

Applications of
Comparative Genomic Hybridization
in a Clinical Cytogenetics Laboratory

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April 10, 2000

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ABSTRACT

Comparative Genomic Hybridization (CGH) provides a method to rapidly detect amplifications and deletions of chromosomal material as small as 10 Mbp (Bentz et al. 1998) in an entire genome. Thus, this modern molecular cytogenetic technique was investigated in order to optimize a working protocol. DNA from a test sample and a reference sample was isolated, fluorescently labeled, and hybridized to normal male metaphase spreads. The spreads were then viewed using a fluorescent microscope and imaged using Perceptive Scientific Instruments' (PSI) *MacProbe* fluorescence imaging program. Predicted results were found in some cases, but not in others, indicating that further testing is needed before CGH can be reliably used for clinical testing of patients.

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INTRODUCTION

Fetal loss has plagued the human population throughout history. Even in the midst of major advancements in medical care, 20% of human oocytes and 10% of sperm have chromosomal abnormalities. More than 90% of the abnormalities in oocytes and about 50% of those in sperm are numerical (Gersen and Keagle 1999). The term aneuploidy defines these numerical aberrations in oocytes and sperm as the abnormalities are passed on to offspring. The incidence of autosomal aneuploidy in newborns is estimated at 0.2% and because many autosomal aneuploidies cause spontaneous abortion 27-30% of spontaneously aborted fetuses have autosomal aneuploidies (Gersen and Keagle 1999). These startling statistics have compelled scientists to develop methods to predict the chromosomal content of unborn fetuses. The information provided by these prenatal diagnoses allows parents to take appropriate actions to prepare for the inevitable outcome of their pregnancy. More often than not the prenatal testing allows for parents to be confident that their baby will be healthy. Thus, prenatal testing has undergone considerable advancements in the last century, allowing clinical genetics laboratories to predict accurately and rapidly the presence of aneuploidy in an unborn fetus.

In 1900, following the rediscovery of Mendel's research in inheritance, W.S. Sutton developed a "chromosome theory of inheritance." Sutton hypothesized that observed nuclear structures, called chromosomes, are used to transmit genes. Chromosomes appeared to occur in pairs with each somatic cell having both copies of each chromosome. Gametes, on the other hand, have only one chromosome from each pair (Sutton 1903, cited in Gersen and Keagle 1999). Thus when gametes fuse, each parent passes on half of his or her genetic material to the zygote. Sutton called the study

of chromosomes cytogenetics, combining the fields of cytology and genetics (Gersen and Keagle 1999).

The field of cytogenetics has advanced tremendously since Sutton's time. Tjio and Levan (1955, cited in Gersen and Keagle 1999) discovered that the diploid number for humans is 46. Their findings shook the genetics community; previously geneticists believed that the human diploid number was 48 (Gersen and Keagle 1999). When Ford and Hamerton (1956, cited in Gersen and Keagle 1999) confirmed these results, clinical genetics began. It became possible for cytogeneticists to identify chromosomal abnormalities based on differences in number of chromosomes in human cells (Gersen and Keagle 1999). However, many abnormalities could not be identified because techniques were yet unavailable to recognize hypothesized inversions of a single chromosome, translocations involving chromosome pieces of similar size, or even small deletions and duplications (Gersen and Keagle 1999).

These problems did not persist long. Torbjörn Caspersson (1968, cited in Gersen and Keagle 1999) developed a method of staining chromosomes, referred to as quinacrine banding, that produced a banding pattern unique to each chromosome pair. Quinacrine banding uses quinacrine dihydrochloride, which preferentially binds to A-T regions of the chromosome to produce this banding pattern. The quinacrine banding pattern allowed for a standard karyotype to be developed. Each chromosome was assigned a number based on its size and the position of its centromere (Gersen and Keagle 1999).

Quinacrine banding eventually proved to be cumbersome because it required fluorescence microscopy and a darkened room, but these difficulties were overcome when Drets and Shaw (1971, cited in Gersen and Keagle 1999) produced similar banding

patterns using a Giemsa stain. Giemsa was originally developed for identification of the protozoan that causes malaria, but it also stains A-T regions of DNA. Thus, banding of chromosomes and subsequent analysis of the banding pattern for each chromosome became feasible for any facility that was willing to train individuals in this analytical technique, and to develop a protocol for banding metaphases under the conditions in their lab. The development of Giemsa banding allowed chromosomal analysis to become a routine method in labs throughout the world (Gersen and Keagle 1999).

Eventually, precise identification of gene locations on chromosomes became possible using techniques that combined molecular genetics with cytogenetics. Once molecular geneticists were able to sequence genes, and to clone specific segments of DNA, these segments could be labeled with fluorescently labeled nucleotides, creating fluorescent DNA probes that could be hybridized to metaphase spreads (Gersen and Keagle 1999). Because the sequences were complementary to only one part of the human genome, certain areas on chromosomes could be identified as either absent or present and the copy number of each area could be elucidated. This procedure is known as fluorescence in situ hybridization (FISH). FISH probes provide important information if a patient is at high risk for an abnormality. Because FISH takes only 1-2 days (preparation of metaphase spreads for quinacrine or Giemsa staining takes 5-7), FISH allows for rapid detection of chromosomal abnormalities in high-risk patients (Gersen and Keagle 1999).

However, FISH does not provide a complete genome analysis. Consequently, FISH is used only when certain abnormalities are suspected, and is always used in conjunction with Giemsa banding analysis. Down's syndrome is a genetic disorder

developed as a consequence of having 3 copies of a specific region of chromosome 21. A FISH probe for this region of chromosome 21 can be used to determine if the patient has trisomy 21. But this procedure monitors only one segment of a single chromosome. If FISH indicates that a patient does not have Down's syndrome, the patient may have other abnormalities not screened for in the FISH assay. Because of these circumstances Giemsa banding is done in addition to FISH to reveal the full complement of chromosomal abnormalities in the fetus. For a pregnant woman carrying a baby at high risk for Down's syndrome or other trisomies, it is a relief to know the outcome in 1-2 days even if Giemsa banding will provide more information in 5-7 days.

The advances in FISH formed the basis of other molecular cytogenetic developments; one of these FISH-based technologies is Comparative Genomic Hybridization (CGH). CGH provides for complete analysis of deletions or additions of chromosomal material to any genome (Kallioniemi et al. 1992). CGH utilizes nick translation to label total genomic DNA of unknown composition, referred to as test DNA, and total genomic DNA of known composition, referred to as reference DNA. Nick translation involves the replacement of dTTP's in the DNA with fluorescently labeled dUTP's. The reference DNA and the test DNA are labeled with differently colored fluorochromes. For this study SpectrumRed-labeled dUTP (Vysis) was used to label the reference DNA, while SpectrumGreen-labeled dUTP (Vysis) was used to label the test DNA. Following nick translation, the test and reference DNA are mixed, and the DNA mixture is hybridized to normal metaphase spreads. Because the reference DNA contains the same DNA content as the metaphase spreads, any given metaphase on the spread has the potential to be completely labeled with the nick translated reference DNA. However,

because the reference DNA is mixed with the labeled test DNA, the labeled reference DNA must compete with the test DNA for places to hybridize to on the metaphase spreads. Thus, the different fluorochromes are randomly spread throughout the metaphase spread. CGH utilizes fluorescence microscopy and computer imaging programs to determine the average green-to-red ratio for each chromosome in any given assay. If the reference DNA and the test DNA are normal male, the green-to-red ratio of a given chromosome will be 1:1. However, if there is an extra chromosome or even a piece of extra chromosomal material as small as 15 Mbp (Parente et al. 1997) in the test DNA, there will be extra green probe for that region (i.e., green-to-red ratio will be greater than one). In contrast, if a part of a chromosome is deleted in the test DNA, that region of the chromosome will have a lower green-to-red ratio than one because less green probe for that region is present in the DNA mix than the red probe. Figure 1 shows a generalized protocol for CGH and the typical results of the procedure.

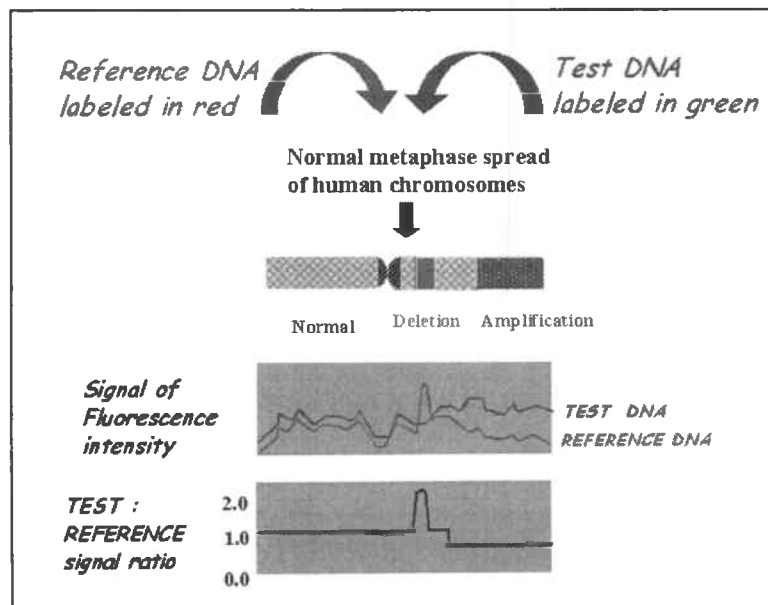


Figure 1: A generalized protocol for CGH and typical results of the procedure

Traditional Giemsa banding can not always elucidate the chromosomal origin of extra chromosomal material or the absence of deleted chromosomal material because banding analysis is limited to recognition of the limited number of bands banding produces. However, comparative genomic hybridization (CGH) has the potential to provide a complementary method to determine chromosomal origin of these aberrations because the chemistry of CGH and subsequent image analysis allows for identification of additions or deletions in any part of the genome.

CGH can also be used to determine chromosomal content of many tissue types (Table 1), including tissue samples that can not be cultured and thus are not available to be analyzed using Giemsa banding analysis. CGH utilizes only the DNA component of the test tissue. Because DNA can be extracted from almost any tissue, and CGH can utilize this DNA to define the chromosomal content of a test sample, chromosomal content of tissues that are incapable of dividing to form metaphases can be defined.

Danielly et al. (1999) used CGH to study numerical chromosome aberrations in placental samples of spontaneously aborted fetuses. Twenty-seven of fifty samples studied could not be analyzed by traditional cytogenetic methods. They cited culture failure, external contamination, and metaphase spreads of poor quality as major contributing factors in the inability to perform traditional cytogenetic methods. All fifty samples were analyzed using CGH, and, in almost all of the cases that traditional methods were used, CGH correctly identified the abnormality of the tissue. Those abnormalities not correctly identified were attributed to mosaicism of the tissue. CGH measures only the average red-to-green ratio for each chromosome using the ratios for each chromosome from each cell in the tissue. Consequently, mosaicism can not be

identified. Assuming no mosaicism, CGH identified the abnormalities associated with the 27 tissues that could not be analyzed using traditional cytogenetic techniques (Danielly et al. 1999). Similarly, Breen et al. (1999) correctly identified 19 of 19 chromosomal abnormalities using CGH. They confirmed their results using fluorescence in situ hybridization probes specific to the location of the genome that CGH identified as either added or deleted. Bryndorf et al. (1995) successfully analyzed 11 fetal samples using CGH, and Levy et al. (1998) accurately identified the origin of extrachromosomal material of 12 samples. Both studies were confirmed by FISH or DA-DAPI staining (Breen et al. 1999; Levy et al. 1998).

Most notably, CGH has been used to analyze tumor cells that are known to contain numerous chromosomal aberrations. The chromosome aberrations typical of tumor cells are difficult to identify using traditional cytogenetic methods. CGH can be used to characterize regions of chromosomes that are most likely to have unbalanced genomic rearrangements in specific tissue types. This information can be used to define genes involved in copy number alterations for specific types of cancer. CGH has also been used to follow the progression and clonal evolution of cancer (Forozan et al. 1997). Kallioniemi et al. (1992) analyzed tumor cell lines and primary bladder tumors and revealed 16 different regions of amplification. Many of these amplifications were in regions not previously known to be amplified in these types of tumors. Summersgill et al. (1998) used CGH to identify increases in chromosomal material from the p-arm of chromosome 12 in undifferentiated tumors that had been paraffin-embedded. Paraffin-embedded samples are notoriously difficult to analyze using FISH and impossible to analyze with Giemsa staining. Many other studies have revealed positions of oncogenes

and/or frequent chromosomal abnormalities using CGH on tumor cells (Edstrom et al. 2000; Jay et al. 1999; Barnard et al. 2000; Aust et al. 2000).

TABLE 1: Different types of tissues that can be analyzed using CGH and the success certain studies have had

Tissue Type	References	Success
B-cell leukemias	Bentz et al. 1998	5 of 5
Lymphocytes	du Manoir et al. 1992	1 of 1
	Breen et al. 1999	10 of 11
	Levy et al. 1998	12 of 12
Renal papillary carcinoma	du Manoir et al. 1992	1 of 1
T-PLL cells	du Manoir et al. 1992	1 of 1
Placentae of spontaneously aborted fetuses	Daniely et al. 1999	8 of 9
Fetal samples	Bryndorf et al. 1995	11 of 15
Solid tumors	Kallioniemi et al. 1992	12 of 12
	Edstrom et al. 2000	34 of 34
	Jay et al. 1999	1 of 1
	Barnard et al. 2000	4 of 4
	Aust et al. 2000	74 of 74
Paraffin-embedded solid tumors	Summersgill et al. 1998	4 of 4

Overall, there are many advantages to making CGH an integral part of a cytogenetics laboratory. To utilize CGH the laboratory technician needs no prior information about the specimen's genetic content; so test DNA can be analyzed without preparing metaphase spreads. CGH has additional advantages over the more traditional

FISH analysis because specific probes are not needed to elucidate the target region unless additions or deletions are smaller than can be detected by CGH (Kallioniemi et al.1992). FISH necessitates a preconceived notion of an abnormality to discover which parts of the genome are added or deleted. It would be very time consuming to produce FISH probes for every location of the genome. In addition to these benefits, CGH provides rapid identification of amplifications or deletions as small as 10 Mbp in the genome. CGH takes only 3-4 days to complete, whereas traditional banding methods can take several days to weeks to acquire enough metaphase spreads to analyze (Haag et al. 1995). FISH is also a relatively fast method, but only a few genomic locations can be tested in the time it takes to perform CGH. Finally, CGH accurately locates the amplified regions on normal chromosomes, so clinical implications can be more readily assessed. Traditional cytogenetic methods do not always give a definitive origin of extra chromosomal material because Giemsa banding does not distinguish chromosomal origin in many cases.

Despite the many advantages to the use of CGH in a cytogenetics laboratory, there are also limitations to the procedure. First, the DNA sample size (post nick translation) has specific requirements (i.e., size and homogeneity). Fragment sizes must be of a certain size to hybridize adequately. Second, CGH cannot detect structural rearrangements, point mutations, small intragenic rearrangements, or complete ploidy changes (Kallioniemi et al.1994). However, this limitation applies to traditional cytogenetic techniques as well in some cases (Haag et al. 1995). Also, at this time CGH only provides a resolution of 15Mbp (Parente et al. 1997). However, advancements in the technique are expected to resolve genome segments as small as 3-5Mbp (Kallioniemi et al.1992) and at least one study elucidated deletions down to a size of 10-12 Mbp

(Bentz et al. 1998). Finally, CGH provides poor resolution in polymorphic regions (i.e., centromeric, heterochromatic, telomeric), so abnormalities in these regions of the chromosome can not be detected (Kallioniemi et al. 1994).

The goal of this study is to develop the CGH technique clinical application in a genetic laboratory with respect to prenatal studies of aneuploidy. Because CGH techniques must be adapted in each lab, methods best suited for conditions in the laboratory at Shodair Hospital must be determined prior to incorporating CGH into the cytogenetic analyses of the laboratory. My goal is to optimize this procedure and compare the results of CGH studies with the results of routine cytogenetic methods. Ultimately, I would like to develop a protocol to obtain reliable reproducible results with CGH so that the method can be used to complement the traditional cytogenetic methods performed at Shodair.

MATERIALS AND METHODS

Preparation of Metaphase Slides

Ten milliliters of whole blood from a normal (known chromosomal makeup) human male were drawn into sodium-heparinized vacutainers. Then the blood was mixed well. Seven-tenths of a milliliter was aliquoted into each of two cultures. One culture contained 10mL medium made of 199+5% fetal bovine serum (FBS) with antibiotic and antimycotic supplements (+Ab/Am), and L-glutamine +2.0% phytohemagglutinine (PHA). The second culture contained 10mL of medium made of RPMI 1640+ 20% FBS +Ab/Am, and L-Glutamine +2.0% PHA. The cultures were then incubated at 37°C for 72 hours in a CO₂ incubator. The cells in each culture were arrested in metaphase at 72 hours by adding 0.1 mL colcemid (10 µg/mL) for 15 minutes at 37°C. Next, the samples were centrifuged for 10 minutes at 800xg. Supernatants were removed, and pellets were resuspended in 10-13 mL of a hypotonic solution (0.057M KCl (2.12g/500 mL)) prewarmed to 37°C, then incubated for 15 minutes in a 37°C CO₂ incubator. Two milliliters of 3:1 absolute methanol:glacial acetic acid fixative were added to the hypotonic solution. The tubes were mixed well by inversion, then spun again. The supernatants were removed and pellets were resuspended in 10 mL of fresh fixative. The samples were centrifuged at 800xg for 10 minutes and pellets were rinsed two more times using 5 mL of fixative each time. Approximately 10 slides were prepared from each culture by drop method. Slides were stored in the freezer for future use.

Not all assays used slides prepared in the lab. One assay used normal male metaphase slides ordered from Vysis as controls.

Isolation of DNA

Originally, DNA was isolated from blood samples from the same male individual and an additional normal (known chromosomal makeup) female. DNA was isolated from leukocytes using the Puregene (Gentra) isolation kit (D-5000). The manufacturer of the isolation kit provided the following procedure.

The blood was poured from the collection tube into a 15-mL sterile plastic centrifuge tube with volume markers on the side. The volume of blood was then estimated to the nearest 0.5 mL. This estimation became one “volume.” Then, three “volumes” of RBC lysis solution (Gentra) were added to the tube, the contents of the tube were mixed well by inversion, and the tube was then allowed to stand for ten minutes. Next, the tube was centrifuged for 10 minutes at 3200 RPMs, and the supernatant was removed, leaving only a white cell pellet. Then the tube was vortexed rigorously to evenly resuspend the cell pellet, and one volume of cell lysis solution (Gentra) was added. Pipetting the solution in and out of the tube enhanced lysis of the white cells. Next, 0.005 mL RNase solution (Gentra) per 1 mL of original blood volume was added, and the tube was incubated in a water bath at 37°C for 15 minutes. After incubation, the sample was cooled to room temperature, and one-third volume of Protein Precipitation Solution (Gentra) was added to the lysate. The sample was then vortexed for 20 seconds and centrifuged at 3200 RPMs for 10 minutes. During centrifugation, one volume of isopropanol was poured into a new labeled tube matched to each specimen. Following centrifugation, the supernatant out of the centrifuged tube was poured into the isopropanol and the pellet was discarded. Next, the supernatant/isopropanol mix was gently inverted until visible DNA strands formed a coherent gelatinous mass. A sterile

inoculating loop was then used to transfer the DNA into a 1.5-mL microfuge tube containing 1 mL of 70% ethanol in water. Then, the ethanol/DNA solution was microfuged for one minute, and the extra ethanol was removed using a sterile swab. The pellet was allowed to air dry for 10 minutes. Finally, 1/10 volume DNA hydration solution (Gentra) was added to the DNA. The solution was then mixed and heated at approximately 50°C for one hour in a multi-blok heater. Following isolation, the DNA was then quantitated by its absorption at 260 nm (50 µg/mL DNA has an OD of 1.0 at 260nm).

Because various labs are using CGH, numerous groups have developed protocols for the CGH techniques. I chose to use the protocol and reagents provided by Vysis as a baseline for my protocol because Vysis' reagents and protocols have a good reputation. The following are the protocols outlined by Vysis with minor modifications.

Nick Translation

Nick translation was performed using the following reagents: 0.2 mM SpectrumGreen dUTP (for labeling test samples) or SpectrumRed dUTP (for labeling standard samples) prepared by adding 10 µL of 1 mM dUTP to 40 µL nuclease-free water; 0.1 mM dTTP prepared by adding 10 µL of 0.3 mM dTTP to 20 µL nuclease-free water; 0.11 mM dNTP mix prepared by mixing 10 µL each of 0.3 mM dATP, 0.3mM dCTP, and 0.3 mM dGTP; and 0.2 µg/µL to 1 µg/µL solution of extracted DNA in Tris EDTA (10 mMTris, 1 mM EDTA, pH8.5) buffer.

A microcentrifuge tube was placed on ice and allowed to cool; then the following components were added to the tube in the order listed:

(17.5-x) μL	nuclease-free water for 1 μg extracted DNA
x μL	for 1 μg extracted DNA
2.5 μL	0.2 mM SpectrumGreen or SpectrumRed dUTP
5 μL	0.1 mM dTTP
10 μL	dNTP mix
5 μL	10X Nick Translation buffer
(Briefly vortex and centrifuge)	
<u>10 μL</u>	Nick Translation Enzyme
50 μL	total volume

The tube was then briefly vortexed and centrifuged and placed in a refrigerator at 15°C for 8-16 hours. Finally, the reaction was stopped by heating the tube in a 70°C water bath for 10 minutes and placed on ice.

Electrophoresis

Gel Electrophoresis was used to check the size of the DNA. All gels used to check the size of nick translated DNA were 1.5%LE (FMC) with Tris Acetate EDTA (TAE) as a buffer. Electrophoresis was done at ≈ 70 Volts and 40mA. The gels were stained with ethidium bromide after 30 minutes of electrophoresing by placing the gels in a container with the stain. A 100 bp-gel marker was used as a standard size marker on all gels, and a Hind III digestion of lambda DNA was used as a second size marker on some of the gels.

Purification

Some samples were purified to remove unincorporated dUTP. One sample was purified using the QIA PCR Purification kit. The manufacturer provided the protocol for this gel column purification. The protocol involved column filtration of the DNA solution and subsequent elution of double stranded DNA. (The complete protocol is not

outlined because this purification proved ineffective.) The other samples were filtered using ethanol precipitation. One-tenth of a volume of 3M sodium acetate was added to the DNA samples, followed by 2.5 volumes of 100% ethanol were added. The samples were vortexed and centrifuged, then placed on an ice block in the freezer (-20°C) for 45 minutes. Samples were moved to a -70°C freezer for 15 minutes and centrifuged at 13,000xG for 10 minutes. The pellets were then dried by discarding the ethanol. Excess ethanol was removed with a sterile swab. The DNA samples were then dissolved in 10mM Tris-Cl, pH 8.5. Both precipitated samples and unprecipitated samples were electrophoresed under the conditions described above to determine probe size.

Co-precipitation

Next, the CGH probe mixes were made. The procedure outlined by Vysis for co-precipitation is as follows. Ten microliters (200 ng) of SpectrumGreen (nick translated labeled) test DNA, 100 ng of SpectrumRed (nick translated labeled) standard DNA, and ten microliters (10 µg) of Human Cot-1-1 DNA, to prehybridize the numerous repetitive sequences in the human genome, were combined in a 1.5-mL microcentrifuge tube, (total volume of the previous three reagents equals one “volume”). Then, 0.1 volume of 3M Sodium acetate, and 2.5 volumes of 100% ethyl alcohol were added to precipitate the DNA. The solution was vortexed briefly and placed in a -70°C freezer for 30 minutes. Next, the mix was centrifuged at 12,000xg for 10 minutes to pellet the DNA. The ethanol was poured off and excess ethanol was removed using a sterile swab. Finally, the pellet was resuspended in 3 µL purified water and 7 µL CGH hybridization buffer.

Slide Preparation and Hybridization

The metaphase slides prepared previously were viewed using phase microscopy to find an area with numerous metaphases. This area was marked using a diamond tipped scribe. Next, the slides were pretreated in 2xSSC + 1%NP40, pH 7.0 at 37°C. Pretreatment time was varied. Next, the slides underwent a dehydration series of 2 minutes each in 70%, 80%, and 90% ethanol, (the pretreatment and following dehydration are additions to the Vysis protocol). Then, the slides were placed in denaturation solution (49 mL formamide, 7 mL 20xSSC (pH5.3) and 14 mL purified water, (pH 7.0-7.5)) at 70°C for 5 minutes. Following denaturation, the slides were dehydrated for 1 minute in a 70% ethanol solution, followed by 1 minute in an 85% ethanol solution, and 1 minute in a 100% ethanol solution. Then, the slides were dried by touching the bottom edge to a blotter and wiping the bottom edge with a paper towel. During this series of dehydrations, the probe mix prepared above was placed in a 73°C water bath for 5 minutes. Once the slide was thoroughly dried and the probe mix removed from the water bath and centrifuged briefly, 10 µL of the denatured probe mix was placed on the slide. Finally, the slide was placed in a sealed, humidified box and placed in a 37°C incubator for 48-72 hours for hybridization.

Post-wash and Microscopy

Following the 48-72 hour incubation, most slides were washed in a 0.4xSSC/0.3% NP-40 wash solution at 73°C for 2 minutes. Next, these slides were placed in a 2xSSC/0.1% NP-40 wash solution at room temperature for one minute (Vysis, CGH protocol). However, a few assays were done using slides that were washed using a method described by Kallioniemi et al. (1994). These washes involved a series of 3 washes in 50% formamide/2xSSC, pH 7 at 45°C for 10 minutes each. The formamide

washes were followed by two 10-minute washes in 2xSSC, one at 45°C and the other at room temperature. Finally, the slides were placed in distilled water for 10 minutes at room temperature.

After either type of wash, 10 µL of DAPI II counterstain and a coverslip were added to the hybridized area. Hybridized metaphases were then viewed and imaged using fluorescence microscopy and the *MacProbe* (PSI) imaging program. The following tables describe the assays performed and the variations in the above procedure attempted.

TABLE 2: Probes prepared in the lab by nick translation

Nick Translation	Sample	Enzyme Volume	Incubation Time	dUTP	Purification
1	Normal Female	10 µL	8 hours	SpectrumRed	-----
2	Normal Female	10 µL	8 hours	SpectrumGreen	-----
3	Normal Male	10 µL	16 hours	SpectrumRed	QIA
4	Normal Female	10 µL	16 hours	SpectrumGreen	QIA
5	Normal Male	5 µL	16 hours	SpectrumRed	EtOH
6	Normal Female	5 µL	16 hours	SpectrumGreen	EtOH
7	Normal Female	10 µL	16 hours	SpectrumGreen	EtOH
8	+13	5 µL	16 hours	SpectrumGreen	EtOH
9	+21	5 µL	12 hours	SpectrumGreen	-----
10	+13	5 µL	18 hours	SpectrumGreen	EtOH
11	+18	5 µL	12 hours	SpectrumGreen	EtOH

TABLE 3: Variations for co-precipitation, pretreatment, and post-wash. All assays used lab-prepared slides with the exception of Assay #13 which used Vysis slides. Incubation time was 72 hours for all assays except assay #10 which was done for 48 hours.

*TMG=Total Male Genome

**TFG=Total Female Genome

***(NT#) refers to the nick translation numbers indicated in Table 2

Assay #	Standard: SpectrumRed (NT#)***	Test: SpectrumGreen (NT#)	Pretreatment	Wash
1	Vysis TMG* 2X	-----	Yes	Vysis
2	Vysis TMG	Normal Female (NT#6)	Yes	Vysis
3	Normal Male (NT#5)	Normal Female (NT#6)	Yes	Vysis
4	Normal Male (NT#5) 2X	-----	Yes	Vysis
5	Normal Male (NT#5)	Normal Female (NT#7)	Yes	Vysis
6	Normal Male (NT#5) 2X	Normal Female (NT#7) 2X	Yes	Vysis
7	Vysis TMG	Normal Female (NT#7)	Yes	Vysis
8	Normal Male (#5) 2X	-----	Yes	Kallioniemi
9	Vysis TMG	+13 (NT#8)	No	Vysis
10	Vysis TMG	+21 (NT#10)	Yes	Vysis
11	Vysis TMG	+18 (NT#11)	Yes (no NP40)	Vysis
12	Vysis TMG	+18 (NT#11)	Yes (no NP40)	Kallioniemi
13	Vysis TMG	Vysis TFG**	Yes	Vysis

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Hybridization/Microscopy

Following nick translation of each DNA sample, DNA, consisting of SpectrumRed-labeled standard DNA, SpectrumGreen-labeled test DNA, and unlabeled Cot-1 DNA, was co-precipitated. The Cot-1 DNA prehybridizes to the repetitive sequences of the human genome. The various combinations for each co-precipitation are described in Table 3. Each DNA mix was denatured and hybridized to normal male metaphase spreads that had also been denatured. The DNA was allowed to hybridize to the spreads for approximately 72 hours; then the metaphase spreads were counterstained with DAPI II. Next, the slides were viewed using a fluorescence microscope. The results of hybridization and microscopy are described in Table 5. Microscopy for all assays showed expected results unless no hybridization occurred. For any chromosome that was known to be in excess in the test DNA (i.e., +13, +18, etc.), that particular chromosome appeared greener to the naked eye than the other chromosomes in the metaphase spread (Figure 2).

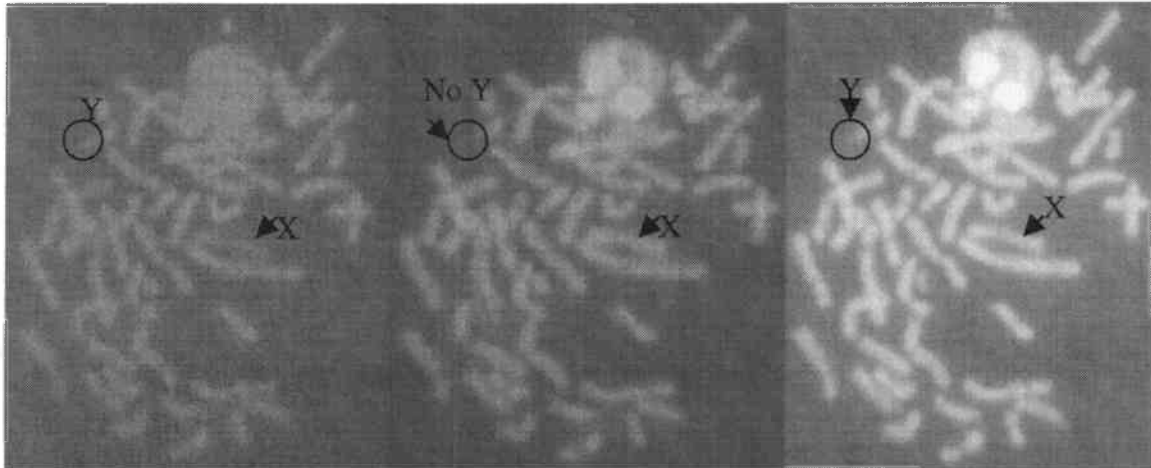


Figure 2: SpectrumRed, SpectrumGreen, and SpectrumRed/SpectrumRed/DAPI fluorescent images of one cell from Assay #2. The cell is a male cell (XY) while the test DNA is female so the SpectrumGreen-labeled female DNA does not hybridize to the Y chromosome while the SpectrumRed-labeled male DNA does. In addition, the X chromosome only hybridizes half as much SpectrumRed-labeled DNA (male) DNA as SpectrumGreen-labeled female DNA because the female test DNA contains two X chromosomes (XX).

Imaging/Computer Analysis

Following hybridization and microscopy, the metaphases were imaged using the Perceptive Scientific Instrument's (PSI) *MacProbe* fluorescence-imaging program. The banding pattern created by the DAPI II counterstain was used to identify each chromosome in the metaphase spreads. Then, the program assigned a red-to-green ratio for each chromosome of each cell. The ratios for one cell in assay number 2 can be seen in Figure 3 below. Following analysis of multiple cells, the program then averages the ratios for each chromosome. The average ratios and their standard deviations for all the cells analyzed in assay number 2 are shown in Figure 4 below.

Only assay numbers 2 and 13 demonstrated hybridization that gave computerized statistical results consistent with expected results. Both assays involved a normal male

(standard) to normal female (test) cross. The Y-chromosome shows an elevated red-to-green ratio indicating less than the diploid copy number of the Y chromosome in the test DNA (Figures 3 and 4). Because the test DNA was female for each of these assays and therefore contained no Y-chromosome, these results were expected. These assays also showed a diminished red-to-green ratio for the X-chromosome indicating excess X chromosome in the test DNA. These results were also expected because the test DNA contained one more X-chromosome than the standard DNA. Further experimentation with the computer program is expected to bring better results for other assays.

TABLE 5: Results of hybridization, microscopy, and imaging for each Comparative Genomic Hybridization assay. Descriptions for the hybridization of the standard DNA and the test DNA refer to appearance of the fluorescence markers and consistency of expression throughout the chromosomes. DAPI II descriptions refer to ability to assign chromosome numbers based on the banding pattern created by the DAPI II. Assay numbers refer to the numbers from Table 3.

Assay #	Standard DNA (SpectrumRed)	Test DNA (SpectrumGreen)	DAPI II
1	Very grainy; a great deal of nonspecific binding	-----	OK
2	Good hybridization	Good hybridization	OK
3	No hybridization	Good hybridization	OK
4	Mostly nonspecific binding	-----	Good
5	Grainy	Grainy	Good
6	Mostly nonspecific binding	Mostly nonspecific binding	OK
7	No hybridization	No hybridization	OK
8	Grainy; some nonspecific binding	-----	OK
9	Grainy; markers hybridized to different parts than green	Grainy, hybridized to different parts than red	OK
10	Very grainy; a great deal of nonspecific binding	Very grainy; a great deal of nonspecific binding	Bad
11	Very grainy	Mostly nonspecific binding	OK
12	No hybridization	No hybridization	OK
13	Good hybridization	Good hybridization	Good

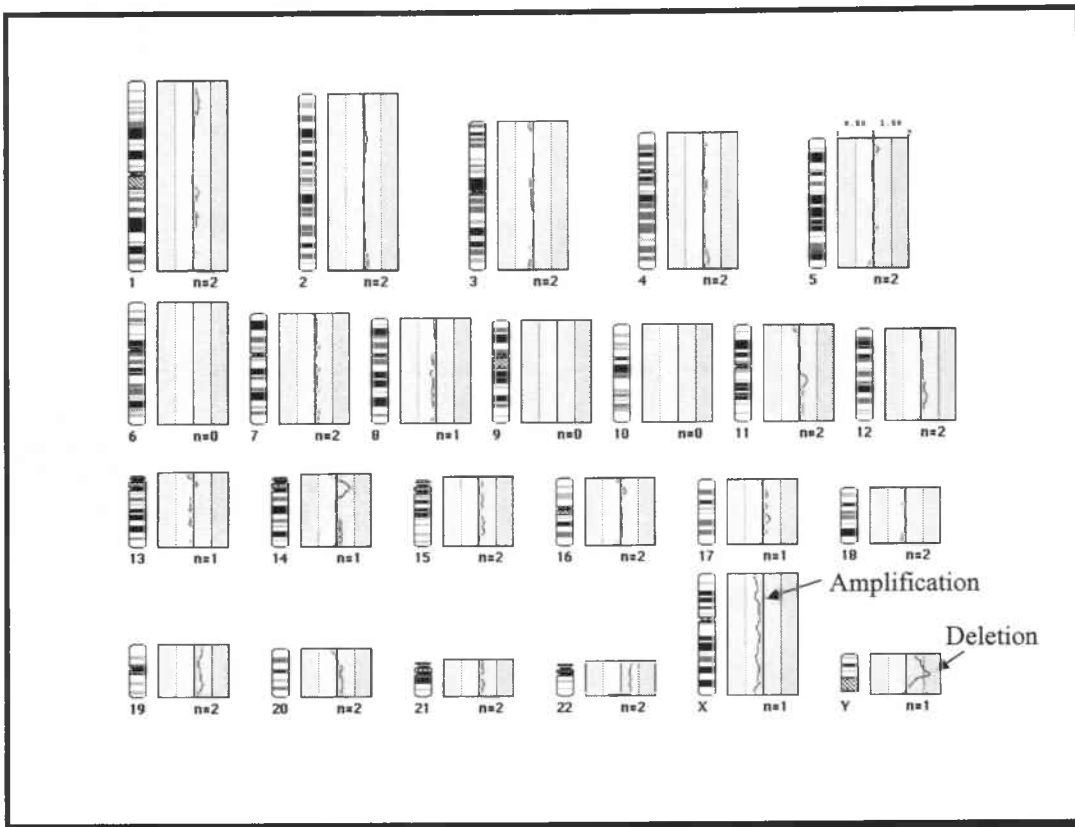


FIGURE 3: Results of computerized statistical analysis of one cell from Assay #2. The test DNA shows amplified X material, and deleted Y material as is expected in a normal female (test)-normal male (standard) cross. Chromosomes 6, 9, and 10 were not analyzed in this cell due to overlapping or insufficient DAPI staining.

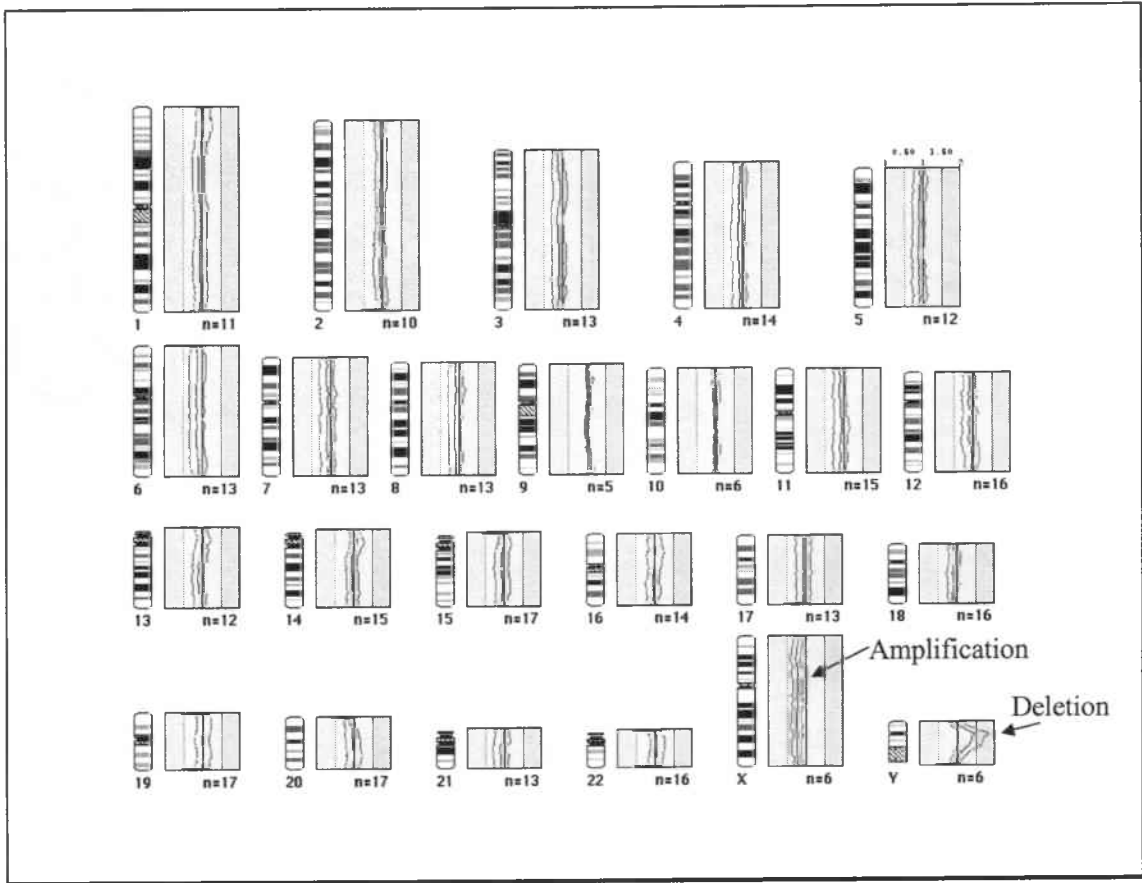


FIGURE 4: Results of the computerized statistical analysis of all the cells analyzed from Assay #2. The middle line represents the mean ratio of SpectrumRed/SpectrumGreen. The side lines represent the standard deviation of the ratio.

DISCUSSION

The goal of this study was to develop the CGH technique for clinical application in a genetics laboratory with respect to prenatal studies of aneuploidy. Because CGH techniques must be adapted in each lab, methods best suited for conditions in the laboratory at Shodair Hospital must be determined prior to incorporation of CGH into the cytogenetic analyses of the laboratory. My goal was to optimize this procedure and compare the results of CGH studies with the results of routine cytogenetic methods.

When this research was begun, the success of other labs in developing a protocol for Comparative Genomic Hybridization (CGH) provided a positive outlook for the eventual incorporation of CGH into the standard protocols at Shodair Hospital in Helena, Montana. Thus far, consistent success has not been achieved, but further optimization and eventual incorporation is anticipated.

Initially, nick translation proved to be the biggest obstacle. Because enzyme activity can not always be predicted, determining the quantity of enzyme and the length of time needed for nick translation to produce optimal DNA fragments proved to be difficult. The enzyme used for all nick translation reactions came from the same aliquot. Yet, even if nick translation was performed under the exact same conditions for two samples, it did not produce DNA fragments of the same length. For instance, at one point, test and reference DNA (from assay #3) were nick translated simultaneously, and DNA fragments for the two samples proved to be in quite different size ranges. In addition, when conditions were changed to produce suitable DNA fragments (i.e., longer time and more enzyme for shorter fragments) results were the opposite of what one would expect. From these unexpected results we can assume that despite a common

source and common conditions, enzyme activity can not be predicted with accuracy. Therefore, emphasis needs to be placed on the electrophoresis following nick translation. Most likely, it will never be possible to nick translate and assume DNA fragments are of optimal size even if identical conditions are maintained for each nick translation. However, with further experimentation a range of conditions that will give optimal size fragments could be developed.

The electrophoresis of nick-translated samples also proved to be challenging. Unfortunately the probe used to label the DNA is approximately the same molecular weight as the optimal-sized DNA fragments. Unincorporated probe in a nick-translated DNA sample masked the DNA smear on the gel following electrophoresis. Therefore, it was mandatory to purify the DNA to eliminate the unincorporated probe. The first method tested was QIA filtration. This method proved to be completely ineffective as no DNA was obtained following filtration. Ethanol precipitation, on the other hand, proved to be quite successful. The only drawback to the procedure was the lack of access to a refrigerated centrifuge. It is possible that room temperature centrifugation could cause some DNA to remain in solution due to the heat produced from friction in the centrifuge. The supercooling of the DNA solution was used to help eliminate this problem, and because a DNA pellet was always obtained, it is assumed that the method allows for very little loss of the sample. Once the DNA purification was added to the protocol, electrophoresis proved to be an accurate method to determine not only the size of the probes but also the probe ratios between the test and standard samples. This ratio was used to determine the relative amounts of standard and test DNA needed for co-precipitation.

Co-precipitation of the test and standard DNA was susceptible to the same problems as the ethanol precipitation used to purify the DNA samples for electrophoresis. Once again, a DNA pellet was always obtained, so DNA remaining in solution was assumed to be minimal.

Hybridization of the probe mix to the metaphase slides also proved to be an important factor in the production of good CGH results. As time went on, the hybridization lost quality. The loss of quality can most likely be attributed to aging of the slides. Presently, different storage techniques are being tested to slow the aging of the slides. One such storage technique is ethanol immersion which preserves slides for longer periods of time than simple freezer storage. Eventually, when a protocol is developed, each batch of slides will be tested for hybridization quality prior to being used for future CGH analysis. Many other factors have contributed to poor hybridization. Pretreatment of the slides is often done with a solution that contains a detergent. After several hybridizations showed excessive non-specific binding, the detergent was removed from the pretreatment solution. However, the removal of the detergent proved to be ineffective in eliminating non-specific binding. The use of the Kallioniemi et al. (1994) post-wash, which does not have a detergent while the Vysis post-wash does, was also ineffective in eliminating non-specific binding. The source of the non-specific binding is yet to be determined.

In addition to excessive non-specific binding, the chromosomes showed considerable granularity. Other researchers have found that probe size is the chief contributor to granularity, and presently nick translation is being varied to produce variable probe size. The aging of slides could also contribute to the granularity as

chromosomal DNA becomes harder to manipulate with age. It is hoped that the new storage conditions will help eliminate granularity.

Finally, with regard to hybridization, it was found that often only one probe will hybridize to the metaphases. The source of this problem is unknown, but it may be related to co-precipitation. If one of the DNA samples does not pellet, it will not be represented in the mixed probe and therefore can not hybridize to the metaphase. Other labs co-precipitate overnight, so this method is an option if hybridization of one probe continues to be limited. Testing the DNA solutions post nick translation and ethanol precipitation for the presence of probe is also an option.

The last factor that has contributed to CGH quality is imaging. The CGH portion of the *MacProbe* program (Perceptive Scientific Instruments) is not especially user friendly and the manual does not provide many useful suggestions. Presently, I have received information from other labs using this imaging system for CGH and imaging techniques are being fine-tuned. The most difficult factor that needs to be considered in order to achieve quality images is capture time. However, careful examination of the histograms for each probe color that the imaging program produces has helped other labs determine optimal capture time. This method has not been overly successful for me. Perhaps my lack of success in using histogram analysis to determine capture time is due to poor hybridization quality, and it is hoped that both problems will be alleviated simultaneously.

Overall, the results of this research thus far have been mildly discouraging, but the problems are identifiable, and I am optimistic that they are solvable because other labs have successfully overcome similar problems. Most importantly, some success has

been achieved and the development of a working protocol appears to be within reach. Thus, the prenatal detection of aneuploidy for patients at Shodair is on the verge of advancement. When CGH is incorporated, rapid aneuploidy assessment for the entire genome will be achievable and the goals of this study will have been reached.

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