Comparative Genomic Hybridization to Detect Unbalanced Chromosomal Rearrangement

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Comparative Genomic Hybridization to Detect Unbalanced Chromosomal Rearrangement

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Natural Sciences at Carroll College, Helena, Montana

Laura Jane Peila
April 9, 2001
This thesis for honors recognition has been approved for the Department of Natural Sciences by:

Dr. Marilyn Schendel

Dr. Mary Haag

Mrs. Joan Stottlemeyer

April 9, 2001
Throughout my research experience at Shodair Children's Hospital, several individuals, to whom I am grateful, have provided me with their help and expertise. First, I would like to thank Dr. Mary Haag for inviting me to do research at Shodair and for always providing guidance throughout this project. Additionally, I would like to thank my peer, Leslie Phillips, for spending all summer familiarizing me with CGH in the lab and for passing her CGH research on to me. Also, I thank Sandy Phillips and Amy Zearfoss for their assistance with \textit{MacProbe 4.21} computer software, especially with karyotyping on it. I thank Linda Bieschel and Nicole Davis for their expertise regarding DNA, Matt Horvath for teaching me how to drop slides, and Theresa Boomer for sharing her knowledge of CGH. Finally, I would like to thank Dr. Marilyn Schendel, my thesis advisor, and Mrs. Joan Stottlemyer for their assistance in the writing of my thesis.
ABSTRACT

Comparative genomic hybridization (CGH) is a technique used to detect unbalanced chromosome rearrangements based on the use of in situ hybridization of differentially labeled DNA. This technique can be used to analyze complex clinical cases which have constitutional chromosomal abnormalities that do not lend themselves to routine chromosomal analysis. CGH was examined in order to develop a reliable and reproducible protocol that can be used as an additional diagnostic tool in Shodair Hospital's clinical lab. CGH involves the isolation of both test and reference DNA and the differentially labeling of the different DNA with fluorescent probes. Then those samples of DNA were hybridized onto a normal metaphase spread. The slide was examined under a fluorescent microscope and analyzed using Perceptive Scientific Instrument's MacProbe fluorescent imaging software. It appears that a slightly modified version of the published Vysis Protocol (1998) yields the best CGH results in our clinical diagnostic genetics laboratory.
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INTRODUCTION

Over the past ten years, the combined knowledge of molecular biology and cytogenetics has resulted in important advancements in the diagnostic capabilities in the field of human genetics. Scientists have been able to identify the DNA sequences at specific gene locations and synthesize and fluorescently label the complementary DNA segments. This provided fluorescent DNA probes which could be hybridized to metaphase spreads. The probes consist of short, complementary sequences of single-stranded DNA. After hybridization, the metaphase spreads are observed with a fluorescence imaging system and imbalances for the region can be detected (Phillips, 2000). This is one of the most common molecular techniques for chromosomal analysis and is called fluorescence in situ hybridization (FISH). FISH uses fluorescent molecular probes that target a specific chromosome region based on its unique DNA sequences.

There are three different types of FISH probes: locus specific probes, alphoid or centromeric repeat probes, and whole chromosome probes. The locus specific probe hybridizes to a particular area on a chromosome. It is helpful when scientists want to find out on which chromosome an isolated gene is located. The centromeric repeat probes are created from repetitive sequences that are found at centromeres of chromosomes. These probes are useful in determining whether or not an individual has the correct chromosome number (NHGRI, 2001). Finally, the whole chromosome probe is used to paint an entire chromosome. This allows the scientist to examine chromosomal abnormalities, such as in the case of a translocation (NHGRI, 2001).

However, FISH does not provide a complete genome analysis, and it is necessary to have some insight into the suspected chromosomal abnormality. For example, if the
suspected chromosomal abnormality is on chromosome 21, a specific probe for chromosome 21 would be used. However, if the abnormality turned out not to involve chromosome 21, but another chromosome, the FISH results may be uninformative. Therefore, due to the chromosome-specific nature of this analysis, FISH could be quite time consuming when the chromosomal origin of a genetic disorder is unknown.

However, comparative genomic hybridization (CGH), a relatively new technique, is advantageous because it examines the entire genome instead of a particular DNA sequence on a specific chromosome. CGH was first described by Kallioneimi et al in 1992 (du Manior et al., 1992) and is an alternate technique based upon fluorescence in situ hybridization (FISH). Using FISH to analyze the entire genome of complex cases would be more time-consuming and laborious than CGH. An additional advantage of CGH is that it is not dependent on the success of cells dividing in the laboratory to do metaphase chromosome analysis (Levy et al., 1998). In fact, CGH allows for an analysis of most specimens regardless of their mitotic activity (Kallioniemi et al., 1993). When researchers investigate a difficult case that gives few clues as to a specific chromosome abnormality, using CGH to study the whole genome simultaneously has the potential to be more time and cost efficient.

The theory behind CGH involves differentially labeling a sample of test DNA and reference DNA. This can be accomplished by first nick translating the DNA. Nick translation is a procedure that allows fluorescent label to be incorporated into the DNA by substituting labeled dUTPs for dTTPs. Nick translation is dependent on two enzymes, DNA polymerase I and DNase I. DNase I nicks the DNA, so the DNA ends up in various sized fragments depending on the DNase I concentration and on the length of time nick
translation is occurring. DNA polymerase I adds nucleotide residues to the 3'-end of one strand of a DNA molecule while it simultaneously removes nucleotides at the 5'-end of the adjacent region (Maniatis, 1982). This allows for nucleotide replacement using the fluorescently labeled dUTPs in place of the dTTPs, which ultimately labels the DNA. The test DNA and reference DNA are labeled with either red or green proprietary fluorescent DNA labels (Vysis). For CGH, the nick-translated DNA fragments should be about 200-600 bp.

After nick translation, the test DNA and reference DNA are co-hybridized onto a normal metaphase spread and incubated for at least 48 hours. After co-hybridizing the DNA, researchers can view the slide using fluorescence microscopy and analyze it via computer imaging software such as PSI's MacProbe. The first step involved in the computer imaging program requires about 10 different cells in metaphase to be captured via a digital gray-scale camera and stored in the computer. Analysis is based upon the different color intensities of the fluorescently labeled chromosomes. The ratio of the probe intensity of SpectrumGreen-labeled reference DNA to SpectrumRed-labeled test DNA can be used to determine the presence or absence of DNA segments. SpectrumRed and SpectrumGreen are proprietary fluorescent DNA labels that correspond to Texas Red and fluoresceine, respectively (Vysis). For example, a normal test DNA sample compared to a normal reference DNA sample will show a ratio of one because their respectively colored chromosomes will show equal intensities. However, if an amplification is present, the chromosomes of the test DNA will show a higher intensity than those of the reference DNA. Therefore, a ratio of greater than one will be present.
Likewise, a deletion would show a ratio of less than one. Figure 1 graphically shows the methodology of CGH.

![Figure 1. Typical reaction of a CGH protocol (Kallioniemi, 1993).](image)

Unfortunately, there are limitations to CGH analysis. First, only unbalanced insertions, deletions, and amplifications can be detected using CGH. Structural rearrangements such as translocations, inversions, insertions, and small DNA mutations cannot be detected. With translocations, inversions, and sometimes insertions where the genetic material has changed its location on the chromosomes, all of the genetic material is still present. When the DNA is nick translated, co-precipitated, and hybridized, the rearranged chromatin (now in fragments) will still be able to hybridize to the correct area on the normal metaphase. Therefore, balanced genetic abnormalities cannot be detected (Kallioniemi et al., 1993). Likewise, point mutations cannot be detected because it is simply too small to disrupt the hybridization of the fragment. It would probably be necessary to have much smaller DNA fragments (around 10-15 bp) in order to detect a
point mutation. However, CGH requires the use of much larger DNA fragments. Secondly, CGH cannot determine ploidy changes (Kallioniemi et al., 1993). If the case being studied is triploid or tetraploid, CGH could not analyze it accurately because a ratio of 1 would still be found regarding the red-to-green color intensities. Additionally, CGH does not analyze peri-centromeric or heterochromatic regions because these regions contain repetitive sequences that are blocked by the Cot-1 DNA during hybridization (Kallioniemi et al., 1993). Also, the fluorescence intensities diminish at the chromosome telomeres, so it is difficult to obtain an accurate ratio for these regions (Kallioniemi et al., 1993). It is important to be aware of these problems when analyzing a diagnostic sample.

CGH has been a useful research tool for studying cancer specimens in which complex chromosomal abnormalities are common. Unbalanced chromosome rearrangements, including insertions, deletions, and amplifications, have been characterized in many types of malignant tumors such as breast cancer, bladder and ovarian tumors (du Manoir et al., 1993). One study tested the CGH technique by studying eleven different cancer cell lines of known amplifications with varying origins including bladder, breast, colorectal, neuroblastoma, and lung tissues (Kallioniemi et al., 1992). In five of the eleven samples, more than one locus showed amplification. In four of the samples, two or three separate loci on the same chromosome were amplified (Kallioniemi et al., 1992).

By 1997, CGH was being used for cancer research involving about 1500 different tumor studies. The applications of CGH include detecting unbalanced chromosomal rearrangements as well as analyzing the progression and clonal evolution of cancer
(Forozan et al., 1997). These unbalanced rearrangements are prevalent in solid tumors, such as carcinomas.

CGH has also been helpful in locating which genes are aiding DNA amplification in cancerous tissue. For example, it has been found that up to 30 different chromosomal regions play a role in the progression of breast cancer (Forozan et al., 1997). This is a much higher number than previously thought according to research done using DNA probes to known oncogenes. Specifically, CGH analysis aided in the discovery of amplified sequences originating from 20q13 that are often found in breast cancer tissue (Kallioniemi et al., 1993). The number of regions detected by FISH and the use of specific probes to known oncogenes has been much lower than those pinpointed using CGH. In fact, CGH has helped discover at least six genes that show amplification in cancer cells (Forozan et al., 1997).

The concept of tumor progression recognizes the gradual change from a slow-growing tumor to the metastasized cancer. It is believed that the accumulation of these genetic changes becomes critical. When researchers analyze cancerous tissue with CGH, different stages of the cancer can be examined including primary tumors and the latter metastasized cancer cells (Forozan et al., 1997). Such cancer research using the technique of CGH has great implications in the study of the clonal evolution of cancer that may assist research toward cancer diagnosis and treatment.

Another study used CGH to determine the germ cell origin of undifferentiated tumors by showing a gain in the 12p region in more than 80% of the patients having testicular germ cell tumors (Summersgill et al., 1998). This study used CGH analysis with both paraffin-embedded and snap-frozen samples. Both types showed the gain in
the 12p region, but it appeared that the frozen samples gave more definitive results (Summersgill et al., 1998). The paraffin-embedded samples may not have been as sensitive as using DNA from the frozen samples, perhaps because of poorer quality of the DNA template created from the fixed material (Summersgill et al., 1998).

Additional research that utilizes CGH analysis includes the investigation of spontaneous fetal abortions. Since 50% of all first trimester spontaneous abortions are caused by chromosomal aneuploidy, CGH can be used to describe the genetic basis for the fetal demise (Daniely et al., 1998). In the past, fetal aneuploidies have been detected by obtaining tissue samples, culturing the cells, preparing metaphase spreads, and using chromosomal banding patterns for analysis. However, informative results may be limited due to the possibility of culture failure or selective growth of maternal cells (Daniely et al., 1998). One study applied the CGH technique to 50 placentae of spontaneously aborted fetuses and was able to detect different types of genetic defects in 18% of the samples. The unbalanced chromosomes included a trisomy 8, 15, 16, 18, 21, 22, one double trisomy involving chromosomes 14 and 21, and one monosomy X (Daniely et al., 1998). CGH failed to detect a chromosomal abnormality in only one sample where conventional analysis was able to determine a mosaicism for a trisomy.

Additionally, about 3% to 4% of newborn babies have congenital disorders, including unbalanced chromosomal rearrangements that occur in an estimated 1 out of every 250 live births (Levy et al., 1998). One study performed CGH analysis on eleven postnatal clinical samples that originated from patients that were shown to have extrachromosomal material detected by conventional cytogenetic analyses. In all eleven samples, CGH was able to successfully detect the chromosomal origin of the extra
chromosome marker (Levy et al., 1998). Another study used CGH to identify the origin of chromosomal abnormalities in seven prenatal samples. Traditional cytogenetic analyses were unsuccessful in determining the origin of the abnormality. However, CGH accurately detected the origin of all seven abnormalities, confirmed using FISH (Bryndorf et al., 1995). In this same study CGH was used to detect four common chromosome abnormalities in both cultured and uncultured samples (Bryndorf et al., 1995). This research implies that CGH could be a valuable tool in prenatal genetic testing and other clinical diagnostics.

The above observations and information have sparked an interest in perfecting the technique of comparative genomic hybridization at Shodair’s clinical cytogenetics laboratory. The unambiguous genome screening and discovery of chromosomal abnormalities could be pinpointed in a timely manner. The use of CGH would be helpful in analyzing complex cases that do not lend themselves to routine chromosomal analysis such as FISH. Eventually, CGH could be used to screen comprehensively for chromosomal abnormalities on appropriate samples. Based on the principal of co-hybridizing differentially labeled DNA and analysis via computer imaging, it is my hypothesis that CGH will consistently show ratios unequal to one in cases where an unbalanced chromosome rearrangement, including deletions, unbalanced insertions, and amplifications, is evident. To test this hypothesis, CGH will be performed using male as the reference sample and female DNA as the test sample. CGH should show a ratio equal to one for all autosomes but an imbalance of the sex chromosomes X and Y. Once the CGH analysis accurately shows an amplification or excess of the X-chromosome and a loss of the Y-chromosome, then further studies will be done using the CGH method on
abnormal DNA samples. I will test a case which is known to show a full trisomy of chromosome 18 and of 21, a case of XXX, and DNA from the MPE 600 cancer cell line. Upon obtaining consistent results, I believe Shodair Children’s Hospital could confidently apply this technique to other complex cases involving constitutional chromosomal abnormalities.
MATERIALS AND METHODS

Preparation of Metaphase Slides

The majority of the slides used in the CGH assays performed were ordered from Vysis. However, some of the assays required the use of slides which were prepared in the lab. To accomplish this, ten milliliters of blood from either a normal male or normal female were drawn into sodium-heparinized vacutainers. After the blood was thoroughly mixed, seven-tenths of a milliliter was added to each of the following solution cultures. One culture consisted of a 10 mL medium containing 199+5% fetal bovine serum (FBS) with antibiotic and antimycotic supplements (+Ab/Am) and L-Glutamine +2.0% phytohemaglutanine (PHA). The other culture consisted of a 10 mL medium made of RPMI 1640+20% FBS +Ab/Am and L-Glutamine +2.0% PHA. Next, the cultures were incubated at 37°C for 72 hours in a CO\(_2\) incubator. At 72 hours, 0.1 mL of colcemid (10ug/mL) was added to each culture for 15 minutes at 37°C in order to arrest the cells in metaphase. The cultures were centrifuged at 800xg for 10 minutes, after which the supernatants were removed, and the pellets were resuspended in about 10 mL of a prewarmed (37°C) hypotonic KCl solution (0.057 M). The cultures were incubated at 37°C for 15 minutes in a CO\(_2\) incubator. Next, 2 mL of 3:1 absolute methanol:glacial acetic acid fixative were added to the KCl solution. The culture tubes were inverted five times to mix the solution and were centrifuged for 10 minutes at 800xg. The supernatants were removed and the pellets were suspended once again in 10 mL of fixative. The cultures were centrifuged, and the pellets were rinsed two more times using 5 mL of fixative. Slides were prepared using the drop method. The slides were stored in the freezer for future use.
Isolation of DNA

The DNA used for CGH was isolated from blood samples from normal male and female subjects. DNA was isolated from leukocytes following the protocol given in the Puregene (Gentra) D-5000 isolation kit (Davis, 1980; Buffone, 1985). The DNA lab technologists isolated all of the DNA necessary for this research project.

Nick Translation

A modified nick translation procedure was performed in order to label the DNA used for CGH (Maniatis, 1982). To label the test DNA, 0.2 mM of SpectrumGreen dUTP was prepared by adding 10μL of 1mM dUTP to 40 μL of nuclease-free water. SpectrumRed labeled reference DNA was purchased from Vysis. Next, 0.1 mM dTTP was prepared by adding 10μL of 0.3 mM dTTP to 20 μL of nuclease-free water. Finally, 0.1 mM dNTP mix was made by mixing 10 μL of 0.3 mM dATP, 10 μL of 0.3 mM dCTP, and 10 μL of 0.3 mM dGTP.

The following reagents were added to a microcentrifuge tube that was placed in a frozen Eppindorf carrier.

<table>
<thead>
<tr>
<th>μL</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>1 ug Extracted DNA</td>
</tr>
<tr>
<td>(17.5-x)</td>
<td>Nuclease-free water</td>
</tr>
<tr>
<td>2.5</td>
<td>0.2 SpectrumGreen or SpectrumRed dUTP</td>
</tr>
<tr>
<td>5</td>
<td>0.1 mM dTTP</td>
</tr>
<tr>
<td>10</td>
<td>dNTP mix</td>
</tr>
<tr>
<td>5</td>
<td>10X Nick Translation Buffer</td>
</tr>
<tr>
<td>10</td>
<td>Nick Translation Enzyme (DNA Polymerase I and DNase I)</td>
</tr>
<tr>
<td>50</td>
<td><strong>Total volume</strong></td>
</tr>
</tbody>
</table>
The tube was briefly vortexed and centrifuged at 6200 xg and placed in 15° C for 4 hours. Either a water bath or a dry chamber can be used. The reaction was stopped by placing the microcentrifuge tube in a 75° C water bath for 10 minutes. Finally, the microcentrifuge tube was placed in a covered container to protect the sample from light and was stored in a -20° C freezer.

Purification

The DNA was purified to eliminate unincorporated dUTP. The DNA was precipitated by adding 0.1 volume (5 uL) of 3M sodium acetate and 2.5 volume (125 uL) 100% cold ethanol. The DNA was then placed in a -20° C freezer for about 30 minutes and transferred into a -75° C freezer for 30 minutes. Next, the DNA was centrifuged at 13,000 xg for 10 minutes. The supernatant (ethanol) was removed, and the pellet was allowed to dry. Finally, the pellet was resuspended in 50 μL of TAE buffer (Vysis 1998).

Gel Electrophoresis

Upon purification, gel electrophoresis was used in order to confirm that the DNA fragment sizes were between 200 and 500 bp. In a sterile microcentrifuge tube, 5 μL of the sample DNA, 4 μL of TAE buffer, and 1.5 μL of gel-loading dye were added, lightly vortexed, and briefly centrifuged at 6200 xg. Subsequently, 7 μL from each tube was placed into its own well in the gel. The gel consisted of 1.5% agarose, and the buffer used was Tris Acetate EDTA (TAE). The gel was run for approximately 2 hours at 70 Volts and 40 mA. The gel was stained for 10 minutes in ethidium bromide and finally
viewed under ultraviolet light. A Hind III digestion of lambda DNA was used as a marker for gel size.

Co-precipitation

The CGH probe mixes were made by adding 10 μL (200 ng) SpectrumGreen nick-translated DNA, 1 μL (100 ng) SpectrumRed reference DNA, and 10 μL of Human Cot-1 blocking DNA, which prehybridizes the repetitive sequences in the human genome. A 0.1 volume 3M sodium acetate (2.1 μL) and 2.5 volume cold 100% ethanol were added to precipitate the DNA. The samples were briefly vortexed and centrifuged at 6200 xg. They were placed in the -75°C freezer for 30 minutes. The sample was centrifuged at 13,000 xg for 10 minutes, and the ethanol was removed. The pellet could be resuspended in 3 μL of purified water and 7μL of CGH hybridization buffer.

Slide Preparation and Hybridization

First, the metaphase slides that were prepared earlier were viewed using phase microscopy in order to find an area containing numerous metaphases. The designated area was marked with a diamond-tipped pen. The slides were put into a dehydration series of 70%, 80%, and 95% ethanol at room temperature for two minutes each. Next, they were placed in a prewarmed (75°C) denaturation solution (28 mL Formamide, 4 mL 20xSSC, and 8 mL purified water, pH 7.2) for 5 minutes. The slides underwent another dehydration series consisting of 2 minutes in each of 70%, 80%, and 95% cold ethanol. While the slides were undergoing their second dehydration series, the CGH probe mix made earlier was denatured in a 75°C water bath for approximately 5 minutes. After the
slides were dehydrated, the slide bottoms were blotted and the tops were left to air dry in darkness. When the slides were dry, the probe mix was removed from the water bath and was briefly vortexed and centrifuged at 6200 xg. Ten μL of the probe mix was added to each slide. A coverslip was placed over the slide and was sealed with rubber cement. The slide was placed in a covered humidified chamber within a 37° C CO2 incubator for approximately 48 hours.

Post-Washing the Slides

After being removed from the incubation chamber, the slides were briefly (2 seconds) agitated in a prewarmed (75° C) solution of 0.4x Saline Sodium Citrate (SSC)+0.3% NP-40 and then let stand for 2 minutes in the solution. Next, the slides were washed in a 2xSSC + 0.1% NP-40 wash solution for 1 minute at room temperature.

Fluorescence Microscopy and Computer Imaging

After it was washed to eliminate background (unhybridized probe), 10μL of DAPI II counterstain and a 22x40mm coverslip were placed over the hybridized area. This stain causes bands to appear on the chromosomes which can then be karyotyped. Upon staining the slide, the hybridized metaphases could then be viewed under the fluorescent microscope and were imaged using the MacProbe 4.21 Perceptive Scientific Instruments’ (PSI) imaging program. Approximately 10 well-spread metaphases were captured onto the computer per case. Although each cell was karyotyped, not every chromosome in the cell could be used in the analysis. The computer cannot accurately detect intensity ratios on chromosomes that are in overlap or on chromosomes that are not
well aligned along its vertical axis. From the karyotypes, CGH profile ratios could be generated.

The histograms will show an increase or decrease of chromosome specific DNA dependent upon the presence of unbalanced genetic material in the sample being tested. For instance, if the test DNA is labeled in green, an addition to the genetic material will show a shift to the left. If chromosome specific DNA is deficient in the test DNA, the histogram will show a shift to the right. If the test DNA is labeled in red, then shifts in the opposite direction will be seen on the histogram for both additions and deletions in the genetic material.
RESULTS

Nick Translation

The first step in developing a reliable and reproducible protocol for CGH is the production of uniformly fragmented and labeled DNA using nick translation. The results of nick translation depended on both the length of time the DNA was nick translated as well as the type of label used (SpectrumRed or SpectrumGreen). Table 1 summarizes data from numerous nick translation attempts. The DNA was analyzed using gel electrophoresis after performing nick translation in order to determine fragment size and incorporation of the fluorescent label into the DNA. The optimum fragment length of nick translated DNA is between 200 and 600 base pairs (Vysis 1998).
<table>
<thead>
<tr>
<th>Experiment</th>
<th>DNA</th>
<th>Label</th>
<th>Time (hours)</th>
<th>Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NF</td>
<td>SG</td>
<td>9.83</td>
<td>No smears on gel</td>
</tr>
<tr>
<td>2</td>
<td>NF</td>
<td>SG</td>
<td>12.13</td>
<td>No SG apparent</td>
</tr>
<tr>
<td>3</td>
<td>NF</td>
<td>SG</td>
<td>12.00</td>
<td>Excess SG~500 bp</td>
</tr>
<tr>
<td>4</td>
<td>NF</td>
<td>SG</td>
<td>11.75</td>
<td>No smears on gel</td>
</tr>
<tr>
<td>5</td>
<td>NF</td>
<td>SG</td>
<td>4.00</td>
<td>200-500 bp</td>
</tr>
<tr>
<td>6</td>
<td>NM</td>
<td>SG</td>
<td>4.00</td>
<td>200-500 bp</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>SG</td>
<td>4.00</td>
<td>200-500 bp</td>
</tr>
<tr>
<td>8</td>
<td>NF</td>
<td>SG</td>
<td>4.00</td>
<td>200-500 bp</td>
</tr>
<tr>
<td>9</td>
<td>NM</td>
<td>SG</td>
<td>4.00</td>
<td>200-750 bp</td>
</tr>
<tr>
<td>10</td>
<td>NM</td>
<td>SG</td>
<td>4.00</td>
<td>200-750 bp</td>
</tr>
<tr>
<td>11</td>
<td>NM</td>
<td>SR</td>
<td>4.00</td>
<td>Ambiguous</td>
</tr>
<tr>
<td>12</td>
<td>Vysis Positive Control (MPE 600 cell line)</td>
<td>SG</td>
<td>4.00</td>
<td>Ambiguous</td>
</tr>
<tr>
<td>13</td>
<td>Trisomy 21 M</td>
<td>SR</td>
<td>4.00</td>
<td>200-525 bp</td>
</tr>
<tr>
<td>14</td>
<td>Trisomy 18 F</td>
<td>SR</td>
<td>4.00</td>
<td>150-525 bp</td>
</tr>
<tr>
<td>15</td>
<td>Trisomy 21 M</td>
<td>SG</td>
<td>4.00</td>
<td>200-500 bp</td>
</tr>
<tr>
<td>16</td>
<td>Trisomy 18 F</td>
<td>SG</td>
<td>4.00</td>
<td>200-500 bp</td>
</tr>
<tr>
<td>17</td>
<td>Triple X</td>
<td>SG</td>
<td>4.00</td>
<td>200-500 bp</td>
</tr>
<tr>
<td>18</td>
<td>Triple X</td>
<td>SR</td>
<td>4.00</td>
<td>200-500 bp</td>
</tr>
</tbody>
</table>

SG=SpectrumGreen fluorescent label  
SR=SpectrumRed fluorescent label  
NF=Normal female  
NM=Normal male  
M=Male  
F=Female  
*All assays were incubated at 15°C.
Hybridization

Hybridization of the co-precipitated test DNA and reference DNA to the normal metaphase spread was performed. The slide was viewed under fluorescence microscopy, and was analyzed on *MacProbe 4.21* in order to detect the color-intensity ratios of the test and reference DNA. The results obtained from hybridizing the DNA samples are shown in Table 2. The standards for the ratings poor, marginal, and good are stored in PSI's *MacProbe* computer software, and the computer issues a rating to the sample being analyzed. The length of time the DNA was hybridized to the metaphase spread was varied in order to find optimum conditions for the CGH procedure. Although the range of hybridization time between 66 and 88.5 hours showed good hybridization according to the computer analyses, it is subjectively thought that the time of 66 hours allowed for the most reproducible results.
Table 2. Length of incubation for optimal hybridization

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Length of Incubation (hours)</th>
<th>Hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>168</td>
<td>Poor</td>
</tr>
<tr>
<td>2</td>
<td>41</td>
<td>Marginal</td>
</tr>
<tr>
<td>3</td>
<td>41.5</td>
<td>Marginal</td>
</tr>
<tr>
<td>4,5</td>
<td>88.5</td>
<td>Good</td>
</tr>
<tr>
<td>6-8</td>
<td>120</td>
<td>Marginal</td>
</tr>
<tr>
<td>9,10</td>
<td>78</td>
<td>Good</td>
</tr>
<tr>
<td>11-14</td>
<td>66</td>
<td>Good</td>
</tr>
<tr>
<td>15-18</td>
<td>66</td>
<td>Good</td>
</tr>
</tbody>
</table>

All were incubated at 37.5°C

**Poor:** Ratio of the homogeneity of image >5; one color overshadows the other; chromosomes appear grainy (>1.0); low signal-to-background ratio (between 0 and 3.0)

**Marginal:** Ratio of homogeneity of image 4-5 (still uneven labeling); granularity of chromosomes between 0.8-1.0; signal-to-background ratio 3.0-4.5

**Good:** Even labeling with SpectrumRed, SpectrumGreen, and DAPI with the ratio of homogeneity of image being < 4.0; chromosomes appear yellowish/purple and show granularity of chromosomes between 0-0.8; signal-to-background ratio >4.5
Computer Imaging and Analysis

The results of computer imaging and analysis are shown in Table 3. The table shows the type of slides used and provides the corresponding results concerning the signal-to-background ratio, hybridization, homogeneity and granularity of chromosomes. The computer generated ratings of poor, marginal, or good depending on the quality of each of the categories described in Table 3. The signal-to-background ratio examines the amount of specific binding of the labeled DNA to the chromosomes compared to nonspecific binding on the slide. The quality of hybridization depends on the degree to which the differentially labeled test DNA and reference DNA hybridize onto the metaphase spread. The homogeneity of the chromosomes depends on whether or not the test and reference DNA samples are hybridized onto the metaphase spread in a homogeneous fashion, and the granularity of the chromosomes examines whether or not the chromosomes appear grainy. The less granular chromosomes provide a more accurate CGH analysis.
Table 3. Results obtained from Perceptive Scientific Imaging’s (PSI) *MacProbe 4.21* computer software.

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Slide Preparation</th>
<th>Signal to Background Ratio</th>
<th>Hybridization</th>
<th>Homogeneity of Chromosomes</th>
<th>Granularity of Chromosomes</th>
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<tr>
<td>1</td>
<td>Vysis</td>
<td>Poor</td>
<td>Poor</td>
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<td>Marginal</td>
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<tr>
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<td>Good</td>
<td>Good</td>
<td>Marginal</td>
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<tr>
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<td>Good</td>
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<td>Good</td>
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<tr>
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<tr>
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<td>Good</td>
<td>Marginal</td>
<td>Good</td>
</tr>
<tr>
<td>14</td>
<td>Fresh Shodair</td>
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<td>Good</td>
<td>Good</td>
<td>Good</td>
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<tr>
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<td>Good</td>
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<td>Good</td>
<td>Good</td>
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<td>Good</td>
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<td>Good</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
</tr>
</tbody>
</table>

Vysis=Purchased from vysis; stored in -20°C freezer  
Shodair=Made at Shodair Children’s Hospital and stored in -20°C freezer  
Fresh Shodair=Made at Shodair Children’s Hospital and used immediately thereafter  
All DAPI banding was okay.

Specific Case Studies

The CGH studies involved using varying test DNA samples. Each of the results from these case studies occurred by using the optimum nick translation time of 4 hours and the hybridization time of 66 hours. The first experiments tested normal female DNA
against normal male reference DNA. In this instance, the results should show all of the autosomes being normal, and thus having a ratio of 1:1 green to red. The sex chromosomes will show an amplification of the entire X chromosome and a deletion of the Y chromosome. Figure 3 shows the computer-generated average profile for the normal female test DNA against a normal male reference DNA hybridized onto a normal male metaphase spread. Notice that chromosomes one through twenty-one all show a ratio of one, while the sex chromosomes do not. The blue line shows the average and the surrounding brown lines show the standard deviations of each ratio. Figure 2 shows the chromosomes labeled in green, red, and also in DAPI II counterstain which is blue in color. Notice that there are no Y chromosomes labeled in green, as that is the the normal female test DNA. The chromosomes labeled in red show both an X and a Y chromosome, as that is the reference DNA of a normal male. The DAPI labels both the normal female and normal male DNA. Therefore, both X and Y chromosomes can be seen in blue.

The next DNA sample that we tested was that of a triple X. This case study showed a greater amplification in the X chromosome than a normal female would display. Figure 4 shows the metaphase spread onto which test and reference DNA were hybridized.

A female trisomy 18 was also examined using CGH. Figure 5 shows the computer-generated average profile for this individual. In this case, the reference DNA was labeled in green and the female trisomy 18 test DNA was labeled in red. Therefore, the histogram for chromosome 18 shows more red, which means that chromosome 18 is amplified. Likewise, the Y chromosome shows more green, which means that the ratio is
less than 1 for the Y chromosome. Note the centromeric regions where Cot I DNA has
bound. The centromeres consist of repetitive sequences complimentary to the Cot I
DNA. Therefore, we are not analyzing this portion of the chromosome. Chromosome 18
shows an amplification due to the extra chromosome 18. The Y chromosome shows a
deletion because the test DNA is female, and thus is missing a Y chromosome.

Another interesting case studied was DNA from the MPE 600 cancer cell line.
The hybridized test, reference, and DAPI II labeled cells can be seen in figure 6. Notice
the green regions which are characteristic of amplifications and the red regions which are
regions that show deletions. Figure 7 shows the average profile for this cancer cell line.
Note the major amplifications and deletions in chromosomes 1p, 1q, 9p, 11q, 15p, 16q,
17p, and the Y chromosome. Cells from the MPE-600 line should show extra copies of
1q, 11, and 13, and they should show deletions in copies of 1p, 9p, 11q, 15p, 16q, 17p,
22p, and the Y chromosome (Vysis 1997). Figure 8 shows the MPE-600 cell profile that
was provided by the vysis company. The test DNA in Figure 7 is labeled in red, which is
opposite from Figure 8 in which the test DNA was labeled in green.
Figure 2. The green cell is that of a normal female. The red labeled metaphase spread shows the normal male. The DAPI labels both the male and female DNA.
Figure 3. This is the computer-generated average CGH profile for a normal female test DNA against a normal male reference DNA. Note that each of the autosomes shows a ratio of one while the X chromosome shows an amplification and the Y chromosome shows a deletion.
Figure 4. These four quadrants show a normal male metaphase spread with test DNA from an individual with 47, XXX hybridized to it. The first quadrant shows the SpectrumGreen labeled +X DNA. Quadrant 2 shows the SpectrumRed labeled normal male DNA. Quadrant 3 shows the DAPI II stained chromosomes. Quadrant 4 shows the hybridization of the SpectrumGreen, SpectrumRed, and DAPI II labeled cells.
Figure 5. The computer-generated average profile for a 47, +18 Female. Note the centromeric regions where Cot I DNA has binded. Also note chromosome 18 and chromosome Y. The reference DNA is labeled in green and the female trisomy 18 test DNA is labeled in red.
Figure 6. This is the image created on PSI's MacProbe 4.21 for the MPE 600 cancer cell line. Notice the green regions which are characteristic of amplifications and the red regions which are regions that show deletions.
Figure 7. The computer-generated average profile for the cell line MPE 600. Note the major amplifications and deletions in chromosomes 1p, 1q, 9p, 11q, 15p, 16q, 17p, 22p, and the chromosome. The test DNA is labeled in green and the reference DNA is labeled in red.
Figure 8. This is the histogram of the MPE-600 cancer cell line provided by Vysis.
DISCUSSION

The purpose of this research was to optimize a protocol for CGH that could be used in the clinical cytogenetics laboratory at Shodair Children’s Hospital. CGH could then be applied to cases having constitutional chromosomal abnormalities in order to detect unbalanced chromosomal rearrangements.

Getting good incorporation by substituting SpectrumGreen-labeled dUTPs in place of the dTTPs into the test DNA required varying the length of nick translation from 16 hours to 4 hours. The optimal length of nick translation appears to be 4 hours. When the DNA was nick translated for this length of time, the DNA fragments were roughly between 200 and 500 bp, which is expected to be the optimal size (Table 1).

Additionally, the overall procedure seemed to give better results when the test DNA was labeled with SpectrumRed dUTPs instead of SpectrumGreen dUTPs and commercially labeled SpectrumGreen reference DNA was used. The intensity of the SpectrumRed was twice as great as that of SpectrumGreen when nick translating the test DNA. Therefore, only half the concentration (100ng) of SpectrumRed is used compared to the amount of SpectrumGreen used (200ng).

The pellet obtained during co-precipitation of the test DNA and reference DNA was initially quite small and sometimes almost nonexistent. This problem may have been due to the unavailability of a refrigerated centrifuge. A freezer technique was adapted to our methods in order to compensate for the lack of a refrigerated centrifuge. It appears that 30 minutes in a -75°C freezer is the optimum time necessary to allow for a visible pellet to be formed.

The majority of the slides used were purchased from Vysis and stored in the
-20°C freezer until use. These slides gave results that showed little background staining. Freshly made slides give the best hybridization results. However, if the metaphases on the slides age too long before they are used for CGH, the hybridization is poor. It was found that using slides no more than 8 hours after they were made provided the best hybridization results. Also, the signal to background ratio decreases when aged slides are used. This results in the computer's inability to differentiate between intensities of the chromosomes versus the background signal.

The best hybridization seems to have occurred when the test DNA was nick translated for 4 hours and the incubation time was 66 to 88.5 hours. The hybridization was somewhat good (the test DNA and reference DNA were not completely evenly hybridized) when the incubation time ranged from 41 to 88.5 hours (Table 2). The case studies that were hybridized for 66 hours showed better overall results than did those hybridized up to 88.5 hours. This may be due to the size of DNA fragment that is created during nick translation. As the length of nick translation time of the DNA samples increases, shorter DNA fragments are produced. The DNA fragment size that is produced during a 4-hour nick translation hybridizes optimally over a period of 66 hours.

There appeared to be a lot of background on the first assays performed. Initially we used a pretreatment using SSC before dehydrating the slides. However, not using a pretreatment seemed to give better results. The salt in the SSC pretreatment solution may have caused an increase in background signal. Background on some slides was due to oil getting under the coverslip. The use of 22x40 mm coverslips instead of 22x22 mm coverslips prevented oil from contacting the sample.
This research has shown that a slightly modified version of the published CGH protocol can be used in a clinical genetics laboratory. This study found a four-hour nick translation time to be optimal, as opposed to the 8-16-hour recommendation in the published protocol. Also, nick translating the test DNA with SpectrumRed and using commercially labeled SpectrumGreen reference DNA seem to give better analyses. This study also showed that a 66-hour hybridization time provided the ideal binding of the labeled DNA samples to the metaphase chromosomes. Freshly made slides, instead of older slides that have been stored in the -20°C freezer, appear to show the best results. This is because the freshly made slides seem to acquire less background signal. The fresher slides may eliminate the possibility of having deposits on the slide due to normal wear. Additionally, the salt in the SSC post-washing solution may have deposited on the older slides, interfering and causing an increased background signal. Finally, in aged metaphase spreads, chromatin naturally deteriorates which lessens the overall quality of the slide. Random fragments of chromatin on older slides may allow for background signal as well.

The research done in the cytogenetics laboratory at Shodair Children’s Hospital could affect the overall diagnostic capabilities of the lab. For instance, as mentioned in (Levy et al., 1998), appropriate prenatal and postnatal samples could be tested for extrachromosomal material using CGH analysis. In cases where the origin of the abnormality is unknown, CGH may be more successful in providing comprehensive chromosomal analyses than would traditional cytogenetic analyses. The overall implications of this research is extraordinary in that it provides a time and cost-efficient method for analyzing difficult cases. In clinical labs it is necessary to be efficient
because the results of a certain chromosomal analysis directly affects the individual who has chromosomal abnormalities, and efficient lab work along with the ability to obtain results within a short length of time allows for quicker treatment for the individual case. Because previous research has noted that CGH may more accurately detect the origin of chromosomal abnormalities than FISH (Bryndorf et al., 1995), it is relevant to try to incorporate CGH analyses in diagnostic laboratories. Additionally, CGH is a technique that does not require the presence of dividing cells; therefore, a wider range of successful diagnoses can be made on complex cases in a timely fashion. The impact of this new technological research has the potential to enhance the quality of diagnoses made in the lab by using CGH as a supplemental diagnostic tool.

CGH is a valid diagnostic tool for unknown abnormalities that are complex due to unbalanced translocations, duplications, amplifications, and deletions of a larger size. Such complex cases cannot be resolved using traditional methods of analysis. For instance, the cancer cell line MPE 600 has several abnormalities difficult to resolve by G-banding. CGH can highlight additions and deletions to refine a karyotype. CGH gives us targets for further characterization by more definitive methods such as locus specific probes, centromeric repeat probes, or whole chromosome probes.

Overall, CGH provides impressive benefits. When a karyotype is complex and unbalanced, CGH is most efficient in screening the sample to determine where to look for the origin of the abnormality. Additionally, CGH targets chromosomes that are involved in complex duplications and deletions. Finally, CGH is not dependent on the presence of metaphases from fixed tissue for analysis. CGH has limitations as well. First, CGH does not analyze for structural rearrangements; the chromosomes must be unbalanced. The
limit of resolution for CGH is 5-10 Mb (Forozan, et. al, 1997) while the resolution for FISH is 2-60 Kb. Finally, follow-up confirmatory studies such as FISH probes or DNA marker analysis is necessary after CGH analysis.
REFERENCES


