytran™ membrane using the Southern Blot Apparatus (figure in Appendix D). Following the twenty-four hour transfer, the Nytran membrane was washed in 5x SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4) for five minutes at 60°C. The DNA was immobilized by crosslinking in the Stratagene UV Stratalinker 2400 (autocrosslink at 1200 μJ(x100) for 25-50 seconds).

III. RESULTS

III.A. *POLYMERASE CHAIN REACTION.*

PCR reactions were performed on two individual cosmids, c170G6 and c128D6, and a set of cosmid pools during this project. Results were successful in that the two individual cosmids were mapped to STS markers on chromosome 12 and c128D6 was used to determine the location of one particular STS within the region, D12S1914. PCR was also used to label STS markers in the region with digoxigenin for testing with cosmids in hybridization procedures.

III.A.1. *Preliminary Experiments.*

The preliminary PCR reactions were used to verify the protocols being used and to test the accuracy of the techniques. Using cosmid 170G6 and primers 950F and 950R, ten repetitions of the replication cycle (94°C, 58°C, and 72°C for thirty seconds each) were attempted with no resulting positive band when electrophoresed. A repeat of the experiment using thirty repetitions of the replication cycle gave a positive band for both the cosmid and the total human DNA columns (FIGURE 16).

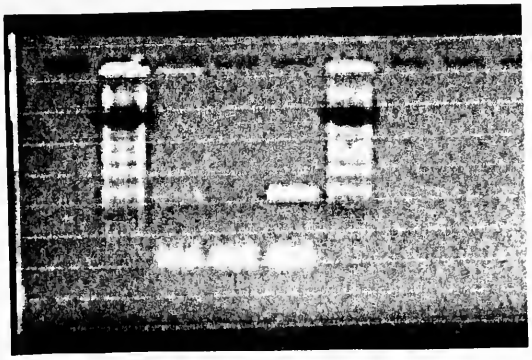


FIGURE 16. PCR of Cosmid 170G6 with Forward and Reverse 950 Primers. Positive band for D12S950. Lane 1 is empty, Lane 2 contains 1 KB ladder, Lane 3 contains Total Human DNA (K562), Lane 4 contains Chromosome 12, and Lane 5 contains cosmid 170G6.

There was no band for chromosome 12 as was expected following observation. The signal from chromosome 12 DNA, obtained from a rodent-human cell hybrid, should have shown

half the signal of K562 DNA because it contained only one copy of the chromosome where K562 DNA contains two copies. The positive band for c170G6, a cosmid that was previously mapped to the same STS marker, allowed for verification of the methods being used. These being verified, further testing could be undertaken.

III.A.2. *Determining the Placement of Marker D12S1914.*

To determine the relationship between markers D12S950 and D12S1914, a PCR reaction was run using c170G6 with primers 950F, 950R and c128D6 with primers 1914F, 1914R. A positive band was observed for c128D6 at approximately 75 base pairs but no band was observed for c170G6 (FIGURE 17).

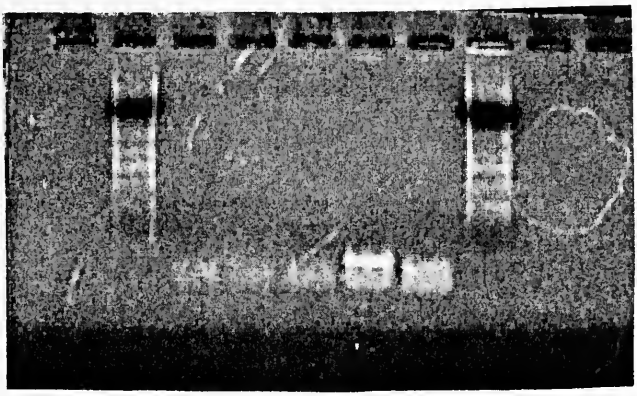


FIGURE 17. PCR of c170G6 with forward and reverse 950 primers and of c128D6 with forward and reverse 1914 Primers. Lanes 2 and 8 contain 1KB ladder (0.5 μg), Lane 3 contains c170G6, Lane 4 is a negative control of TE for 950 primers, Lane 5 contains an unknown cosmid suspected of being c170G6, Lane 6 has c128D6, and Lane 7 contains a TE negative control for 1914 primers. In Lane 6 there is a visible positive band for c128D6 with marker D12S1914 at approximately 75 base pairs.

III.A.3. *PCR with Cosmid Pools.*

In order to process the cosmid DNA more efficiently, cosmid pools of 5 μL each of five different cosmids were prepared for 94 of the available cosmids for the section of chromosome 12 being mapped. In general testing with cosmid pools was unsuccessful. Positive controls did not show bands at migration distances corresponding to the STS markers being tested. Agarose gels did not run smoothly or photograph clearly, giving fuzzy banding overall or light primer bands and no marker bands at higher molecular

weights. The primers were found to degrade over time so new aliquots had to be made and the concentration checked periodically to insure proper concentration for the experiments. The *Taq* DNA polymerase also seemed to stop annealing properly during this portion of the project.

III.A.4. *Digoxigenin Labeling of K562 DNA for STS markers of 12p13 region.*

Although attempts to test cosmid pools for the presence of STS markers were unsuccessful, the polymerase chain reaction was used to effectively replicate and label STS markers with digoxigenin for use with DIG hybridization procedures. K562 DNA was used as a template for labeling STS markers D12S950, D12S1914, and D12S1987 with digoxigenin. When digoxigenin was properly incorporated into the marker sequence the molecular weight of the marker was slightly increased. This was due to the increased mass of the labeled uracil over that of the original thymidine of the sequence. STS marker D12S950 showed a faint band visible at approximately 396 or 344 bases following labeling (FIGURE 18). PCR was also used to label STS markers D12S1987 and D12S1914 producing bands of approximately 298 bases and approximately 134 bases, respectively (FIGURE 19). Normal PCR amplification would result in fragments of 280, 224 and 91 bases for D12S950, D12S1987 and D12S1914 respectively.

TABLE 1. STS Markers of the 12p13 region tested during the course of this project.

STS Marker	Minimum Marker Length	Positive Cosmid Control
D12S950 (c170G6)	0.280 KB	c170G6
D12S1914 (SHGC-10331)	0.091 KB	c128D6
D12S1987 (SHGC-9936)	0.224 KB	c128D6

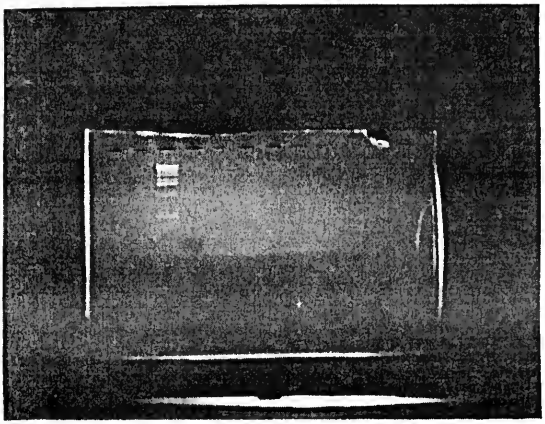


FIGURE 18. 1.5% agarose gel electrophoresis of a digoxigenin labeling PCR procedure using Forward and Reverse Primers for STS D12S950. Lane 3 contains 0.5 μg 1KB ladder and Lane 4 contains K562 labeled DNA. A faint band is visible in Lane 4 at approximately 396 or 344 bases corresponding to the labeled STS marker.

FIGURE 19. 1.5% agarose gel electrophoresis of digoxigenin labeled K562 DNA with Forward and Reverse Primers for D12S1987 and D12S1914. Lane 3 contains 0.5 μg of 1KB ladder, Lane 4 has K562 DNA labeled for D12S1987, and Lane 5 has K562 DNA labeled for D12S1914. The band in Lane four is approximately 298 bases while the band in Lane 5 is approximately 134 bases in length.

III.B. *HYBRIDIZATION.*

Hybridization using the digoxigenin system was found to produce successful results during this project. The three STS markers D12S950, D12S1987, and D12S1914 were labeled with PCR and were bound to DNA dot blots containing all the available cosmids. For each STS marker tested there were several very darkly labeled positive cosmids observed following incubation with x-ray film. Hybridization using psoralen biotin derivatives was not successful other than those procedures run as part of the control procedure.

III.B.1. *Control Hybridization.*

A control hybridization was performed using DIG labeled template DNA, pBR328, supplied with the kit and self-labeled during PCR. Unlabeled pBR328 DNA was bound to a nylon membrane to form a dot blot. Probe concentration used for the procedure was 5 μL/mL hybridization solution of which there was 2.5 mL per 100 cm^2 dot blot membrane. The control hybridization procedure revealed binding down to a $5x10^{-7}$ μg/μL concentration of template DNA (FIGURE 20). Mock hybridizations were carried out to determine the optimal probe concentration for experimental hybridizations. These hybridizations used small unlabeled pieces of membrane exposed to 1 μL/mL, 2 μL/mL, 3 μL/mL, or 5 μL/mL probe concentration during the overnight incubation. Following luminescence detection only the 5 μL/mL concentration showed too high a background for use in experimental hybridizations. The 2 μL/mL probe concentration was used for the experimental hybridizations.

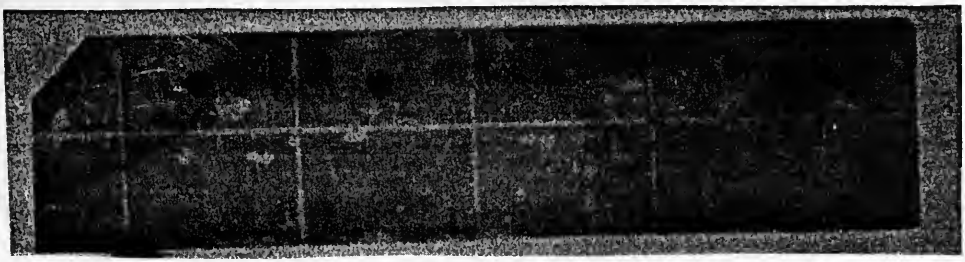


FIGURE 20. Control Digoxigenin Hybridization using unlabeled pBR328 as the target DNA. DIG labeled pBR328 DNA was used as the probe. The probe produced a visible label for unlabeled DNA dilutions of $1x10^{-5}$μg/μL, $5x10^{-6}$μg/μL, $1x10^{-6}$μg/μL, and $5x10^{-7}$μg/μL.

III.B.2. *Sequence-Tagged Site Marker D12S950.*

Digoxigenin labeling of human DNA, K562, with primers 950F and 950R produced visible product at approximately 344 or 396 base pairs when electrophoresed. This demonstrated that the DNA for STS marker D12S950 was effectively labeled with digoxigenin. The labeled sequence was then used as a probe for STS sequences within the cosmid dot blot during hybridization. Following film exposure eleven cosmids showed strong labeling reactions (FIGURE 21). Those cosmids with strong labeling were c64A5, c66G2, c133A5, c164G4, c167F1, c170G6, c175F11, c189C3, c203F5, c205G9, and c221H6. These cosmids are most likely positive for the STS marker sequence. A positive control of c170G6, already known to contain the STS marker through PCR, showed a strong labeling reaction on the blot, providing assurance that the system is accurate and conclusive.

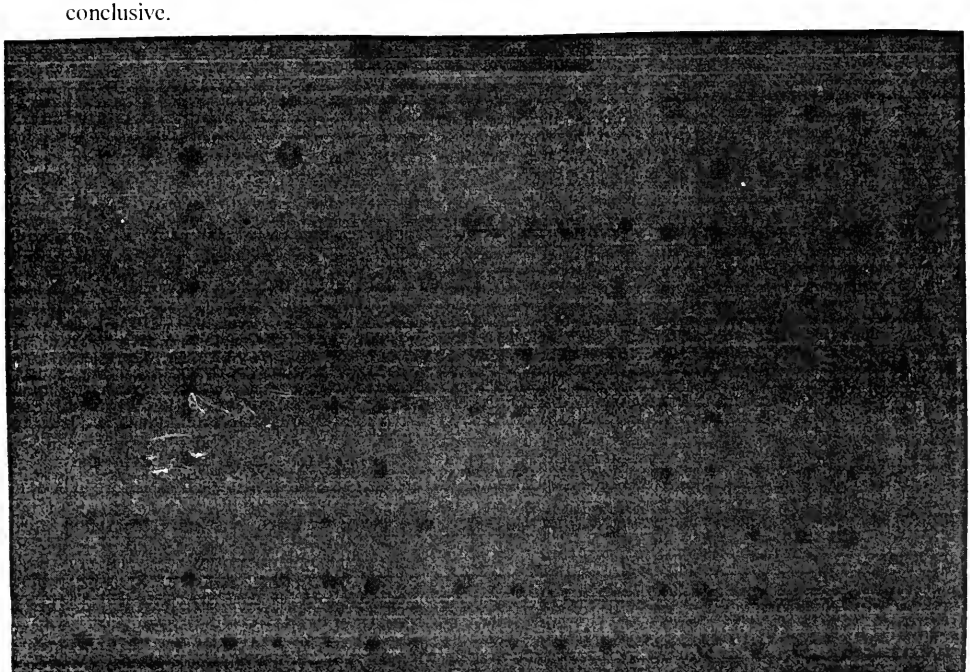


FIGURE 21. Dot Blot Hybridization of cosmid DNA with digoxigenin labeled D12S950 marker. The most distinct and darkest labeled dots were used to determine the identity of cosmids positive for the marker. These cosmids were c64A5, c66G2, c133A5, c164G4, c167F1, c170G6, c175F11, c189C3, c203F5, c205G9, and c221H6. Cosmid 179G6 was a positive control for the hybridization.

III.B.3. *Sequence-Tagged Site Marker D12S1987.*

Digoxigenin labeling during PCR of K562 DNA with primers 1987F and 1987R was successful as shown in the approximately 0.298 KB band on gel electrophoresis, an unlabeled marker band would have been at 0.224 kilobases. Following hybridization of the labeled probe with a cosmid DNA dot blot, seven cosmids showed strong labeling reactions (FIGURE 22). Cosmid 128D6 was a positive control for the marker and it also strongly labeled in the hybridization. Positive cosmids were as follows: c72G11, c128D6, 168C12, c192A8, c192F12, c203F5, and c213E4.

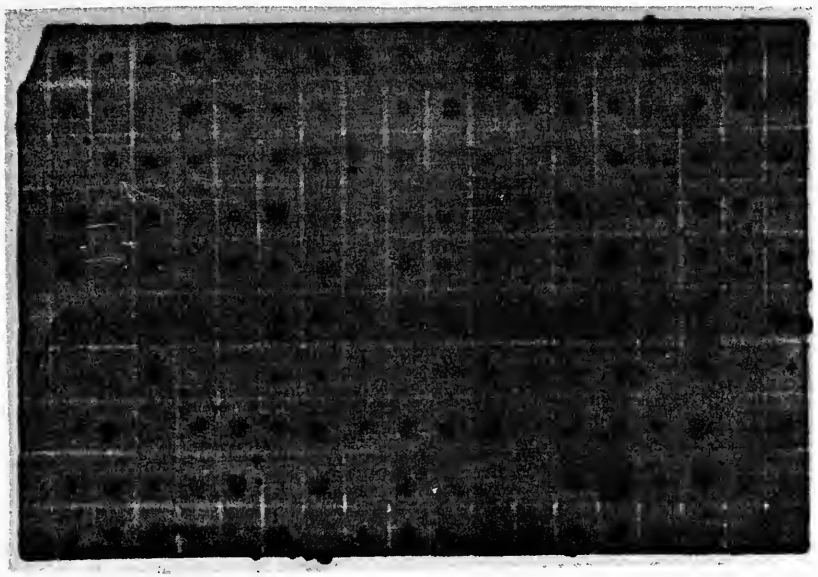


FIGURE 22. Digoxigenin Labeled D12S1987 STS marker hybridization to cosmid dot blot. Seven cosmids showed strong labeling reactions: c72G11, c128D6, c168C12, c192A8, c192F12, c203F5, and c213E4. Cosmid 128D6 was a positive control for this hybridization.

III.B.4. *Sequence-Tagged Site Marker D12S1914.*

An approximately 0.134 kb band on agarose gel electrophoresis demonstrated the positive digoxigenin labeling of K562 DNA with primers 1914F and 1914R for STS D12S1914 during PCR. PCR of the unlabeled marker would have yielded a band length

of 0.091 kilobases. There were eight cosmids on the dot blot that demonstrated strong labeling reactions when exposed to the labeled probe during hybridization (FIGURE 23). The positive cosmids were c1C6, c69B7, c88A9, c167H8, c179F11, c189G12, c192A8, and c200B8. Cosmid 128D6 was a positive control, as determined by PCR mapping methods, but did not label in this hybridization.

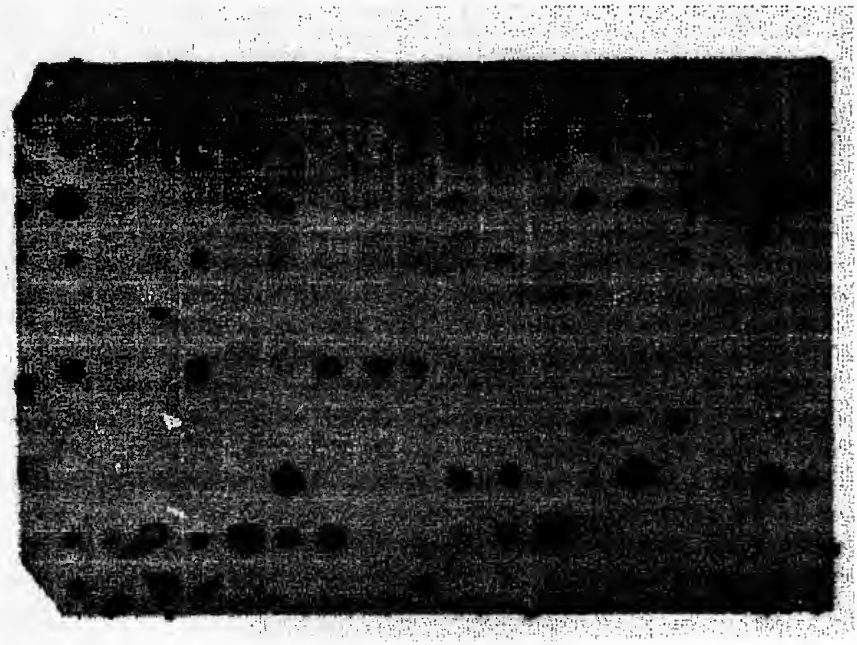


FIGURE 23. Hybridization of digoxigenin labeled D12S1914 STS marker to cosmid dot blot. Eight cosmids labeled most strongly in this procedure. These were c1C6, c69B7, c88A9, c167H8, c179F11, c189G12, c192A8, and c200B8.

III.C. *RESTRICTION ENZYME DIGESTIONS.*

Restriction endonucleases are often used to fragment DNA for studying the relationships between several different pieces of DNA of interest. Restriction enzyme digestions were used here to map the relationships between cosmids that were positive for particular STS markers. Three sets of cosmids were tested using restriction

endonucleases, those positive for D12S950, D12S1914, and D12S1987. Endonucleases Eco RI, Bam HI, Bgl II, and Eco RI with Bam HI were used to fragment the cosmids. A preliminary cosmid map, or contig, was developed for one of the markers tested (D12S1914).

III.C.1. *Endonuclease Digestion for Cosmids Positive for STS D12S1914.*

Cosmids positive for D12S1914 were cut with Eco RI, Bam HI, Eco RI and Bam HI together, and Bgl II (FIGURES 24 AND 25). The positive cosmids were c1C6, c69B7, c88A9, c167H8, c179F11, c189G12, c192A8, and c200B8. Mapping migration distances of the cut cosmids in comparison with the migration of 1KB ladder DNA and bacteriophage lambda Hind III markers allowed a determination of the approximate sizes of the cosmid fragments (FIGURE 26). Using a combination of each digestion it was possible to produce a map of the cosmids relative to each other for the region of the STS marker.

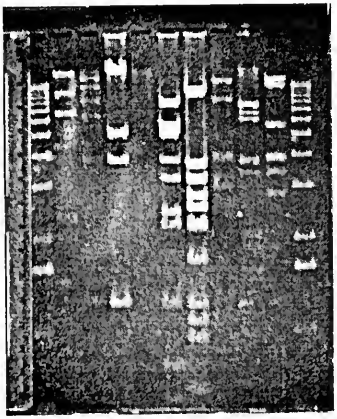


FIGURE 24. Eco RI endonuclease digestion of cosmids positive for STS marker D12S1914 as electrophoresed on a 0.8% agarose gel. Lanes 2 and 12 contain 0.5 µg 1KB ladder, Lane 3 contains 0.42µg λ Hind III DNA, Lane 4 has c1C6, Lane 5 has c69B7, Lane 6 has c88A9, Lane 7 has c167H8, Lane 8 has c179F11, Lane 9 has c189G12, Lane 10 has c192A8, and Lane 11 has c200B8.

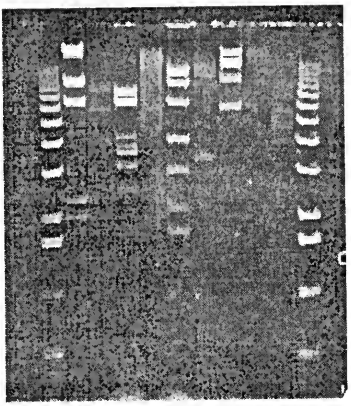


FIGURE 25. Bgl II endonuclease digestion of cosmids positive for D12S1914 electrophoresed in a 0.8% agarose gel. Lanes 2 and 12 contain 0.5 μg 1KB ladder, Lane 3 has 0.42μg λ Hind III DNA, Lane 4 has c1C6, Lane 5 has c69B7, Lane 6 has c88A9, Lane 7 has c167H8, Lane 8 has c179F11, Lane 9 has c189G12, Lane 10 has c192A8, and Lane 11 has c200B8.

TABLE 2. Results of Eco RI digestion of cosmids positive for D12S1914. Starred boxes represent positive bands shared between cosmids with the band size along the top. Sizes were determined by plotting the migration distance of the markers with their sizes in a logarithmic graph.

	3100	2200	1300	3900/4000	11000	6200	2600	3300
c1C6				****	****	****		
c69B7			****	****				
c167H8	****	****	****	****				
c179F11	****	****	****	****			****	
c189G12	****			****	****	****		
c192A8				****				****
c200B8				****	****		****	****

TABLE 3. Results of Bam HI, Eco RI double digestion of cosmids positive for D12S1914. Starred boxes represent positive bands shared between cosmids with the band size along the top. Sizes were determined by plotting the migration distance of the markers with their sizes in a logarithmic graph.

	11000	3300/3400	3900/4000	4300	6000	6800
c1C6	***	***	***			
c69B7	***	***	***			***
c167H8		***	***	***		***
c179F11		***	***			
c189G12			***	***	***	***
c192A8					?	
c200B8	***	***	***	***	***	

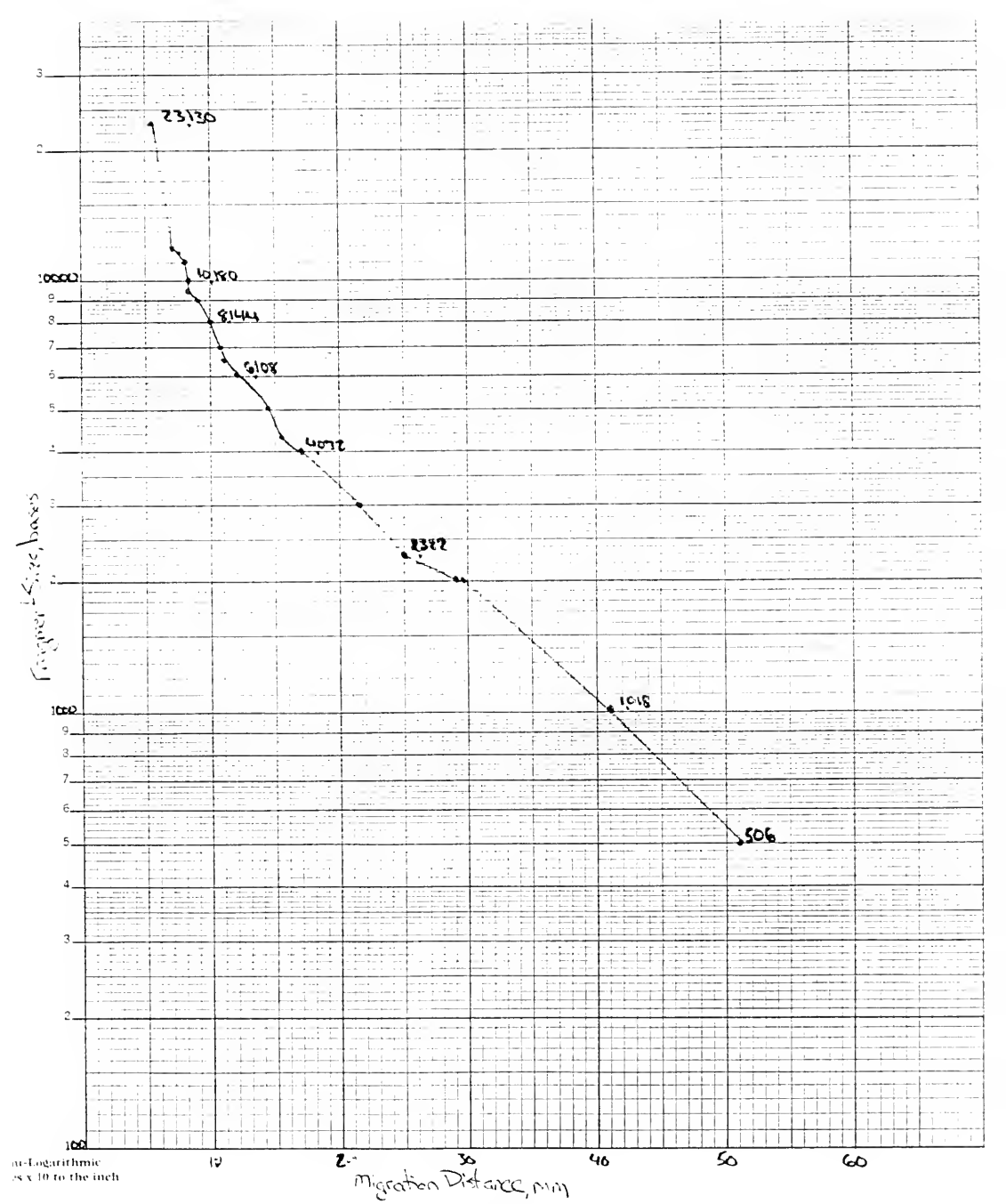


FIGURE 26. Logarithmic plot for 1 KB ladder and λ Hind III fragments on the double endonuclease digestion gel for D12S1914.

III.C.2. *Endonuclease Digestion for Cosmids Positive for STS D12S950.*

The positive cosmids for D12S950 were tested with Eco RI, Eco RI and Bam HI, and Bgl II endonucleases. Of the eleven cosmids initially determined as strongly labeling during hybridization there were several that did not digest well as seen by the very faint or nonexistent banding (FIGURE 27). Only those eight, which showed distinct banding patterns, were digested with Bgl II: c64A5, c133A5, c164G4, c167F1, c189C3, c203F5, c205G9, and c221H6. Many of these digestions did not produce clear, distinct banding when electrophoresed and, as such, a clear, distinct could not be determined.

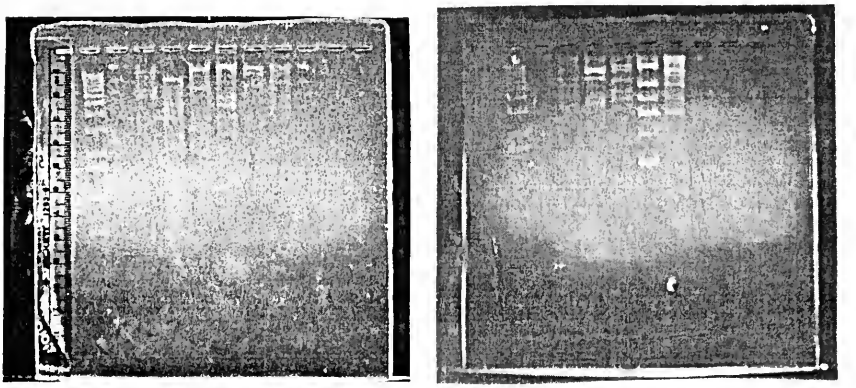


FIGURE 27. Restriction Enzyme Digestion with Eco RI and Bam HI of Cosmids Positive for Marker D12S950 as electrophoresed on a 0.8% agarose gel. For gel 1: Lane 2 contains 0.5 μg 1KB ladder, Lanes 3 and 10 have 0.42μg λ Hind III DNA, Lane 4 has c64A5, Lane 5 has c66G2, Lane 6 has c133A5, Lane 7 has c164G4, Lane 8 has c167F1, and Lane 9 has c170G6. For gel 2: Lane 2 contains 0.5 μg 1KB ladder, Lanes 3 and 9 have 0.42μg λ Hind III DNA, Lane 4 has c175F11, Lane 5 has c189C3, Lane 6 has c203F5, Lane 7 has c205G9, and Lane 8 has c221H6.

III.C.3. *Endonuclease Digestion for Cosmids Positive for STS D12S1987.*

No endonuclease digestions were carried out upon these cosmids. Further testing would use the same technique as the digestions carried out upon STS D12S1914 and D12S950 in hopes of producing a cosmid map of the region surrounding the marker.

III.D. *PRELIMINARY COSMID MAPS.*

Preliminary cosmid contigs were produced for STS marker D12S1914 following restriction enzyme digestion of the cosmids positive for the marker (FIGURES 28 AND 29).

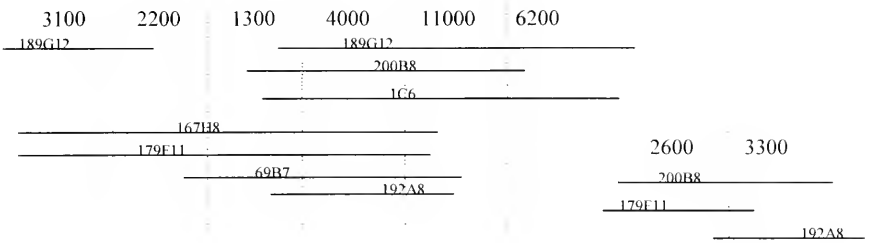


FIGURE 28. Contig for STS marker D12S1914, Eco RI digestion. Solid horizontal lines represent the cosmids.

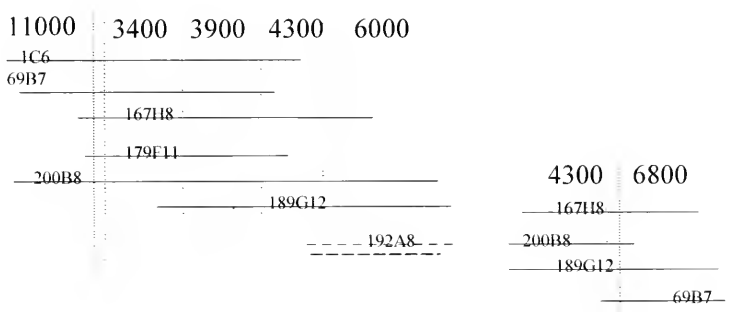


FIGURE 29. Contig for STS marker D12S1914, Eco RI and Bam HI digestion. Solid horizontal lines represent the cosmids. The dotted line for cosmid 192A8 represents an uncertainty of the existence of a band in that position.

III.E. *SOUTHERN BLOT.*

Southern blot procedures were carried out on the Eco RI digestion of cosmids positive for D12S950, the Eco RI and Bam HI double digestion of D12S950 cosmids, and the Eco RI digestion of D12S1914 cosmids. The blot membrane for the D12S1914 cosmids underwent DIG hybridization following the dot blot procedures. Following a six-hour film exposure and development there were no visible bands. A twenty-four hour exposure also produced no visible results. A second hybridization with the same

restriction enzyme digestion of D12S1914 showed some light bands but the background was too great to determine the position of the marker within the digestion fragments.

III.F. *Overview of Results in Terms of Mapping Roadmap.*

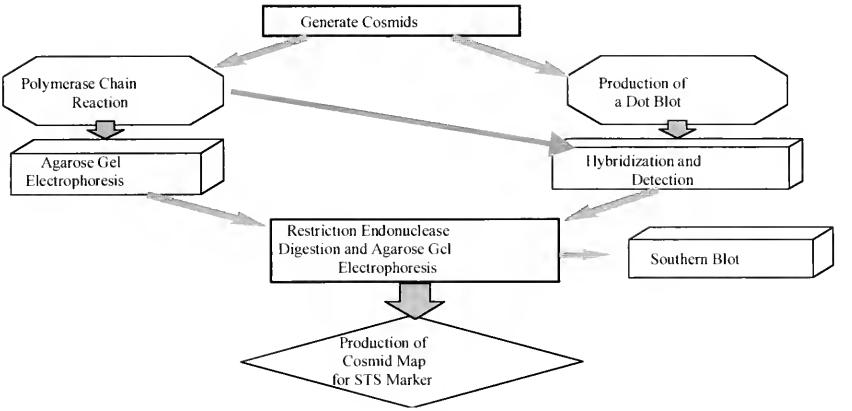


FIGURE 30. Roadmap of steps required to produce a physical map, or cosmid contig of a chromosome based on STS markers.

Cosmids were generated through several cosmid preparations for use in making the dot blot and for restriction endonuclease digestions. The cosmids were also initially used in PCR testing for STS markers but after DIG was implemented as the more effective method of testing for marker sequences the template DNA was changed to K562 human DNA. PCR amplified and DIG labeled STS marker sequences were confirmed using agarose gel electrophoresis and then the labeled STS was used as a probe for hybridization to the dot blots of cosmid DNA. Hybridization with DIG labeled D12S950, D12S1987, and D12S1914 STS markers produced eleven, seven and eight strongly labeling positive cosmids, respectively. Restriction endonuclease digestions and agarose gel electrophoresis produced a way of analyzing the positive cosmids for overlaps when migration distances of the fragments were compared with those distances found for the fragments of known size. Arranging the determined fragment sizes in such a way as to maximize overlap between the cosmids allowed the production of two possible cosmid contigs for the region of the genome spanning the STS marker.

IV. Discussion

The production of a cosmid map for the 12p13 region was successfully begun with this project. Two possible contigs were described from the restriction enzyme digestions of Sequence-Tagged Site marker D12S1914 (Figures 28 and 29). The contigs contained many overlaps that are described with high confidence. For the Eco RI digestion contig the confidence stemmed from the concentration of cosmids at the fragments 3900 and 4000 bases in length (Table 2). Seven cosmids contained these fragments; c1C6, c69B7, c167H8, c179F11, c189G12, c192A8, and c200B8. The 3100 and 11000 base fragments also matched for three of the cosmids in the region. Uncertainty arose in the 2600 and 3300 base fragments for c179F11, c192A8, and c200B8 as well as the 3100 fragment for c189G12. The contig based on the double endonuclease digestion of D12S1914 cosmids with Eco RI and Bam HI also had several reliable overlaps (Table 3). The overlap of cosmids for 3400 and 3900/4000 base fragments was very strong. Five cosmids overlap for the 3400 base fragment and six cosmids overlap for the 3900/4000 base fragment pair. Of the eight cosmids digested only c88A9 and c192A8 did not span the region. These two cosmids did not produce clear banding on the gel though so their results are not reliable. The relationship of the 6000 and 6800 base fragments to the contig was not definite. Without Southern Blot results to label the exact STS marker location, it was not possible to produce a strong overlap between the two contigs.

Other goals were reached as progression was made toward the development of a cosmid contig. DIG labeling of STS markers via PCR was performed successfully and visibly detected via agarose gel electrophoresis. DIG hybridization techniques were followed to produce positively labeling cosmid dot blots. It was possible to determine strongly labeling cosmids for use in restriction enzyme digestions and they were carried out with much success, contributing to the two cosmid contig productions. The two STS markers whose contigs have not yet been produced are much closer to that goal because of the assignment of cosmids to the markers via hybridization with DIG labeled marker sequences that were performed in this project.

Not only was PCR used to label STS markers with DIG for hybridization but it also determined the position of STS marker D12S1914 by the matching of c128D6 to its

sequence. Since the cosmid was already matched to markers D12S1987 and D12S928 found to the right of D12S1914, the match allowed for D12S1914 to be accurately placed to the left of D12S928 and to the right of D12S950 (FIGURE 31). It was D12S950 and D12S1914 whose exact locations had been previously unknown (See FIGURE 5 in the introduction.). However the exact positions of D12S1914 and D12S1987 then remained to be determined.

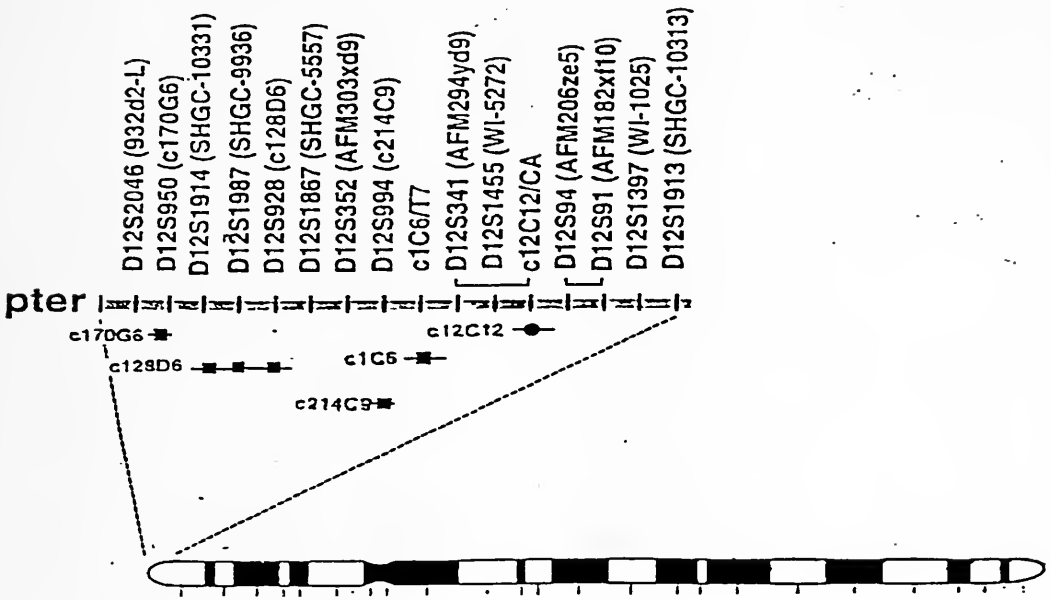


FIGURE 31. Modified STS map of Chromosome 12p13. The five cosmids are shown mapped to their respective markers.

Studying the relationships between positive cosmids in the three DIG hybridizations showed two cosmids that were positive for more than one STS marker (TABLE 4). This relationship enabled the exact positioning between STS D12S1914 and D12S1987 on the STS map of chromosome 12 (FIGURE 32).

TABLE 4. Cosmids that are multiply positive for STS markers as determined by DIG hybridization.

STS Marker	Positive Cosmid Control	c192A8	c203F5
D12S950 (c170G6)	c170G6 (+)		+
D12S1914 (SHGC-10331)	c128D6 (-)	+	
D12S1987 (SHGC-9936)	c128D6 (+)	+	+

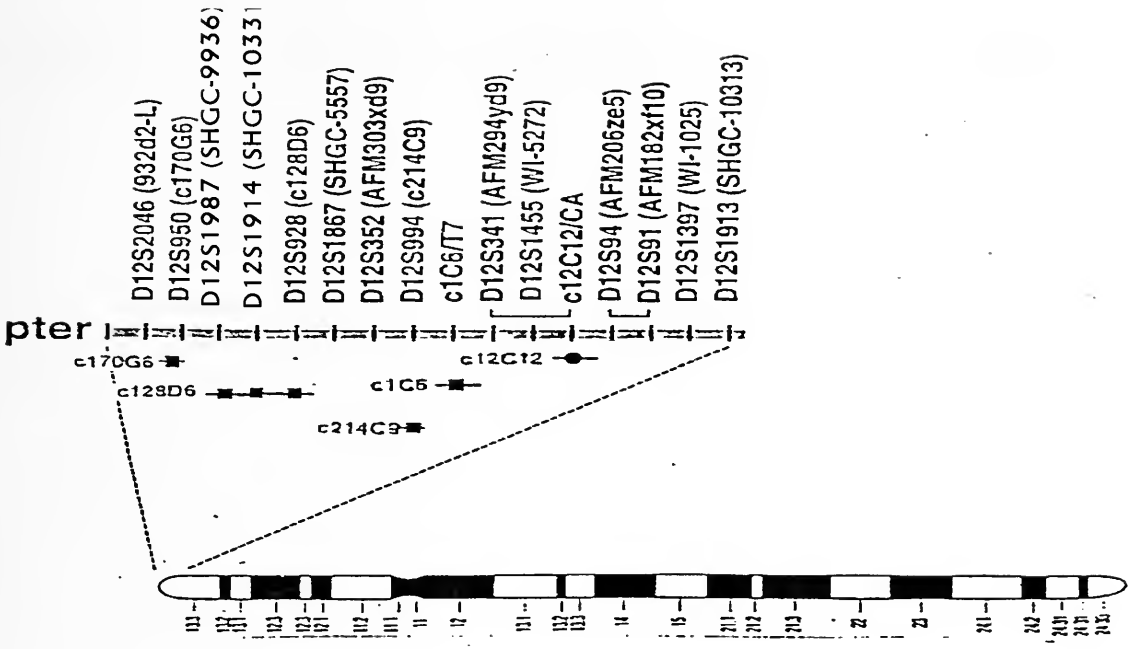


FIGURE 32. Modified STS map of Chromosome 12p13 showing the positions of D12S1914 and D12S1987.

Along with the successes of a project, there are many possible problems that can arise, but, in the Human Genome Project, there seem to be many more chances for error because of the sheer magnitude of the goals. Each technique used in this project, polymerase chain reaction, hybridization, and restriction enzyme digestion, had its own unique set of guidelines for optimization of the results and its own set of difficulties that were encountered. Many of the difficulties were alleviated upon closer examination but

some resulted in the surrendering of one technique for another which might be more successful, as was the case with PCR-based cosmid pool testing and psoralen biotin labeling for DIG labeling procedures.

Polymerase chain reactions were very sensitive to the running conditions. The most common problems were an undetectable or low yield of product, the presence of nonspecific background bands due to mispriming of the primers, and mutations due to misincorporation of deoxynucleotide triphosphates.[16] Other causes of failure were due to incorrect concentrations of dNTPs, *Taq* DNA polymerase, or magnesium. The recommended concentrations of dNTPs, *Taq*, and magnesium will vary some according to the primers being used in the reactions but there are some basic guidelines to be followed.[16] The recommended *Taq* concentration are between 1 and 2.5 units per 100 μL reaction. If the enzyme concentration is too high an enzyme concentration is used there may be an accumulation of nonspecific background products caused by mispriming and extension of the incorrect sequence. If the concentration is too low there may be an insufficient amount of product. The concentration of deoxynucleotide triphosphates to produce optimum results has not been determined but it is known that 20 μM of each dNTP in a 100 μL reaction is enough to synthesize 2.6 μg DNA or 10 pmol of a 400 base pair sequence. Two and six tenth micrograms of DNA is plenty of material for use as a probe for hybridization or to be detected via electrophoresis, considering 100 to 500 ng of DIG labeled probe is all that is necessary for hybridization with 100 cm^2 dot blot membrane. The lowest possible concentration that can produce results should be used in order to prevent mispriming. The concentrations of each individual deoxynucleotide should be equivalent to prevent possible misincorporation. The amount of magnesium necessary for proper running is 0.5 to 2.5 mM over the total dNTP concentration used in the reaction, usually around 200 μM. Optimal primer concentrations are between 0.1 to 0.5 μM. Concentrations higher than this may promote mispriming or accumulation of nonspecific products.

Incorrect temperature or timing for annealing, extension, and denaturing of products during cycling can also cause failure. The temperature and length of time required for primer annealing depends on the base composition, length, and concentration of amplification primers in the reaction.[16] The annealing temperature is typically 5°C below the

T_m of the primers.[16] Temperatures between 55° and 72°C yield the best results and the higher temperatures have been shown to enhance the discrimination against incorrectly annealed primers. The temperature used for extension is typically 72°C.[16] Extension time depends on the length and concentration of the target sequence. A time of one minute is sufficient for products less than or equal to 2 kb and it should be kept in mind that longer times may be helpful in early cycles if substrate concentrations are low or in late cycles when product concentration exceeds enzyme concentration. The most likely cause for PCR failure, though, is incomplete denaturation.[16] Typical conditions for positive results are 95°C for thirty seconds or 97°C for fifteen seconds. Too high temperatures or denaturation carried out for too long a period of time will reduce enzyme activity unnecessarily. It is a good idea to monitor the temperature inside the reaction tubes by measuring the temperature inside a tube containing a thermocouple probe, and an inert material such as mineral oil. Optimization of the number of cycles depends mainly on the starting concentration of target DNA but one should remember that too many cycles may increase the amount of nonspecific background products.[16] The phantom bands of about 12 kilobases in length could be the result of mispriming of primers to the extended template DNA or they could be cosmid DNA. Mispriming would result in the extension of a target sequence that was longer than the one desired. The phantom bands were most likely cosmid DNA.

In order to be more efficient in testing cosmids for particular STS markers via PCR pools of five cosmids each were first tested during PCR and in those pools that tested positive the cosmids were tested individually for the STS marker. The results of testing the nineteen cosmid pools were less than desirable. The testing of cosmid pools with c170G6 yielded no positive results, even for the positive c170G6 control. The testing of pools with primers 1914F and 1914R yielded only possible positive banding because of the unclear and faint bands. Testing of the pools with this cosmid should be repeated to obtain clearer results. Another possibility was to test the cosmids from the possible positive pools by restriction enzyme digestions. This would allow for the detection of an overlap of the cosmids with one another. It remains to be determined why PCR gives positive results with positively matched cosmids only some of the time.

The various negative reactions in PCR have been the result of many possible problems. DNA *Taq* polymerase, the buffer solution, the deoxynucleotide triphosphates and the cosmids and primers can degrade if stored above 0°C or if repetitively frozen and thawed. Some of this degradation cannot be avoided but the freezer used for storage of many of the solutions has a self-thawing cycle that may be causing excess freezing and thawing of the materials. This is most likely the reason that the reactions did not show positive results. Negative results due to improper concentrations of the materials and/or incorrect cycling periods is unlikely in the multiple runs because the same conditions were tested as when the polymerase chain reaction gave positive results. The negative reaction found with the freshly delivered *Taq* was most likely the result of an improperly prepared gel that resulted in some wells with no agarose base

Unclear banding of cosmid pools seen during testing with forward and reverse 1914 primers could have been the result of the use of precipitated Tris Borate in the gel preparation, although it had not caused previous problems. Fresh 5x Tris Borate was prepared and PCR and electrophoresis was repeated with the same results, showing that the Tris Borate solution was not contributing to the problem. Other problems noted include unclear banding of the 1 KB ladder and the covering of primer bands by the reverse migration of ethidium bromide when a double welled gel was used. The KB ladder should be replaced with a fresh aliquot and the gels cut in half when the run is repeated to determine if these steps improved the unclear banding. Other recommendations were that new aliquots of dNTPs should be made regularly as well as aliquots of 10x PCR buffer. Primer and template DNA concentrations should be checked periodically by recording their absorbance to insure the quality of the DNA used in experimentation.

Since the number of reactions that can be carried out at a time with PCR was severely limited, it was thought that an increase in the productivity of cosmid mapping to STS markers would occur if there was an approach that would allow large scale screenings at one time. Dot blot hybridization with the use of oligonucleotide primers as probes offers this possibility. Large numbers of cosmids could be tested in parallel and matched to STS markers by the use of psoralen-biotin labeled oligo primers. Hybridization offered high analysis rates and insensitivity to repetitive sequences since the labeled primers are specific

to sites found in the genome only once.[13] Hybridization with digoxigenin showed positive control luminescence and yielded positive dot blots for the three STS markers being tested and identified several cosmids that contained the marker that could be tested via restriction endonuclease digestions for overlaps. Hybridization using a psoralen biotin was not successful as only the control reaction labeled.

Hybridization can be difficult to perform successfully. There are many factors affecting the rate of hybridization. There are effects of temperature, cation concentration, base mismatching, pH, and probe lengths and base composition. Oligonucleotide probes hybridize to complementary sequences with a high degree of specificity but there can be a problem with self-complementarity of probes and with high guanine and cytosine content (G+C) in base sequences.[4] High G+C content increases the chances of nonspecific labeling occurring but, if longer probe lengths are used, it is possible to make a hybridization both more stable and specific to the sequence desired. The amount of DNA needed to detect a hybridization signal is dependent upon the proportion of genome to complementary probe, the length of the probe, and the amount of DNA transferred to the filter membrane. Under the previously known best conditions, hybridization can detect as little as 0.1 pg DNA that is complementary to a ^{32}P radioactively labeled probe.[25]

Radioactive labels, despite their sensitivity, are difficult to handle and store and are a constant ionizing radiation threat. Radioactively labeled probes also have a short half-life due to degradation of the radioactivity whereas non-radioactive labels have a much longer shelf life over which they may be used. Because of these difficulties in dealing with radioactive labels non-radioactive labeling procedures were explored. There were two methods tested during this project, psoralen biotin labels and digoxigenin thymidine incorporated labels. Psoralen biotin labeling detection of Scheicher and Schnell control DNA under optimum conditions should show a detectable product of as little as 50 pg within 2 to 3 hours of exposure to film.[26] It has been found that the reaction is not affected by a pH from 2.5 to 10.0. The only requirement is that the ionic composition be less than 20 μM.[4] In the digoxigenin labeled control it is possible to detect a DNA concentration down to 0.03 pg of homologous DNA using chemiluminescent detection.[7] This concentration is better than the previous optimum for hybridization and is much

improved upon the concentration able to be detected by the psoralen biotin system. The first detectable results for digoxigenin detection are visible after only fifteen to twenty minutes, depending on the strength of the signal, and are visible for up to two days, allowing for multiple exposures.[7]

Oligonucleotide hybridization using psoralen biotin labeled products showed results that verified all procedures except the actual hybridization of primer DNA to target DNA by hydrogen bonding. The procedures testing for detection of product and for accurate labeling with psoralen biotin were successful but hybridizing labeled primer to a cosmid blot produced no positive results, even at the highest concentrations. Thoughts on the problem of hybridization showed that there might not have been enough time for the labeled primer to migrate to the corresponding area on the cosmid to combine when incubated for only an hour without constant agitation. This conclusion was reached when hybridization was not detected after incubating the dot blot submerged for one hour at the appropriate temperature. The next step would have been to test the incubation period by allowing the labeled primer to hybridize over a twelve-hour period. This should have allowed ample time for migration to occur. If hybridization did not occur after the extended incubation, it was planned to allow for incubation under constant agitation by using a water bath with an agitator or to acquire a hybridization oven for use.

Instead of testing hybridization using a psoralen biotin probe in a water bath equipped with an agitator, it was decided to attempt hybridization by a different method. A digoxigenin probe was used with great success. Three STS markers were successfully labeled as determined by electrophoresis and the probe hybridized to cosmids on the dot blot with high efficiency. Several cosmids were strongly labeled in each hybridization reaction allowing for further testing to take place using restriction enzyme digestions and Southern blot.

This technique was not without difficulties, however. The main problems were high background on some membranes and a high non-specific labeling ratio of all cosmids on the membrane (FIGURE 21). Following film incubation and development it was noticed that nearly every cosmid labeled slightly for the STS marker. These non-specific hybridizations suggest that there might have been excess PCR by-products present that

caused the non-specific binding. Purification of the PCR product after banding in electrophoresis or a decrease in the amount of template used will most likely correct this problem.[7] In the hybridization for STS D12S1914 the non-specific binding may have caused the strong label for c1C6, a cosmid positively linked to an STS much farther away on the chromosome but does not explain the interesting negative for the PCR determined positive cosmid 128D6.

In hybridization, the probe concentration can be very important to the success of the experiment. Too high probe concentration results in non-specific binding of the probe to the membrane, i.e.-high background while too low a concentration could cause lower sensitivity. A good test for determining optimal probe concentration is to perform a 'mock' hybridization using blank pieces of membrane.[7] When performing hybridizations in sealed reaction bags, as was done here, it is important to use at least 3.5 ml hybridization solution per 100 cm^2 of membrane. All air bubbles should be removed prior to bag sealing and the bag should lie flat upon the bottom of the waterbath. Uneven positioning can cause background and sensitivity problems.[7] There were no problems reported with air bubbles though in a couple of instances they may have caused uneven positioning of the membrane on the bottom of the water bath, which in itself could have caused some of the high background problems that were seen.

Detection procedures also have a high influence on the quality of the background in the hybridization reaction. Membranes should be shaken throughout the entire procedure, freshly washed trays should always be used and sterile conditions should be maintained. Chemiluminescent substrates, like CSPD®, need only a dilution factor of 1:10,000 in order to work correctly. Should signal intensification be required 10% dextran sulfate can be added but background would also be increased. If further decreases in background are needed to produce readable results, the stringency and antibody wash steps can be increased to two times twenty-minute durations from two times fifteen-minute washes.

There are several advantages to the decision to change from using the psoralen biotin labeling method to the digoxigenin method, chiefly being sensitivity and convenience of use. The digoxigenin system has a much easier method of labeling using

PCR compared to the long irradiation step and multiple extraction steps required in psoralen biotin labeling. The digoxigenin system used a thermocycler and was nearly all automated for the approximately two hour procedure of labeling while in the psoralen biotin system the samples to be labeled had to be irradiated for an hour then undergo several extraction steps, requiring more handling time and room for errors. Hybridization and chemiluminescent detection procedures are similar in both techniques. In the digoxigenin labeled control it is possible to detect a DNA concentration down to 0.03 pg of homologous DNA using chemiluminescent detection.[7] The first detectable results are visible after only fifteen to twenty minutes, depending on the strength of the signal, and are visible for up to two days, allowing for multiple exposures.[7] Chemiluminescent control labeling with psoralen biotin will detect at least 50 pg of DNA within two or three hours of film exposure.[26] Remember that radioactive labels can detect down to 0.1 pg of hybridized material, making digoxigenin both safer and more sensitive than labeling with radioactive isotopes. The digoxigenin system has 500 times the sensitivity of radioactive labels and 1667 times the sensitivity of the psoralen biotin label.

Restriction endonuclease reactions were carried out to determine the amount of overlap between two fragments of DNA and to aid in the construction of a cosmid contig. Restriction endonuclease digestions of STS positive cosmids produced two convincing contigs and several other strong relationships between the cosmids and their respective markers. Digestion procedures produced clear, distinct bands in electrophoresis for most of the cosmids tested though some examples of broad possibly doubled bands still existed that made mapping fragment size slightly more difficult (FIGURE 24). For the cosmids positive for D12S950 it was difficult to produce a contig with confidence because of unclear banding and many fragments that did not have matching bands in any other cosmid (FIGURE 27).

The non-specific hybridization of the digoxigenin probe may have contributed to some of the problems noticed in restriction endonuclease digestion. For the most part the endonuclease digestions were successful in determining the overlap between the cosmids identified for a particular STS marker. In testing with D12S950 there was indistinct banding and three cosmids that did not seem to show banding despite increases made in

their concentrations. It is possible that the indistinct and missing bands were the result of impure cosmids since some of the ones tested had been stored for long periods of time and their purity was not known because they were produced by others in the laboratory. Cosmid preparations made of some of the cosmids did not correct this problem though, so it is unlikely that it was the total cause. Another possible cause is that these cosmids were not actually positive for the STS marker and may not have contained the restriction sites within their base sequences. The uncertainty here is the result of the non-specific binding of the STS probe to the cosmids that was mentioned previously.

Once the electrophoresis of restriction endonuclease digestions was complete, the band migration had to be measured and plotted against the migration of known fragments of the KB ladder to determine the cosmid restriction fragment sizes. This step had complications also. Sometimes the gels ran slightly unevenly making it difficult to tell if several bands had migrated the same distance or were distinct bands by themselves. Some broad bands may have been two separate bands that were of very similar size or may have indicated a partial digestion of the fragment. In making the cosmid contig for the Eco RI digestion of D12S1914, cosmids several band sizes had to be assumed to be equal for the purpose of constructing a strong relationship within the contig. This was the case with the 3900 and 4000 base pair fragments especially.

Southern blotting of the restriction endonuclease gel for use in hybridization with the STS probe was not successful. Preliminary tests with D12S1914 Eco RI blot showed very faint banding but not enough to be conclusive of which bands contained the STS sequence. The causes of the very faint banding were most likely the result of too little DNA concentration in each individual band for accurate annealing to occur. Running a higher concentration of cosmid initially through enzyme digestion and electrophoresis may solve this problem. Other troubleshooting techniques include all those listed for digoxigenin hybridization, including tests for optimal probe concentration, decreases in background, and maintenance of sterility for the duration of the procedure.

Plans for the future of this project include further testing of the positive cosmids for D12S950 and D12S1987 with restriction endonucleases to determine a possible contig. Following several contig productions it may be possible to link them together

considering two cosmids tested positive via hybridization for two of the STS markers being tested. Cosmid 192A8 tested positive for D12S1987 and D12S1914 while cosmid 203F5 tested positive for D12S950 and D12S1987. The relationship between markers D12S1914 and D12S1987 needs to also be determined conclusively either by these contigs or others built later on.

V. References

[1] 5 Prime→3 Prime, Inc. Phase Lock Gel™ product insert.

[2] http://www.ncgr.org/Index.html

[3] http://www.nhgri.nih.gov/HGP/
http://www.ornl.gov/TechResources/...nome/project/5yrplan/synopsis.html

[4] Berger, Shelby L., ed., Alan R. Kimmel, ed. (1987). Methods in Enzymology. **152**: 393-396, 432-442. Academic Press: Orlando, FL.

[5] Boehringer Mannheim. DIG DNA Labeling and Detection Kit Nonradioactive product insert. (No. 1093 657).

[6] ----------. DIG Luminescent Detection Kit product insert. (No. 1363 514).

[7] ----------. (1995). The DIG System User's Guide for Filter Hybridization. Biochemica: Germany.

[8] ----------. PCR DIG Probe Synthesis Kit product insert. (No. 1636 090).

[9] Brum, Gil, Larry McKane, Gerry Karp. (1994). Biology: Exploring Life. Edition 2. John Wiley and Sons, Inc.: New York.

[10] Cohen, D., I. Chumakov, J. Weissenbach. (1993). "A First-generation Physical Map of the Human Genome," *Nature*. **366**:698-701.

[11] Collins, Francis S. "Evolution of a Vision: Genome Project Origins, Present and Future Challenges, and Far-Reaching Benefits Part 2." *Human Genome News*. **7**:3.

[12] Cooper, Necia Grant, ed. (1994). The Human Genome Project: Deciphering the Blueprint of Heredity. University Science Books: Mill Valley, California.

[13] Craig, A.G., D. Nizetic, J.D. Hoheisel, G. Zehetner, H. Lehrach. (1990). "Ordering of Cosmid Clones Covering the Herpes Simplex Virus Type I (HSV-I) Genome: A Test Case for Fingerprinting by Hybridization," *Nucleic Acids Research*. **18**:2653-2660.

[14] Doggett, Norman A. (1994). "The Polymerase Chain Reaction and Sequence-tagged Sites," The Human Genome Project: Deciphering the Blueprint of Heredity. University Science Books: Mill Valley, California: 128-134.

[15]Gonick, Larry, Mark Wheelis. (1991). Cartoon Guide to Genetics. Harper Perennial: New York.

[16]Innis, Michael A., David H. Gelfeld, John J. Sninsky, Thomas J. White, eds. (1990). PCR Protocols: A Guide to Methods and Applications. Academic Press: San Diego

[17]Knoppers, Bartha Maria, Ruth Chadwick. (1994). "The Human Genome Project: Under an International Ethical Microscope." *Science.* **265**:2035-2036.

[18]Krauter, Kenneth, Kate Montgomery, Sung-Joo Yoon, Janine LeBLanc-Straceski, Beatrice Renault, Ivonne Marondel, Valerie Herdman, Lias Cupelli, Amy Banks, Jonathan Lieman, Joan Menninger, Patricia Bray-Ward, Prakash Nadkarni, Jean Weissenbach, Denis Le Paslier, Philippe Rigault, Iiya Chumakov, Daniel Cohen, Perry Miller, David Ward, Raju Kucherlapati. (1995). "A Second-generation YAC Contig Map of Human Chromosome 12," *Nature.* **377**:321-325.

[19]Lee, Thomas F. (1991). The Human Genome Project: Cracking the Genetic Code of Life. Plenum Press: New York.

[20]Levenson, Corey, Robert Watson, Edward L. Sheldon. (1990). "Biotinylated Psoralen Derivative for Labeling Nucleic Acid Hybridization Probes." *Methods in Enzymology.* **184**:577-583.

[21]Lodish, Harvey, David Baltimore, Arnold Berk, Lawrence Zipursky, Paul Matsudaira, James Darnell. (1995). Molecular Cell Biology. Edition 3. Scientific American Books: New York.

[22]Molecular Probes. SYBR® Gold Nucleic Acid Gel Stain product insert.

[23]Montgomery, Kate T., Janine M. LeBlanc, Peter Tsai, Jennifer S. McNinch, David C. Ward, Pieter J. deJong, Raju Kucherlapati, Kenneth S. Krauter. (1993). "Characterization of Two Chromosome 12 Cosmid Libraries and Development of STSs from Cosmids Mapped by FISH," *Genomics.* **17:**682-693.

[24]Murray, Jeffery C., K.H. Buetow, J. Wever, S. Ludwigsen, T. Scherpbier-Heddema, F. Manion, J. Quillen, V. Sheffield, S. Sunden, G. Duyle, F. Weissenbach, G. Gyapay, C. Dib, J. Morrissette, G. Lathrop, A. Vignal, R. White, N. Matsunami, S. Gerken, R. Melis, H. Albertsen, R. Plaetke, S. Odelberg, D. Ward, J. Dausset, D. Cohen, H. Cann. (1994). "A Comprehensive Human Linkage Map with Centimorgan Density," *Science.* **265:**2049-2054.

[25]Sambrook, J., E.F. Fritsch, T. Maniatis. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY.

[26]Schleicher and Schuell. Rad-Free® Universal Oligo Labeling and Hybridization Kit product insert. (No. 483122).

[27]----------. (1995). Blotting, Hybridization and Detection: An S&S Laboratory Manual. Schleicher and Schuell: Germany. (No. 399005).

[28]Smith, David. (1995). "Evolution of a Vision: Genome Project Origins, Present and Future Challenges, and Far-Reaching Benefits Part 1." *Human Genome News*. 7:2.

[29]Voet, Donald, Judith G. Voet. (1990). Biochemistry. Edition 1. John Wiley and Sons, Inc.: New York.

[30]Voet, Donald, Judith G. Voet. (1995). Biochemistry. Edition 2. John Wiley and Sons, Inc.: New York.

[31]Watson, James D., John Tooze, David T. Kurtz. (1983). Recombinant DNA: A Short Course. Scientific American Books: New York.

[32]Weaver, Robert F., Philip W. Hedrick. (1992). Genetics. Edition 2. Wm. C. Brown Publishers: Dubuque, Iowa.

VI. Appendix A

VI.A. Cosmid Preparation Protocols

Cosmid Preparation

1. Streak cosmids onto 2XTY agar plates with 20 μg/mL Kanamycin added. Incubate at 37°C to obtain single colonies.

2. To sterile 125 mL flask add 30 mL 2XTY broth and 30 μL of Kanamycin, at concentration indicated on vial. Using sterile technique, transfer a single cosmid colony to the flask and incubate overnight (at least 12 hours) at 37°C with vigorous aeration.

3. Centrifuge flask cultures at 2000 RPM for approximately 30 minutes in a tabletop Beckman TJ-6 centrifuge with a TH-4 1-85 rotor. The resulting pellet should be firm.

4. Pour off supernatant and re-suspend pellet in 1.5 mL Solution I. Vortex to suspend completely.

5. Add 3 mL Solution II, freshly prepared the day of the procedure. Invert slowly to mix.

6. After a few minutes add 2.25 ml Solution III. Invert slowly to mix. The viscosity should resemble that of egg albumin.

7. Pour into 50 mL centrifuge tubes. Centrifuge in Sorvall RC-5B centrifuge with SS-34 rotor at 4°C at 17000 RPM for 15 minutes.

8. Pour supernatant through Kimwipes™ into new 50 mL tubes. Make sure Kimwipes™ are rubber banded onto the top of the tube. Wearing new gloves for each cosmid, squeeze the Kimwipe™ to release additional supernatant.

9. Add isopropanol at half volume of supernatant. Seal top with Parafilm™ and invert to mix. Precipitate at room temperature for at least 30 minutes. This step is a good stopping point if needed.

10. Centrifuge supernatant in Sorvall for 30 minutes at 17000 xg to recover the precipitate.

11. Resuspend the pellet in 400 μL TE and transfer to 1.5 mL Eppendorf tubes.

12. Add 2 μl of 10 mg/mL RNAase A and 2 μL of 1000U/ml RNAase T1 to tubes. Incubate in water bath for 30 minutes at 37°C.

13. Add 8.1 μL of 10 mM EDTA and 8.1 μL of 0.2% SDS. Transfer to PhaseLock™ gel tubes from 5'→3' Incorporated. Add an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), invert to mix, and spin at 1400 xg for 2 minutes. Aliquot phenol into tubes first, wearing gloves.

14. Remove top layer from PhaseLock™ tube and add 150 µL of 1 M NaOAc for a concentration of 0.3 M NaOAc. Precipitate with 2.5 volumes of ethanol. Freeze at –80°C for a minimum of two hours.

15. Centrifuge cosmids in Eppendorf 5402 centrifuge at 14000 RPM for 10 minutes. Discard the supernatant; wash the pellet with 1 ml 70% ethanol/30% TE. Pellets are air dried and re-suspended in 90 µL TE and stored at –20°C.

VI.B. Cosmid Preparation Recipes

2XTY Medium (1L)

16 g tryptone
10 g yeast extract
5 g NaCl
Dissolve in 900 mL ddH2O. Adjust pH to 7.0 with 5 N NaOH and adjust volume to 1L. Autoclave in two 500 mL portions.

2XTY Agar (1L)

16 g tryptone
10 g yeast extract
5 g NaCl
7.5 g agar
Dissolve agar then dissolve other ingredients in 900 mL ddH2O. pH to 7.0 with 5 N NaOH and adjust volume to 1L. Autoclave in two 500 mL portions. When pouring plates Kanamycin will be added at a concentration of 20 µL/mL.

Solution I

50 mM glucose
25 mM Tris (pH 8.0)
10 mM EDTA
Adjust to final volume with ddH2O and autoclave.

Solution II MUST BE PREPARED DAY OF PROCEDURE.

0.2 M NaOH
1% SDS
Adjust to final volume with ddH_2O.

Solution III

2.9 M KoAc
1.99 M glacial acetic acid
Adjust to final volume with ddH_2O and pH to 4.8 in hood and autoclave.

VII. Appendix B

VII.A. PCR Protocols

VIII.A.1. Polymerase Chain Reaction

1. Prepare Master Mix, MM, using per tube recipe found below, for n + 1 tubes while keeping all reagents on ice.

2. Separate Master Mix into n sterile 0.5 mL Eppendorf tubes and add DNA template. For individual cosmids, negative control, and Chromosome 12 samples use 19 μL MM and 1 μL of template. For cosmid pools use 18 μL MM and 2 μL template.

3. Place Eppendorf tubes in Ericomp Easy Cycler Series thermal cycler and run on program ten. Program ten consists of one repetition of 2 minutes at 94°C for initial denaturation, thirty repetitions of the replication cycles: 30 seconds at 94°C, 30 seconds at 58°C (or optimum temperature for annealing of primers), and 30 seconds at 72°C, one repetition of final annealing for 15 minutes at 72°C, and a holding phase at 4°C.

4. Centrifuge reaction tubes on the short spin cycle of the Eppendorf Centrifuge 5402 for ten seconds to spin PCR product to bottom of tubes.

5. Add 4 μL of sample buffer to each tube. Load 20 μL of each tube to individual wells of the agarose gel and load 5 μL of 0.1 μg/μL 1 KB ladder to the outer wells as a guide to product size.

6. Run agarose gel as described in agarose gel preparation protocol.

7. When finished running the marker dye of the running buffer (bromophenol blue) will have traveled about half of the distance of the gel. Photograph with Polaroid 667 film using an UV light source and a red filter.

VIII.A.2. Agarose Gel Preparation

1. Wrap doubled tape border on glass plate, making sure to eliminate all wrinkles and air bubbles.

2. Set spacers to allow for wells to be made with bottoms of agarose gel (four layers of index cards is sufficient).

3. Pipette prepared gel solution carefully around the edges of the plate, check for leaks in the tape, and proceed with pouring. Remove air bubbles from gel center and well holes.

4. Allow setting to occur in cold room until solidified. Remove tape from plate edges, load with PCR samples and run on Bio-Rad electrophoresis system, model 1000/500, at 180 V for thirty minutes with a buffer solution of 1x TBE.

VIII.B. PCR Recipes

Master Mix per tube

Tubes = n + 1
12 µL ddH_2O
2 µL PCR Buffer (100 mM Tris HCl, 500 mM KCl)
1.6 µL dNTP (10 mM dATP, dTTP, dCTP, dGTP)
0.255 µL Taq polymerase (5 Units/ µL)
1.2 µL $MgCl_2$ (25 mM)
1 µL Primer Forward (100 ng/ µL)
1 µL Primer Reverse (100 ng/ µL)

PCR DIG Probe Synthesis Mix

ddH20 to total 50µL
5 µL 10x PCR buffer with MgCl2
5 µL PCR DIG dNTP mix (200 µM dNTP)
1 µL Primer Forward (0.1-1 µM)
1 µL Primer Reverse (0.1-1 µM)
Template DNA

10x PCR Buffer (1.5 mL)

100 mM Tris HCl
500 mM KCl
pH to 8.3

Sample Buffer

0.25% bromophenol blue
40% w/v starch

Agarose Gel (150 mL)

2.25 g agarose (1.5%)
120 mL ddH_2O
30 mL 5x TBE
7.5 µL ethidium bromide
Ethidium bromide does not have to be included in the recipe: the gel can be stained after electrophoresis. Wearing gloves, use extreme caution with ethidium bromide, as it is a carcinogen.

Running Buffer: 1x TBE

400 mL 5x TBE
1600 mL ddH_2O

5x Tris Borate (TBE) (1 L)

54 g Tris Base (0.45 M)
27.5 g Boric acid (0.44 M)
20 mL 0.5 M EDTA (pH 8.0)

VIII. Appendix C

VIII.A. Hybridization Protocols for Psoralen Biotin

VIII.A.1. Labeling λ Phage DNA or Oligo Primers with Psoralen Biotin

1. Reconstitute unlabeled λ phage DNA with 40 μL sterile dH_2O.

2. Reconstitute psoralen biotin with 6 μL DMF in hood.

3. Add 10 μL reconstituted DNA to boiling water bath for ten minutes and then place immediately on ice.

4. Place clean 96 well plate on ice.

5. In dim room add 1 μL psoralen biotin to 10λ DNA. Add the mixture to one well, not to exceed 150 μL.

6. Place Rad-Free UV lamp on plate and irradiate for one hour.

7. Add 100 μL TE and remove sample from the well into 1.5 mL Eppendorf tube.

8. Add two volumes dH_2O-saturated n-butanol. Vortex briefly and centrifuge thirty seconds in the Eppendorf Centrifuge 5402.

9. Discard top butanol layer and repeat step 8.

10. Add 990 μL TE to bring to 1 μg/mL.

VIII.A.2. Preparing Cosmid DNA for Hybridization

1. Make dilutions of cosmid DNA in microcentrifuge tubes as described below. Boil tubes for ten minutes. Cool rapidly on ice and then centrifuge on ten-second short spin in Eppendorf Centrifuge 5402.

2. Wet previously gridded Boehringer Mannheim Nylon Membrane by laying on 6x SSC.

3. Drop 5 μL of prepared dilutions between the grids on the membrane. Crosslink in Stratagene UV Stratalinker 2400 on 6x SSC dampened filter paper.

DNA Dilutions

Tube 1	15 µL psoralinated oligo primer
	9 µL 20x SSC
	6 µL dH_2O
Tube 2	6 µL tube1
	7.2 µL 20x SSC
	16.8 µL dH_2O
Tube 3 -7	Repeat as for tube 2.

VIII.A.3. HYBRIDIZATION OF LABELED OLIGO DNA

1. Wet membrane in 1x TBS. Wash in 5x SSC.

2. Place membrane in hybridization bag with 10 mL of thawed pre-hybridization solution per 100 cm^2 of membrane. Incubate for one hour at the temperature for optimal annealing of oligo probe (in this case 42°C).

3. Add 5 µL of labeled primer to 95λ TE (final conc. of primer 25 ng/mL) and boil for ten minutes. Place immediately on ice.

4. Replace pre-hybridization solution with 1.5 mL hybridization solution and primer. Incubate at the temperature used for optimal probe annealing for one hour with gentle agitation.

5. Wash blot in 500 mL Wash Solution I in glass baking tray at room temperature for five minutes with gentle agitation. Repeat once.

6. Wash blot in 500 mL Wash Solution II in glass baking tray in a 40°C water bath for five minutes. Repeat once.

7. Place blot in new hybridization bag with 5 mL detection blocking solution and 50 µL 10% SDS. Incubate at room temperature for one to three hours with gentle agitation.

8. Replace blocking solution with 5 mL fresh blocking solution, 50 µL 10% SDS, and 3 µL streptavidin-alkaline phosphatase (conc. indicated on conjugate vial label). Incubate for one hour at room temperature with gentle agitation.

9. Wash blot in 500 mL Wash Solution III in glass baking tray at room temperature for ten minutes with gentle agitation. Repeat twice.

10. Wash blot in 500 mL1x TBS in glass baking tray at room temperature for ten minutes with gentle agitation.

VIII.A.4. Chemiluminescent Detection

1. Using powder free gloves cut Rad-Free Lumi-Phos 530 substrate sheet to size of membrane blot. Place into side-seal reaction bag, place into imaging cassette and incubate at 37°C until needed.

2. After final membrane rinse remove the substrate sheet unit from imaging cassette. Peel back top layer and place blot nucleic side up on top of the substrate sheet. Replace top sheet layer. Gently roll pipette over top to insure smooth contact between membrane and substrate sheet.

3. Seal the open sides of the side-seal reaction bag. Place into imaging cassette with film and incubate at 37°C for three hours.

4. Develop film with a Konica SRX-101 Processor. Re-expose with clean film and incubate at 37°C for twelve hours.

VIII.B. Psoralen Biotin Hybridization Recipes

10x Tris buffered Saline (TBS) (1L)

0.5 M Tris Base
1.5 M NaCl
pH to 7.5

20x SSC (1L)

3 M NaCl
0.3 M NaCitrate $2H_2O$

10% Sodium dodecyl sulfate (SDS) (500 mL)

0.34 M SDS
Dissolve in 450 mL ddH_2O and Q.S. to 500 mL

Wash Solution I (1L)

20x SSC (0.3 M NaCl, 0.03 NaCitrate $2H_2O$)
3.4 mM 10% SDS
ddH_2O

Wash Solution II (1L)

20x SSC (15 mM NaCl, 1.5 mM NaCitrate $2H_2O$)
3.4 mM 10% SDS
ddH_2O

Wash Solution III (500 mL)

10x TBS (0.05 M Tris Base, 0.15 M NaCl)
3.4 mM 10% SDS
ddH_2O
NOTE: Prepare wash solutions in the order indicated to prevent precipitation of SDS. Prepare immediately before use.

Southern Pre-hybridization/Hybridization Solution (50 mL)

10x SSC (0.75 M NaCl, 0.075 M NaCitrate $2H_2O$)
2.5 g Schliecher and Schuell Rad-Free Blocking Powder TM (5%)
0.035 M 10% SDS
Add SDS and blocking powder directly to SSC and dissolve on stir plate at 40° - 60°C for two hours or until completely dissolved. Store at -18°C. SDS can be resolubilized by heating to 37°C. Be sure to mix well before using.

Detection Blocking Solution (250 mL)

10x TBS pH 7.5 (0.05 Tris Base, 0.15 M NaCl)
7.5 g Schleicher and Schuell Rad-Free Blocking Powder TM (3%)
3.4 mM 10% SDS
ddH_2O

Allow blocking powder to dissolve on stir plate at 40° -60°C for two hours or until completely dissolved. Store base stock at 0° -4°C for up to one week. Add SDS to base stock immediately before use.

VIII.C. Hybridization Protocols for Digoxigenin

VIII.C.1. DIG Probe Synthesis-Incorporation of DIG-dUTP

1. Into a sterile microcentrifuge tube add the following.

   1x PCR buffer with $MgCl_2$
   200 µM dNTP of PCR DIG mix
   0.1–1 µM forward and reverse primers
   2.6 units Expand High Fidelity enzyme
   Template DNA and sterile ddH_2O to total 50 µL

2. Place Eppendorf tubes in Ericomp Easy Cycler Series thermal cycler and run on the program with the corresponding amplification temperature.

   Sample Program:

Cycle 39	94°C	2 min
Cycle 44	94°C	30 sec
(30-35 reps)	56°C	30 sec
	72°C	30 sec
Cycle 41	72°C	5 min
Cycle 42	4°C	18 hours (holding phase)

3. To analyze PCR product for positive amplification use an aliquot of 10 µL to which 4 µL of running buffer is added. Load into 1.5% agarose gel and electrophores. A single band should be visible. Due to incorporation of DIG-dUTP the molecular weight of the PCR product is increased compared to that of the unlabeled product.

VIII.C.2. Hybridization of DIG Labeled DNA

1. Prewarm Standard Hybridization Solution (20 mL/100cm^2 membrane) to 68°C. Incubate filter for 30 minutes with gentle agitation.

2. Denature DIG Labeled DNA probe (5µL/mL) by boiling 5 minutes, rapidly cool on ice.

3. Add DIG probe to prewarmed Hybridization Solution (2.5 mL/100 cm^2).

4. Pour off pre-hybridization solution and add probe/hybridization solution to membrane.

5. Incubate in water bath at 68°C overnight.

6. Wash membrane twice for 5 minutes in 2xSSC, 0.1% SDS at room temperature.

7. Wash membrane twice for 15 minutes in 0.1 SSC, 0.1% SDS at 68°C with constant agitation.

VIII.C.3. Detection

1. Rinse membrane for 5 minutes in washing buffer.

2. Incubate for 30 minutes in 25 mL 1x Blocking Solution.

3. Dilute anti-DIG-AP conjugate to 75 mU/mL in 1x Blocking Solution.

4. Incubate membrane for 30 minutes in 5 mL Antibody Solution.

5. Wash twice for 15 minutes in 25 mL Washing Buffer.

6. Equilibrate for 5 minutes in 5 mL Detection Buffer.

7. Dilute CSPD 1:100 in Detection Buffer.

8. Incubate membrane in sealed hybridization bag for 5 minutes in 0.5 mL CSPD solution.

9. Blot membrane on Whatmann 3MM paper, DNA side up.

10. Seal membrane in hybridization bag and incubate for 5-15 minutes at 37°C to enhance the reaction.

11. Expose 15-20 minutes at room temperature to x-ray film.

VIII.D. Digoxigenin Hybridization Recipes

Maleic Acid Buffer

0.1 M Maleic acid
0.15 M NaCl
pH to 7.5 with solid NaOH. Store at 20°C.

Washing Buffer

Maleic acid buffer
0.3% (v/v) Tween 20
Store at 4°C.

Blocking Solution (10x)

10% (w/v) Boehringer Mannheim DIG blocking reagent in maleic acid buffer
Makes an opaque solution.
Dissolve by microwaving. Autoclave. Store at 4°C.

Detection Buffer

0.1 M Tris-HCl
0.1 M NaCl
pH to 9.5 and store at 20°C.
1M Tris-HCl is 12.11 g Tris base in 100 ml ddH2O, pH to 9.5 and autoclaved.

Standard Hybridization Solution

5x SSC
0.1% (w/v) N-lauryl sarcosine
0.02% (w/v) SDS
1% blocking reagent (1/10 volume of 10x Blocking Solution)

IX. Appendix D

IX.A. Southern Blot Protocols

IX.A.1. Southern Blot

1. Separate DNA fragments via agarose gel electrophoresis.

2. Schleicher and Schuell Nytran membrane is floated on deionized water then submerged before soaking in the 10x SSC transfer buffer.

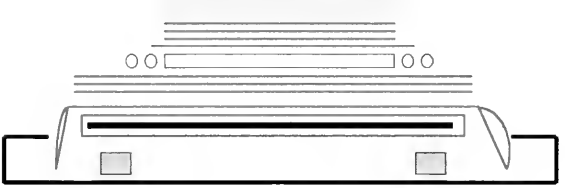
3. After electrophoresis, the gel is subjected to two fifteen-minute washes for DNA denaturation in 1M NaCl, 0.5M NaOH with constant agitation at room temperature. Use 2 mL denaturation solution per mL of gel.

4. Next, the gel undergoes two fifteen-minute washes for DNA neutralization in 1.5 M NaCl, 0.5 M Tris, pH 7.4 at room temperature. Use 2 mL neutralization solution per ml of gel.

5. Transfer DNA onto Nytran membrane by using the following setup. Upsorb material is replaced, as it becomes soaked, until transfer is complete (20-24 hours).

6. Following transfer wash Nytran membrane in 5x SSPE for five minutes at 60°C. UV crosslink to immobilize the DNA to the membrane. Store blot at 4°C.

IX.A.2. Southern Blot Apparatus



Upsorb material
3MM Paper
Nytran membrane
Gel with pipettes alongside
3MM Paper
Glass baking dish and glass plate

IX.B. Southern Blot Recipes

20x SSC

3M NaCl
0.3M NaCitrate $2H_2O$
Store in plastic bottle and dilute 1:2 for use in transfer.

Denaturation Solution

1.0M NaCl
0.5M NaOH
Store in plastic bottle.

Neutralization Solution

1.5M NaCl
0.5M Tris
Adjust pH to 7.4 and store in plastic bottle.

20x SSPE

3.6 M NaCl
200 mM NaH2PO4
20 mM EDTA (pH 7.4)
Adjust pH to 7.4 with NaOH and sterilize by autoclaving.