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Developing and Optimizing Conditions for Single Cell Genetic Analysis

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DEVELOPING AND OPTIMIZING CONDITIONS FOR SINGLE CELL GENETIC ANALYSIS

by

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ABSTRACT

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Preimplantation Genetic Diagnosis (PGD) allows for couples to obtain genetic information at the embryo stage, therefore preventing lethal genetic diseases in their offspring. A recent multi-center study determined a 17% amplification failure rate overall and a 3.4% misdiagnosis rate (ESHRE PGD consortium steering committee, 2002). Experiments presented here were conducted to decrease those rates by optimizing single cell sensitivity and specificity of PGD techniques. Primer extension preamplification (PEP) was used after initial amplification failure, followed by gene specific PCR. Reamplification experiments yielded 54% amplification rates using blastomeres that previously failed amplification. In the clinical trial, four of six blastomeres were diagnosed that had previous amplification failure. PEP and subsequent PCR testing is a useful means to diagnose samples that previously failed amplification. Different lysis buffers/techniques were used to determine the amplification efficiencies of heterozygous single cells (n=100/group). Amplification rates were 38% using Liquid Nitrogen, 97% using Potassium hydroxide/dithiothreitol (KOH), 41% by boiling, and 85% for water. Allele drop-out was minimal in the KOH group, but was high in the water group. KOH lysis is superior in both sensitivity and specificity to other lysis methods tested. Nuclear and cytoplasmic morphology of

biopsied blastomeres was evaluated and correlated with subsequent PCR amplification efficiencies. Cells with a visible nucleus had 100% amplification versus 36% in cells that did not, regardless of the cytoplasmic integrity. Nuclear evaluation studies demonstrate the importance of determining nuclear integrity of biopsied cells. Multiple displacement amplification (MDA) was used to generate sufficient DNA for eventual use in array comparative genomic hybridization (CGH) to determine chromosome complement. Sporadic MDA results occurred at the single cell level; larger cell quantities consistently amplified. MDA can generate sufficient quantities of DNA for array CGH. Preliminary results show promise; further optimization will be required to ascertain the use of array CGH. These studies provide optimization of single cell sensitivity/specificity and lay the foundation for further studies in single cell diagnosis. It is anticipated that an entire array of gene defects and chromosomal conditions can be diagnosed by PGD in the future prevention of severe human disease.

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INTRODUCTION

Preimplantation genetic diagnosis (PGD) is an accepted option for couples at risk of passing inherited diseases on to their offspring. The ability to analyze specific genetic information from a single cell removed from the developing embryo now allows many at-risk parents to decide the clinical outcome of the embryos that they have produced through the in vitro fertilization process. In such cases, only those embryos shown not to have the genetic pre-disposition for the studied condition are transferred to the uterus in the hope of initiating a pregnancy.

Prior to the advent of PGD, couples had several options to minimize the risk of having an affected child: not initiating a pregnancy, using donor gametes, or adoption. However, none of these options result in a biological child, which is fundamentally important for some people. The alternative for many at-risk couples is to initiate a pregnancy naturally and elected to have prenatal testing. This implies that these couples might be faced with the decision of whether to continue or terminate the pregnancy if tests indicated that their child could be born with a devastating genetic disease. For other couples, pregnancy termination is not a morally acceptable option, even if the likelihood of a medical condition is involved.

Those couples that accepted termination as an option were confronted with other factors when trying to conceive again. It became difficult for many to bond with the subsequent pregnancy during those initial prenatal months before chorionic villus sampling or amniocentesis could determine the genetic or chromosomal status of the fetus. This left them with many psychological issues to deal with: not knowing if their child would be healthy so that the pregnancy continued to term, not sharing the joy of pregnancy with family and friends until after prenatal testing, wondering how many times would they gamble with the roll of the genetic dice, even when the odds were in their favor (Pergament, 1991). For these couples, PGD offers a safe option to have genetic information about their offspring earlier in the process, prior to establishing a pregnancy.

The journal, Human Reproduction, was used as a model for this dissertation.

Hundreds of healthy children have now been born as a result of PGD (Verlinsky *et al.*, 2004). Beginning in 2004, the American Medical Association's CPT (current procedural terminology) codebook lists procedural coding for the PGD process. Having CPT codes listed generally sets the standard that a procedure is no longer considered experimental in nature. That being said, multiple aspects of the PGD process continue to be evaluated for safety and efficacy.

Counseling and the informed consent process for patients undergoing PGD focuses on the following aspects of the procedure:

1) The safety of the embryo biopsy procedure. This concern has been previously addressed by other studies both in our institution and elsewhere (Takeuchi *et al.*, 1992; Hardy and Handyside, 1992; ESHRE PGD Consortium Steering Committee, 2002). Those studies support the findings that the embryo survives the biopsy procedure over 98% of the time and that embryo biopsy does not harm the further cleavage of the embryo in the majority of cases.

2) The sensitivity of single cell analysis. This includes discussion on PCR amplification rates; there is a chance of an incorrect diagnosis or a chance of no amplification, and thus, no diagnosis.

3) The specificity of single cell analysis. This includes discussion relating to the risk of allele dropout and contamination of the results by extraneous DNA that could interfere with the ability to obtain the correct genetic answer.

The latter two aspects are the main focuses of the studies undertaken here. In addition, studies were initiated to examine the application of the emerging technologies of microarray analysis for PGD. In the future, these technologies could provide more couples the opportunity to have a healthy child.

SECTION 2
BACKGROUND

The ability to perform PGD emerged as biotechnology advanced in the later part of the 1980's and into the 1990's. The two main components of PGD, embryo biopsy and genetic analysis, are explained further below.

2.1 Embryo Biopsy

Micromanipulation and embryo biopsy were first reported using laboratory animals (Nicholas and Hall, 1942; Tarkowski, 1959, Tarkowski and Wroblewska, 1967). These investigators studied early embryo development after removal of one or more blastomeres. The technique involved the use of a dissecting microscope to visualize the manipulated cell that was being held in place by suction applied to a mouth pipette. Two glass slides were placed at right angles to provide resistance; a glass needle was attached to those pieces and used to pierce the zona pellucida. These methodologies seem primitive when compared to today's technologies, which are listed in the Methods section of this dissertation.

There are three stages at which biopsy for PGD can be performed (De Vos and Van Steirtegham, 2001):

1) Polar Body Biopsy. The first polar body (PBI) of the unfertilized oocyte or the first and second polar body (PBII) of the newly fertilized oocyte is one option for PGD biopsy.

2) Cleavage Stage Embryo Biopsy. A blastomere can be removed from the cleavage stage embryo (generally at 6-8 cells 3 days after oocyte retrieval) as another option for PGD.

3) Trophectoderm Biopsy. Cells from the trophectoderm (TE) layer of the blastocyst (five days after oocyte retrieval) are biopsied as another option for PGD.

1) Polar Body Biopsy

Initially, polar body (PB) biopsy of the unfertilized oocyte appeared to be favorable for couples that wanted to circumvent the ethical issues of biopsying an

embryo (Verlinsky and Kuliev, 1992). Some couples may feel that life begins at the moment of fertilization and could have difficulty discarding the fertilized embryo, even if affected; these same couples may not feel uncomfortable with discarding a potentially affected *unfertilized* egg. However, the moral issue of testing the *unfertilized* oocyte no longer held true when it was realized that the oocyte should be inseminated before the PGD result was available in order to avoid in vitro aging of the oocyte. In addition, many centers that perform PBI biopsy now sample the PBII as well. The rationale for testing the PBII was to improve the accuracy of the genetic diagnosis. The moral objection was then not circumvented since the oocyte was inseminated and fertilization would have occurred at the time of testing (Gitlin *et al*, 2003).

The polar bodies are extracellular material and appear to have no function in embryo development. Removal of the PBI does not appear to interfere with fertilization or subsequent cleavage (Verlinsky and Kuliev, 1992). Lastly, an advantage of PB biopsy is the ability to obtain the genetic information earlier in the IVF culture process (Wells *et al.*, 2002). Newer technologies such as comparative genomic hybridization (CGH) or DNA microarrays require a whole genome amplification (WGA) step in order to have sufficient quantities of DNA template for testing. Most WGA methods take 8-10 hours to perform, in addition to the time required for the CGH or microarray process. The testing window from PB sampling to blastocyst is 4-6 days as compared to 2-3 days when biopsying a 6-8 cell embryo, thus allowing more time for these other techniques. The timing is important for clinical applications when the embryo must be transferred to the uterus within the day 5-6 (after oocyte retrieval) window of implantation (Bergh and Navot, 1992).

The major limitation with PB biopsy is that the genetic material of the PBI and PBII only reflects the maternal contribution to the subsequent embryo; the paternal genetic contribution remains unknown (De Vos and Van Steirteghem, 2001). PB biopsy was initially used for screening the maternal contribution to single gene defects. PB biopsy cannot be used for gender selection in the case of X-linked diseases, conditions that constitute a paternally-inherited dominant

disease, or for the diagnosis of conditions where only the paternal mutation is known. Embryos resulting from oocytes that were diagnosed with an affected female allele would not be transferred, thus reducing the number of embryos eligible for transfer. In most autosomal recessive diseases, oocytes reported to have the mutation still have a 50% chance of resulting in a phenotypically normal embryo, depending upon the subsequent paternal contribution (Gitlin *et al.*, 2003). For most patients undergoing a PGD cycle, the more embryos available for transfer results in a higher chance of conceiving (Vandervost *et al.*, 1998).

Eighty-five percent of chromosomal abnormalities are attributed to maternal origin (Hassold and Chiu, 1985). Therefore, PB biopsy with subsequent PGD has the potential to detect the vast majority of chromosomal errors. However, it is interesting to note that in 75% of Turner syndrome patients (XO), the lone X is *maternal* in origin (Hassold *et al.,* 1988). Thus, even though the overall majority of aneuploidy causes are attributed to maternal nondisjunction, the majority of XO cases are from sperm missing the sex chromosome by either paternal meiotic non-disjunction or anaphase lag. This demonstrates another disadvantage of polar body testing in its ability to detect the majority of sex chromosome aneuploidies (Gitlin *et al.*, 2003).

One of the most comprehensive reports of PB testing for single gene defects was described by Rechitsky *et al.* (2001). Over 1000 oocytes were tested for single gene defects and linked polymorphic markers by using PCR. Two hundred thirty seven unaffected oocytes were identified for potential transfer in 114 couples resulting in 34 pregnancies and the birth of 23 healthy children with no misdiagnoses. These studies demonstrate the successful application of PB testing for at-risk couples to have healthy children.

2) Cleavage Stage Biopsy (Figure 1)

The majority of embryos biopsied for PGD are at the cleavage stage, generally consisting of six to eight cells after three days of embryo culture (ESHRE PGD consortium steering committee, 2002). The biopsy of embryos consisting of less than six cells appears to decrease the developmental potential of the embryo in several animal model studies. Krzyminska *et al.* (1990)

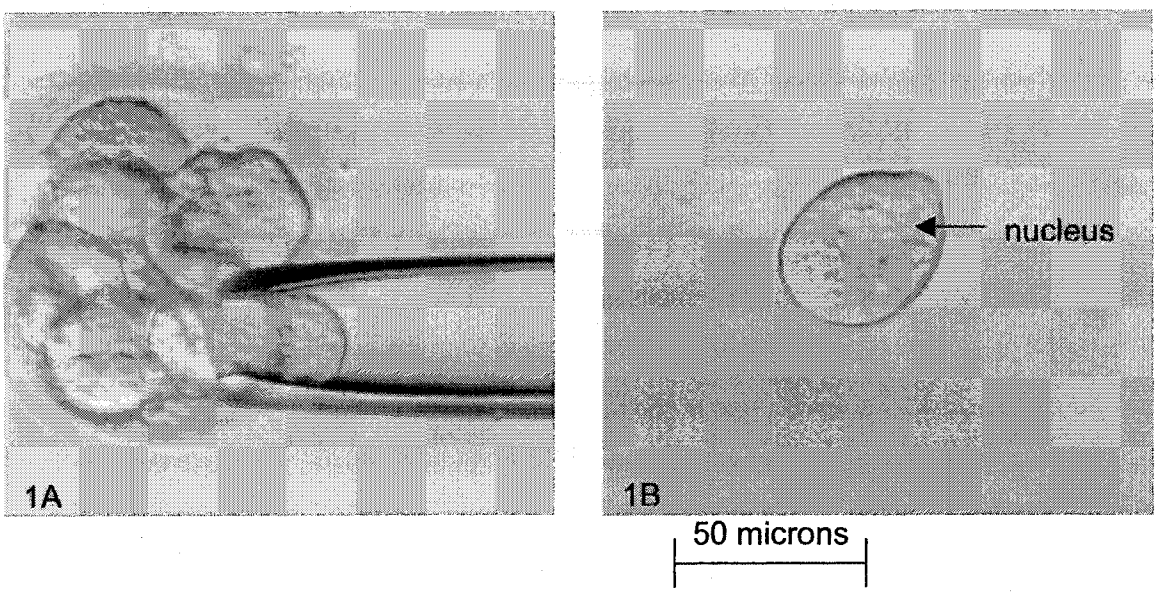

Figure 1. Cleavage stage embryo biopsy. Panel 1A demonstrates the biopsy of a cleavage stage embryo with at least 7 cells visible. The aspiration pipette to the right has an inner diameter of 40 microns. The biopsied blastomere is shown after removal from the embryo (Panel 1B). Note the visible nucleus in the center of the cell.

biopsied mouse embryos at the four-cell, eight-cell, and morula stage. Embryo biopsy at the eight-cell stage did not reduce the blastulation rate (95% versus 99% for controls), nor the implantation rate (biopsy 82% versus 87% for controls). However, at the four-cell stage, the subsequent blastulation rate fell to 76% and the implantation rate decreased to 44%. The authors concluded that embryo biopsy at the 8-cell or greater appeared to be more successful by not affecting the subsequent blastulation and implantation rates.

Since embryo biopsy involves the random selection and removal of one to two cells, totipotency and polarity of blastomeres are important considerations to study. At this early stage, the embryo is still totipotent, that is, each individual cell (blastomere) has the theoretical potential to develop into a healthy offspring (Ziomek *et al.*, 1982). This hypothesis was tested experimentally when Rossant (1976) separated each blastomere from mouse four to eight cell embryos. Each individual cell was inserted into an empty zona and returned to the oviduct. Embryonic development arrested at 5.5 days; the authors suggested that this was due to a reduction in the total number of cells at the time of embryo cavitation. One study provided evidence of the totipotency at an earlier stage in 4-cell cattle embryos (Johnson *et al.*, 1995). Individual blastomeres were isolated and inserted into emptied zona pellucida, then cultured to the blastocyst stage. Two embryos each were transferred into recipient cows, resulting in the birth of two sets of identical twin calves, all genetically from the same 4-cell embryo. Although this study demonstrated the early totipotency of bovine embryos, no additional studies in the literature have been reported.

To determine the existence of polarity in blastomeres in early human cleavage stage embryos, Mottla *et al.* (1995) injected a fluorescent lineage tracer (Texas Red-lysine-dextran) into individual blastomeres of human two- to eight-cell embryos. The embryos were then cultured to the blastocyst stage at which time they were fixed and examined using confocal microscopy to identify the location of the tracer. In successfully injected embryos that developed to expanded blastocysts, randomly injected blastomeres formed both TE and an inner cell mass (ICM); however, more labeled cells were found in TE than in ICM.

These results suggested that early blastomeres are not yet committed to specifically become either TE or ICM.

An interesting study by Gueth-Hallonet and Maro (1992) involving eight-cell mouse embryos demonstrated that blastomere organization changing from symmetrical to polarized led to the two different cell lineages – one giving rise to the TE (generally the cells on the perimeter) and the other giving rise to the ICM (generally the cells on the inside of the embryo that do not border the inside of the zona pellucida). However, blastomeres may behave differently in an intact versus a disrupted embryo. The cell fate may be determined by positioning of the cell in the embryo; however, it is not irreversibly fixed. The researchers found that the inside cells which do not normally contribute to TE could become totipotent if the integrity of the embryo was disturbed. This provided the embryo with a degree of positional flexibility that may play an important role to compensate for damage or loss of blastomeres. In a different study (Ciemerych *et al.*, 2000), 8-cell mouse embryos were injected with a fluorescent protein and similarly, cells were traced through blastocyst development. Their findings concurred with the results of Gueth-Hallonet and Maro (1992) that polarity exists, but is not irreversibly established.

Other investigators have studied polarity as well (Edwards and Beard, 1997). However, the majority of the research was conducted using animal models such as the mouse and rabbit while little work exists with human embryos. Due to difference in the timing of embryonic genome activation (post first cleavage in the mouse compared with post third cleavage in the human, Braude *et al.*, 1988, Heikinheimo *et al.*, 1995), it is difficult to discern if evidence of polarity by the second cleavage stage can be extrapolated to the human. One such study provides evidence of early polarity by the measurement and tracing of two proteins, leptin and Stat3, throughout the oocyte and cleavage to the blastocyst stage in both mice and human (Antczak and Van Blerkom, 1997). Higher concentrations of both proteins were seen in the outer most cells of the embryo and in the TE as opposed to the ICM. Presence of these proteins in

intact ovarian follicles suggest that these proteins may be maternally derived and have a role in embryo development and the establishment of polarity.

The viability of human cleavage stage embryos after biopsy was assessed in several different studies. Hardy *et al.* (1990) examined the effects of the removal of 1, 2, or 3 cells from an eight-cell human embryo. Additionally, nuclei from the ICM and TE were differentially labeled and counted to assess the allocation of cell number. Their studies concluded that removal of two of the eight cells did not affect subsequent blastocyst development. The ratio of ICM:TE cells also was not altered even though there was a reduction in the actual total numbers of cells. Uptake of pyruvate and glucose were also measured in droplets of spent culture medium to assess metabolism in the aforementioned studies (pyruvate is an end-product of metabolism in glycolysis). Even though uptake was reduced in 7/8 embryos and 6/8 biopsied embryos (i.e., 7 of 8 cells and 6 of 8 cells remaining when one and two cells were removed, respectively), this was interpreted to be due to the reduction of cellular mass resulting from the decreased cell number (Hardy and Handyside, 1992). Development to the blastocyst stage did not appear to be adversely affected by the cell removal. Tarin *et al.* (1992) conducted a similar study, removing one quarter of the cells from human four to eight-cell embryos. Pyruvate uptake and cell number were measured at the blastocyst stage. The authors concluded that there was no significant difference in the number of embryos that reached the blastocyst stage as compared to non-biopsied controls. As expected, the total number of cells and the pyruvate uptake were markedly reduced.

From these studies, it is concluded that cleavage stage embryo biopsy can be performed without adversely affecting subsequent embryo development.

3) Trophectoderm biopsy

TE biopsy is another option for embryo biopsy (although rarely used in PGD). Initial work in animal models demonstrated that biopsied blastocysts could implant and lead to pregnancies (sheep, Rowson and Moore, 1966; rabbit, Gardner and Edwards, 1968; mice, Gardner and Johnson, 1972; Monk *et al.*, 1988; cattle Betteridge *et al.*, 1981; marmosets Summers *et al.*, 1988). The

greatest advantages of biopsying at the blastocyst stage are that more cells are available, and that the TE is extraembryonic material (Dokras *et al.*, 1990). TE biopsy may also circumvent ethical issues that a couple may have as only extraembryonic cells are being removed, and the ICM cells that become the fetus remain intact.

There is limited knowledge on how the TE represents the genetic constituency of the remaining embryo cells. There is evidence to suggest that the TE may contain cells that are tetraploid or mosaic from the inner cell mass (it is known that there are multinucleated trophectoderm cells); the genetic make-up of these cells might not reflect the true genetic constituency of the eventual fetus (Everett and West, 1996).

One of the major disadvantages of blastocyst biopsy relates to the timing of the procedure. Blastocyst formation occurs approximately five days after fertilization. Although there appears to be no difference in pregnancy rates in studies of day 5 versus day 6 embryo transfers (de los Santos *et al.*, 2003), there are logistical issues that must be overcome: will the PGD results be available by day 6? If the blastocysts hatch, are they more difficult to transfer? Cryopreservation of the embryos could be considered as an option although there are limited studies with cryopreserved biopsied blastocysts, and the subsequent success rates of survival and implantation are also not known. One anecdotal report described an 80% survival rate with 46 frozen morula/blastocysts; 35 embryos were transferred and 10 implanted for an implantation rate of 28.6% (L Veeck-Gosden, personal communication). Many centers have reported only limited survival at the time of thawing of cryopreserved cleavage stage biopsied embryos (9%, Magli *et al.*, 1999; 15%, Joris *et al.*, 1999). Jericho *et al.* (2003) modified their standard propanediol cryopreservation protocol by elevating sucrose (0.2M) and serum concentrations (20% volume:volume), resulting in a 75% survival rate in 185 frozen/thawed cryopreserved embryos. These results demonstrate promising use of cryopreservation of biopsied embryos in PGD cycles that may allow for greater

flexibility of timing of the embryo transfer during the 5-6 day window of implantation.

2.2 Genetic Analysis

Edwards and Gardner (1967) were among the first to report studies attempting to determine the genetics in a preimplantation embryo. These researchers determined the sex of rabbit embryos by identifying the sex chromatin (Barr body) of the TE from an intact blastocyst. The Barr body, found when more than one X chromosome is present, is the highly condensed chromatin of the inactive X chromosome. The following year, these researchers described embryo biopsy and the ability to excise small pieces of the trophectoderm from a rabbit blastocyst (Gardner and Edwards, 1968). Under the microscope, suction was applied to a micropipette holding the blastocyst in place. A second pipette was placed directly opposite the holding pipette, and suction was applied. This caused a small amount of trophectoderm to be isolated; a microscissors was then used to excise the extruding trophectoderm mass from the blastocyst. The biopsied material was subsequently analyzed by acridine orange staining (a DNA stain) under fluorescent microscopy to determine the presence of sex chromatin. The presence of sex chromatin in a high proportion of trophoblast nuclei is indicative of a female. In those initial studies, 77 embryos were transferred to recipient female does. Only 3 of 77 implanted; the only assessment at day 13 gestation that was given was that the fetuses appeared normal, and no sex results were listed. The low implantation rates were attributed to culture conditions. Embryos were obtained by flushing the blastocysts from the uterus, excising a portion of trophectoderm cells, and holding the embryos in serum for 1-2 hours while the tests were performed. After optimizing the culture conditions, 109 blastocysts were transferred to recipient does, of which 80 (73%) implanted. From those, 18 offspring were born. All 18 (100%) were correctly sexed at the blastocyst stage. Shortly after, changes were made in culture media and culture conditions (including allowing time for the blastocysts to re-expand) that demonstrated improved implantation rates (Edwards, 1969). Despite the low rates of live

offspring, this initial animal work laid the foundation for further animal studies along with human trials almost twenty years later (Monk *et al.*, 1987).

2.2.1 The Polymerase Chain Reaction

PCR revolutionized biotechnology. The discovery of the PCR technique (Mullis *et al.*, 1986; Saiki *et al.*, 1988) allowed for millions of copies of specific genetic information to be replicated from a DNA template. By denaturing the double-stranded DNA, adding specific synthetic oligonucleotide primers bracketing the gene locus of interest, and extending new strands of DNA using a thermostable polymerase, millions of copies of the desired sequence can be amplified.

The story is well documented of Kary Mullis's late night drive into the California mountains where the revelation of the PCR process came to him (Mullis, 1990). During the three-hour drive, he analyzed and reanalyzed each step of the process where he proposed to synthesize millions of copies of DNA from a single template. At one point, he stopped on the roadside, drawing out DNA molecules, the strands denaturing, the primers hybridization, and the new strand extension with DNA polymerase, an enzyme that had been discovered twenty years previously (Kornberg *et al.*, 1964). The next several months after that trip led him on the scientific journey, first searching the literature that revealed no one else had published his idea. Months of laboratory preparation, trial and error with buffer concentrations, different temperatures, all the optimization steps discussed below, followed. Finally, Mullis selected what he called his 'favorite kind of experiment', one that was run in a single tube and involved a yes/no answer. The answer was yes. Yes, segments of DNA could be amplified to produce millions of copies.

The story continues as Science magazine awarded its Molecule of the Year in 1989 to the technique of PCR (Guyer and Koshland, 1989). Mullis received the Nobel Prize in Chemistry in 1993 for developing the polymerase chain reaction. By Mullis's own admission (Mullis, 1990), one of the most crucial keys to success with PCR was the introduction of the thermostable polymerase known as *Taq* (Saiki *et al.*, 1988). The discovery of *Thermus aquaticus* (*Taq*), a thermostable bacteria from which the polymerase is derived was made by Thomas Brock and Hudson

Freeze (Brock and Freeze, 1969), two names that are not as recognizable nor given as much credit as that of Mullis, but who certainly made a significant contribution, nonetheless.

Initially, Brock had the idea to study bacteria in hot springs since at the time, thermophilic bacteria were thought to have an upper temperature limit of 55°C with the upper limit for life at 63°C (Kempner, 1963). His pursuits involved a study of photosynthesis; the hot springs provided a 'steady state' ecosystem that led him to Yellowstone National Park. Due to its altitude, the boiling temperature of the springs is actually 92°C. Although he did find an upper limit of photosynthetic life at 70-73°C, he also found bacteria living at higher temperatures. However, it was difficult to cultivate organisms from these higher temperatures. His focus turned to a spring with the temperature of 73°C. There, *T. aquaticus,* among other bacteria was isolated. Soon after, it was discovered that artificial hot-water systems also housed bacteria. Indeed, bacteria can survive boiling water temperatures (Brock, 1995).

The original name for bacterium *Taq* had been *Caldobacter trichogenes* after an extensive literature search of thermophilic bacterium (Brock, 1995). *Caldo* is Italian for hot, *tricho* is the Greek for filaments, which the bacterium formed under certain conditions. The name was changed to *Thermus Balnearius* (*heat + thermal bath*), before Brock decided upon *Thermus aquaticus* referring to the water (aquaticus) where the bacterium grew. Brock mused that scientists might not have known the enzyme as *Taq* polymerase but rather *Cat* (caldobacter trichogenes) polymerase instead.

David Gelfand of Cetus Corporation presented the idea of using the thermophilic bacterium as the source for DNA polymerase (Saiki *et al.*,1988; Innis, 1988). Similarly, researchers in Cincinnati described a thermostable polymerase from *T. aquaticus* (Chien *et al.*, 1976). Initially, the experiments designed by Mullis used the Klenow fragment *of Escherichia coli* DNA polymerase I as the enzyme to catalyze the extension step of the process. However, fresh enzyme had to be added to each tube after each denaturation step of each cycle since the *E. Coli* polymerase was not heat stable at the high denaturation temperatures. Also,

E.Coli polymerases did not appear to synthesize the entire gene segment desired (Wickner *et al.*, 1972). At that time, the researchers suggested that the temperature range provided a possibility that *Taq* polymerase could be used for gene synthesis. The half-life for *Taq* polymerase at 92.5°C is 130 minutes, 40 minutes at 95°C, and 5-6 minutes at 97.5°C (Saiki *et al.*, 1988). The thermo-stable *Taq* could be added at the beginning and did not need to be replenished as the *E.coli* polymerase had.

The substitution of *Taq* not only simplified the process, it also demonstrated improved sensitivity, specificity and increased yields (Suzuki *et al.*, 1972; Taylor *et al.*, 1972; Verma *et al.*, 1972). This was partially due to the fact that the Klenow fragment was incubated at 37°C while the *Taq* is incubated around 70°C; the increased temperatures increase the specificity. In the initial work by Saiki *et al.* (1988) comparing the two polymerases, samples with the Klenow fragment showed non-specific DNA products, assumed to be due to the decreased stringency at the lower temperature. At the higher temperature of *Taq*, the non-specific priming templates would dissociate, leaving only the complementary primer stands annealed. The higher temperature threshold of the *Taq* made it ideal since it may have been possible to melt the secondary and tertiary structure of the template at those higher temperatures. The increased yield was observed due to the increased efficiency of the *Taq* polymerase.

A disadvantage of the use of *Taq* is the misincorporation rate (the rate at which the wrong nucleoside is inserted into the DNA strand extension). For *Taq*, the misincorporation rates were calculated to be once every 2 $x10^4$ bases while for the Klenow fragment the rate was one in every 8 x 10^5 bases. However, this difference was overcome by analyzing many different aliquots of the same sample and coming to a consensus of the resulting sequence.

There are three basic steps to PCR (Figure 2):

1) The denaturation of the template DNA

2) The annealing of the oligonucleotide primers

3) The extension of the new strands aided by the polymerase and dNTPs. The strand extension involves the coupling of a dNTP to the free 3'OH group at the end of the template. (Tindall and Kunkel, 1988).

1) Denaturation

Prior to the cycling, samples can be heated to 100°C to remove impurities in the DNA sample and proteases (Rolfs *et al.*, 1992). For thermocycling conditions, one cycle of initial denaturation for three minutes at 95°C is performed to separate the double-stranded template. Slightly higher temperatures may be need for G-C rich regions due to the stronger triple bonds of the G-C. If the denaturation temperature is too low, the partially denatured strand will snap back together when slightly cooled and thus the primers could not anneal. Subsequent cycles require less time for denaturation since the template never completely renatures under themocycling conditions.

2) Primer annealing

The second step of the PCR is the annealing of the complementary primers. In the initial cycles of the PCR, the primers act as 'scavengers', performing a genomic screening to find their complementary base sequences. The probability of the primers annealing is determined by several conditions of primer design: annealing temperature, time of reaction, and product concentration (Rolfs *et al.*, 1992). Annealing time and temperatures are discussed here while the primer design and concentration is discussed in the section concerning the components of the PCR.

Annealing time

Annealing time is generally between 20-40 seconds. Longer annealing times are needed for low copy initial templates to allow time for the primers to find their target.

Annealing Temperature

Higher annealing temperatures increase the stringency of the reaction (likelihood of specific hybridization) by minimizing the priming events due to DNA secondary structures such as hairpin loops. However, annealing temperatures

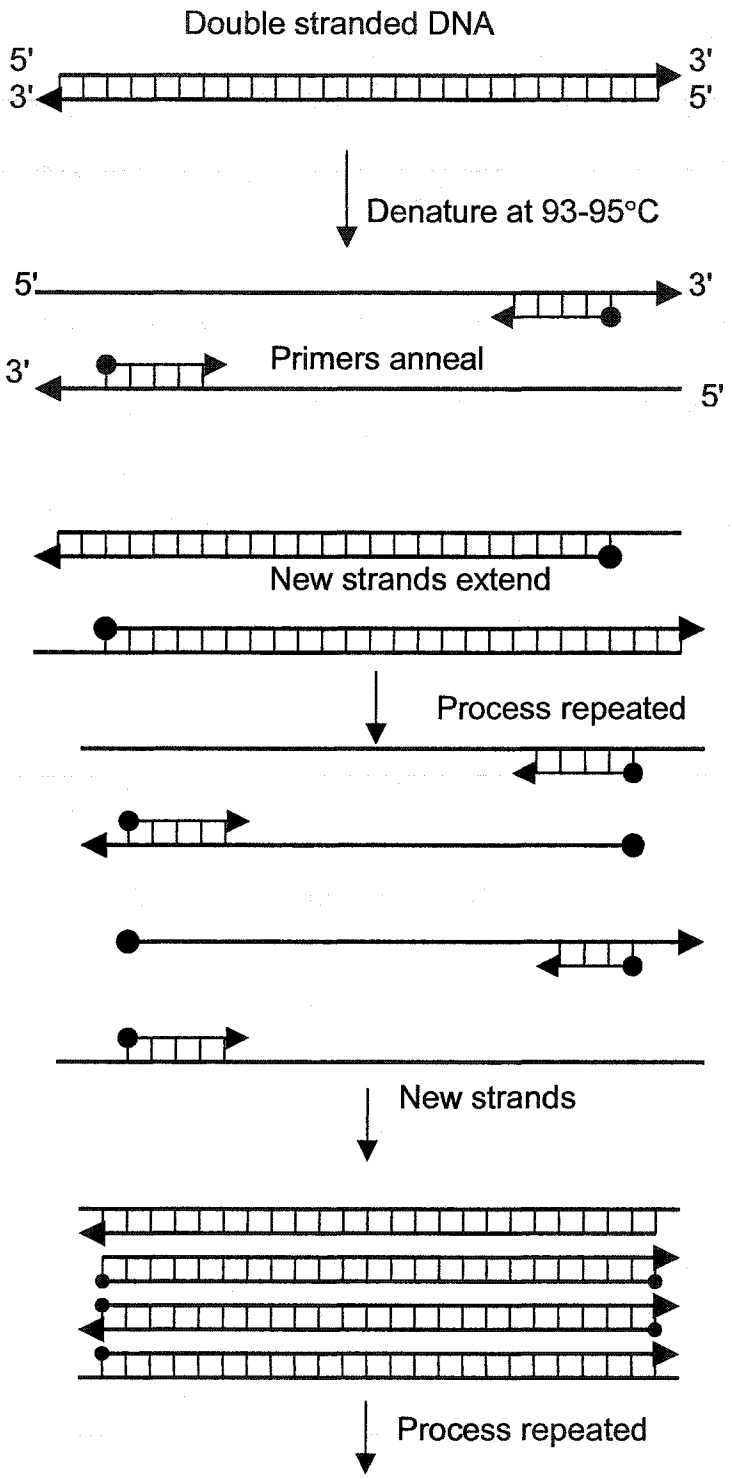

Figure 2. Polymerase Chain Reaction. Double Stranded DNA is denatured. The primers anneal to their complementary sequences. *Taq* polymerase binds and extends the new strand of DNA in the 5'→ 3' direction. The two new strands of DNA will denature and go through the cycle again.

that are too high will reduce the chance of annealing occurring (Innis *et al.*, 1990). Decreasing the annealing temperature lowers the stringency of the reaction, but may also increase the likelihood of amplification occurring. The stringency is decreased because the primers may bind to partial complementarity not involving the 3' end. The highest annealing temperature possible should be used to provide the optimum results of sensitivity and specificity.

There are several methods used to calculate the annealing temperature as described below. The general rule is to start with an annealing temperature that is 5°C lower than the calculated melting temperature, T_m, of the oligonucleotide strand (McPherson *et al.*, 1991). The T_m is defined as the temperature at which 50% of the oligonucleotide is annealed to its complementary DNA strand. There are several formulas to calculate the melting temperature.

The T_m calculation by the nearest-neighbor method is based on similarity of a random object (temperature) to the object studied (Equation 1; Rychlik *et al.*, 1990).

$$T_m = \frac{\Delta H}{(A + \Delta S) + R\ln(Ct/4)} \quad 273.15 + 16.6 \log [salt] \qquad (1)$$

Where:

- ΔH (cal mole-1) is the sum of the nearest-neighbor enthalpy changes for hybrid formation (<0).
- A (cal K-1mole-1) is a constant for helix initiation which is equal to -10.8 cal K-1mole-1 for nonself-complementary sequences and = -12.4 for self-complementary sequences).
- ΔS (cal K-1mole-1) is the sum of the nearest-neighbor entropy changes for hybrid formation (<0)
- R is the molar gas constant (1.987 cal K-1mole-1)

- Ct is the total molar concentration of strands when oligonucleotides are not self-complementary or it is equal to 4 times this concentration in the case of self-complementary sequences.

Another method for calculating the melting temperature is shown as Equation 2 (Sambrook and Russell, 1989).

$$T_m = 81.5 + 16.6\ (\log^{10}[J^+] + 0.41\ (\%G+C) - (600/l) - 0.63\ (\%FA) \quad (2)$$

- Where J^+ = concentration of monovalent ions
- L = length of nucleotide
- FA = formamide

Wu *et al.*, (1991) proposed this calculation for annealing temperature (Equation 3).

$$T_P = 22 + 1.46\ (L_n) \quad (3)$$

- Where T_p = optimized annealing =/2-5C
- L_n= length of oligo

These elaborate formulas have been replaced by the standard method that most researchers use known as the Wallace formula (Equation 4; Thien and Wallace, 1986). This has become a preferred calculation since the T_m is easily calculated and the results serve as a good basis for optimization experiments.

$$T_m = 2°C(A+T)+4°C(C+G) \quad (4)$$

The actual annealing temperature used for the reaction can vary by as much as 12°C due to differences in primer concentration that will favor hybrid formation and result in increased melting temperatures (McPherson *et al.*, 1991). Also, the T_m will be higher with higher salt concentrations due to the high ionic strength as cations stabilize the DNA duplexes.

3) Extension

Strand extension or elongation at 72°C is optimal for reactions using *Taq* polymerase. As little as 20 seconds may be needed for strands shorter than 500 base pairs to elongate, while 40 seconds may be enough for strands up to 1.2 kilobases (kb) (Rolfs *et al.*, 1992). The presence of secondary structures increases elongation time. Many PCR protocols call for a final extension of 5-15 minutes at 72°C after the last PCR cycle to complete extension of all products. Theoretically, it appeared to be more efficient to increase the extension time after each cycle, giving the new product time to extend before further denaturation (a choice employed by our laboratory).

2.2.1.1 The components of the PCR

There are seven essential components of PCR with a discussion of each component to follow (McPherson *et al.*, 1991):

1) The template DNA
2) The use of a thermostable polymerase
3) The oligonucleotide primers
4) Deoxynucleotide triphosphates (dNTP's)
5) Divalent Cations
6) Monovalent Cations
7) Buffer to maintain pH

1) The template DNA

The template DNA can consist of as little as a single copy of the segment of interest. The PCR technique was initially designed with large quantities of starting template in mind. However, variations of the standard PCR protocols can be used to achieve greater sensitivity and specificity even when using a limited number of cells down to the level of a single cell as in PGD (Handyside *et al.*, 1989). One of the modifications that has enhanced the ability to perform PGD is called nested PCR (Oste 1988). Nested PCR uses two primer sets encompassing the target locus in two separate rounds of PCR. The second set is designed downstream (nested) to the first set. The reasons that nested PCR is advantageous when using low amounts of initial DNA template are two-fold:

A) It increases the sensitivity by using two rounds of the amplification reaction. PCR is a finite reaction; beyond the threshold of amplification (which is achieved as reagents are expended), non-specific priming/DNA amplification is seen. By performing approximately 20 cycles of PCR, removing an aliquot of the product, then revitalizing the reaction with additional components of the DNA master mix, exponential amplification can continue from the new reaction starting point.

B) Nested PCR increases the specificity by using two targeted sites on the gene for amplification. Using an 18-25 base primer set in one PCR reaction not only amplifies the target loci, it also allows for the possibility that another homologous sequence in the three billion base pair genome could theoretically amplify. In order to minimize the chance of that occurrence, another primer pair within the same area of the specific locus is used in a second PCR reaction. This substantially decreases the possibility of erroneously amplifying a similar (yet unsought) area of the genome since the template for the second reaction consists of only the segments amplified in the first reaction.

The utility of nested PCR is demonstrated in Figure 3. The upper photo is a gel with samples that were subjected to 50 consecutive cycles of PCR. The lower photo is a gel with the same samples, but after the first twenty cycles of PCR, a 2μl aliquot was removed and subjected to an additional 30 cycles of nested PCR (with the same first primer set). Therefore, both sets of samples were subjected to 50 cycles of PCR. Two additional samples showed amplification in the nested reaction that would not have otherwise amplified with the standard one-step PCR. This gel demonstrates the increased sensitivity seen in nested PCR.

2) The use of a thermostable polymerase

As discussed above, the substitution of *Taq* polymerase from the original *E. coli* polymerase was one of the most important keys to the development of PCR. One unit of enzyme is classified as the amount of enzyme that will catalyze the incorporation of 10nmol of total nucleotide in 30 minutes at 74°C (McPherson *et al.*, 1991). Too much enzyme can lead to a decrease in

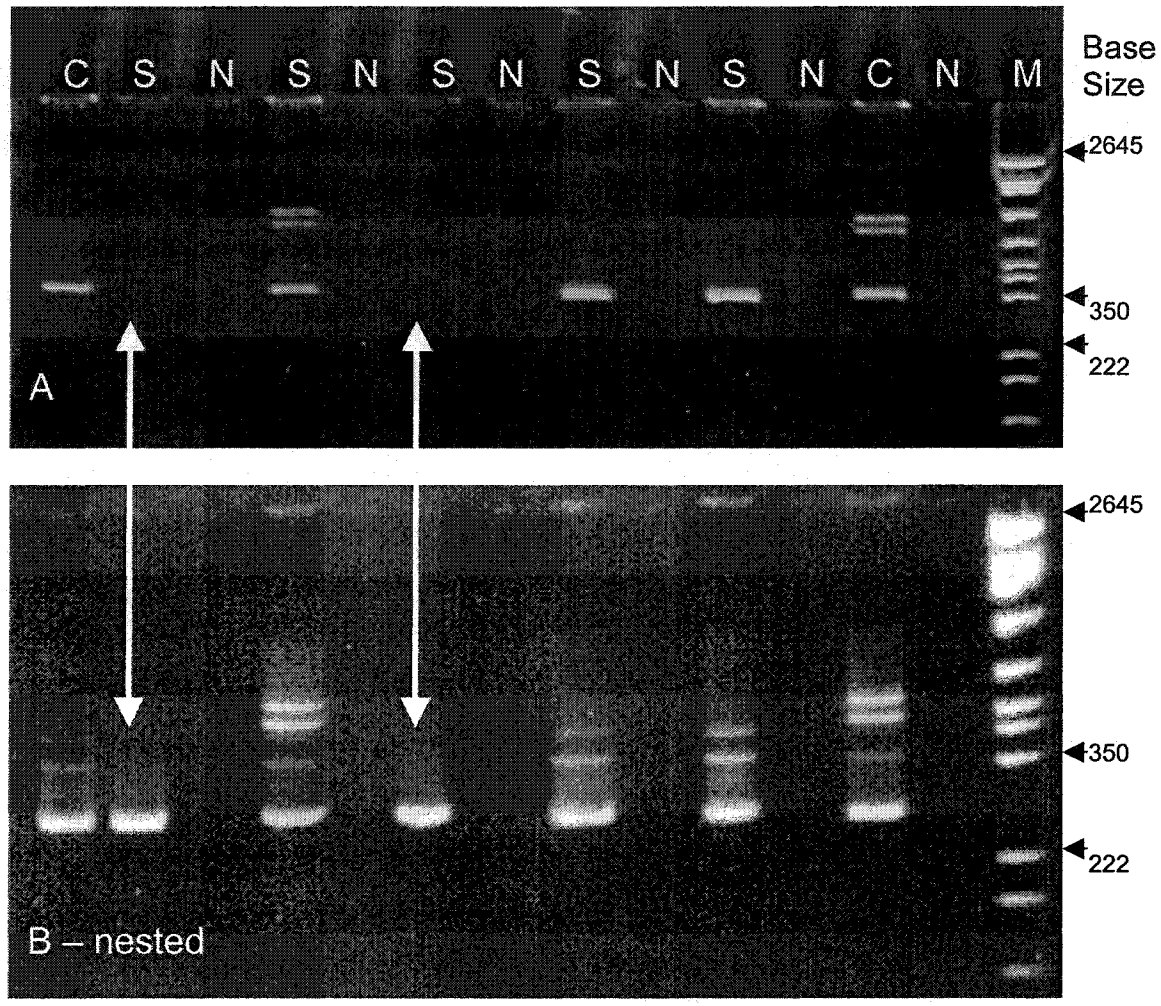

Figure 3. The sensitivity and specificity of nested PCR. Upper and lower photos of 7% polyacrylamide gel lanes contain the same controls (C), single cell samples (S) or negatives (N) along with a DNA marker ladder (M) except that the reaction tubes in panel A were exposed to 50 continuous cycles of PCR while the reaction tubes in panel B contain an aliquot that was removed after 20 cycles of PCR from the corresponding reaction tube. Additional master mix was added and those tubes continued for an additional 30 cycles. Both groups had a total of 50 cycles, but two additional samples amplified in the nested group that did not in the non-nested group.

specificity. When using *Taq* polymerase, the new DNA strand extends at 35-100 nucleosides/second at 70-80°C. As mentioned before, the half-life for *Taq* polymerase at 92.5°C is 130 minutes, 40 minutes at 95°C, and 5-6 minutes at 97.5°C (Saiki *et al.*, 1988).

Processivity of an enzyme is defined as the average numbers of nucleotides synthesized before the enzyme dissociates from the template (McPherson *et al.*, 1991). Fidelity refers to the frequency of enzyme-induced errors. Each error would then be amplified in subsequent PCR reactions. Fidelity is determined by the ability of the enzyme to discriminate between the dNTPs and select the proper complementary dNTP based on the sequence of the DNA template. Fidelity is affected by the balance of the individual dNTP concentration. Proofreading selectively removes misincorporated nucleosides at the 3' end of the primer. High dNTP concentration reduces proofreading activity (Kunkel, 1988). Proofreading is also affected by magnesium (Mg^{2+}) concentration, pH, secondary structure, and the amount of template available. A→C and T→C are the most common errors (Tindall and Kunkel, 1988). *Taq* polymerase has no proof reading (3'→5'exonuclease) activity. However, those errors will for the most part be lost because they do not yield full-length DNA products for further amplification (Tindall and Kunkel, 1988).

Pfu polymerase (*pyrococcus furiosus*) has a twelve-fold higher fidelity than *Taq* polymerase (McPherson *et al.*, 1991). *Pfu* polymerase has 3' to 5' exonuclease activity, which *Taq* polymerase lacks. The temperature range for its use is 72°-78°C. After 1 hour at 95°C, the *Pfu* polymerase still has 95% of its activity. However, it will begin to degrade template DNA in the absence of dNTPs so it must be added last to the reaction mix, after the dNTPs have been added. *Pfu* polymerase requires increased Tris concentrations, and decreased potassium; the lower salt requires a decrease in annealing temperatures from what is used for *Taq*-driven reactions (the importance of these components are discussed further below).

The Stoffel fragment of *Taq* polymerase is a deletion of the 289 N-terminal amino acids of the native *Taq* (Lawyer *et al.*, 1993). It has approximately 2-times

the thermostability of *Taq* polymerase (97.5°C for 20 minutes), but lacks the 5'→3' exonuclease activity. It requires a broader range of magnesium ion concentrations. As with the other polymerases other than *Taq*, inconsistent amplification results and lack of optimized conditions precluded their use. The choice of polymerase is dependent upon the type of amplification reaction along with the sensitivity and reproducibility of the reaction.

3) Oligonucleotide Primers

The oligonucleotide primers are complementary base sequences that anneal to the specific strand of DNA in order to initiate strand replication (Ehrlich, 1989, McPherson *et al.*, 1991, Rolfs *et al.*, 1992). They are generally 18-30 bases in length and are complementary to the DNA sequence of interest. Primers are synthesized using an automated DNA synthesizer to direct each letter (A,T,C,G) in its appropriate place.

Primer optimization is critical for efficient PCR. The primer sequence determines the length of the final PCR product, the annealing temperature, and the ultimate yield of the product (McPherson *et al.*, 1991). The primary goal of primer design is specificity. There are over 3 billion base pairs of DNA in the human genome; a perfect match has to be found in 18-30 bases. Although primer design is somewhat empiric, the guidelines discussed below are based on thermodynamic and structural principles of the DNA. Following the guidelines discussed below does not guarantee success, but disregarding them usually leads to failure.

Computer-assisted programs can aid in primer design. These programs can show complementarity, potential primer-dimer sites, calculate annealing temperatures, and show restriction enzyme sites. An example of primer design and the computer assistance is given in Appendix A. The ideal primers contain 45-55% guanine (G) and cytosine (C) nucleotides due to the more stable triple hydrogen bonds of the GC that will enhance the annealing (Figure 4). This also sets the annealing temperature around 55-60°C. Run-on bases (a string of the same nucleotide) should be avoided; GC stretches can promote non-specific annealing while AT stretches make the primer template complex more vulnerable

to dissociation since the AT double bonds are weaker. This could lower amplification efficiency.

Primers should have one of the following - G or C, CG or GC - at the 3' end (McPherson *et al.*, 1991). This prevents "breathing" of the 3' ends (dissociation) and increases efficiency of priming. The 3' ends of the primer pair should not be complementary to each other, otherwise primer dimers will be extended preferentially to any other product and decrease the yield of the desired product. Primer dimers are so called because of the complex of the oligonucleotide with itself or the other primer in the pair, thus forming the dimer. A primer by itself should also not be self-complementary. This is to avoid having the primer fold back on itself to form secondary structures such as hair-pin loops. In that instance, less primer would be available for the desired reaction.

Primer concentration is easily determined by ultraviolet (UV) spectrophotometry and measuring the optical density at 260nm. One OD is equal to approximately 33ug of primer (Rolfs *et al.*, 1992). Optimally the primer concentration is related to the length of the amplified product and can be calculated as shown in Table I. Generally, it is simpler to dilute the primers to a working concentration of 0.1 to 1μM than it is to calculate the concentration from the table. Primer concentrations should be adjusted to as low as possible for amplification; higher primer concentrations may promote the formation of primer dimers and other non-specific products.

4) Deoxynucleotide Triphosphates (dNTPs)

Concentrations of 200-250mM of each dNTP (dATP, dCTP, dGTP, dTTP) are added to the PCR. The lower the dNTP concentration, the lower the error rate of the base incorporation (Bebenek and Kunkel, 1990). Higher concentrations of the dNTPs can be inhibitory, perhaps by sequestering too much of the $Mg2^{+}$. However, reaction times need to be increased at low dNTP concentration to increase synthesis and minimizes error as T-G mismatches (Bebenek and Kunkel, 1990). dNTPS should be pyrophosphate-free (which can inhibit PCR). The pH of the dNTP solution should be around pH 8.1; the alkalinity

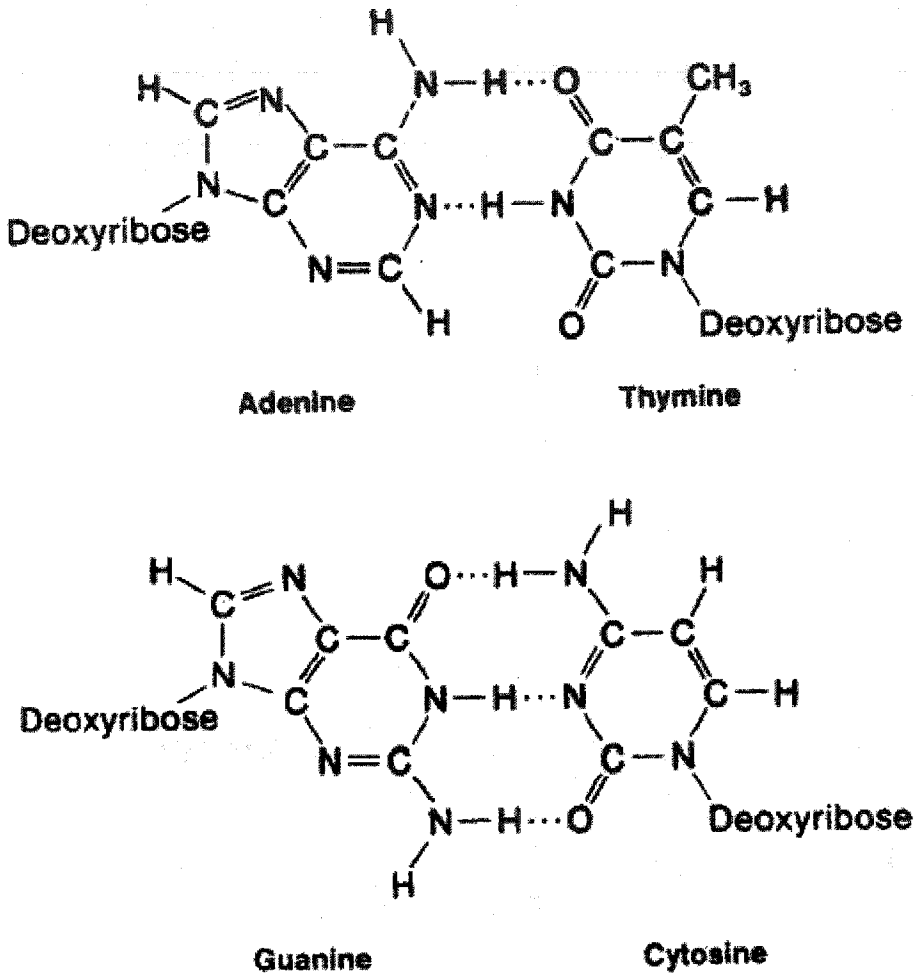

Figure 4. The components of double stranded DNA. The deoxyribose links the bases Adenine (A), Thymine (T), Guanine (G), and Cytosine (C) to the sugar-phosphate backbone of the DNA double helix (not shown). The two strands are linked together with either two or three hydrogen bonds (H) from the complementary bases (AT and GC, respectively).

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Table I. Determination of optimal primer concentrations

	Incorporated primers per strand (molar concentration)		
Yield of produced double-stranded fragment	200 bp fragment	800 bp fragment	Incorporated dNTPs absolute molecular concentration/ACTG irrespective of length
1 μg/100μl	8 pmol (0.08μM)	2 pmol (0.02μM)	3.2 nmol (8μM)
3 μg/100μl	24 pmol (0.24μM)	6 pmol (0.06μM)	9.6 nmol (24μM)

of the solution helps to protect damage during freeze-thaw process. The signal intensity may decrease if the dNTP solution is older than 2 months.

5) Divalent Cations

Mg^{2+} is used to stabilize the polymerase; the divalent cation influences the processivity and T_m of the reaction since oligonucleotides can bind to Mg^{2+} (Eckert and Kunkel, 1990). Mg^{2+} is the most efficient cation; manganese has limited efficiency while calcium is ineffective. Increased fidelity of the reaction can be achieved by decreasing Mg^{2+} concentrations to the total dNTP concentration (Eckert and Kunkel 1990). The concentration of free Mg^{2+} depends upon the dNTP concentration and the EDTA concentration which binds stoichiometrically to the Mg^{2+}. However, too much EDTA or other chelating agents can sequester too much of the Mg^{2+} and inhibit the reaction. Generally, a good starting concentration is 1.5mM $MgCl_2$ although some reactions can require a concentration as high as 6mM Mg^{2+}. The optimal concentration must be determined for each protocol.

6) Monovalent cations

Potassium chloride (KCl) is a component of the PCR buffer that adds a monovalent cation to the reaction (McPherson *et al.*, 1991). KCl concentration should be less than 100 mM or it will decrease the polymerase activity. Polymerase I actually is insensitive to high salt (Chien *et al.*, 1976).

7) Buffer to maintain pH

Control of the pH is accomplished by using Tris buffer. The pH of the reaction should be between 8.3-8.8 at *room temperature* (McPherson *et al.*, 1991). Since the extension step and new strand extension occurs at 72°C, the elevated temperature will effectively lower the pH to around 7.2 for the reaction. Optimal pH increases the polymerase activity and decreases the error rate. An increase in one point of the pH can increase the error by 60 fold (Eckert and Kunkel, 1990). Incubation of DNA at high temp and low pH can change amplification efficiency by inducing DNA damage due to the deamination of cytosine to produce uracil, the same as coding for thymine. Also, DNA damage at

high temperature/low pH can cause spontaneous base release due to increased hydrolysis of the N-glycosylic bond. (Lindahl, 1979).

Generally the reagent components (the above components other than the template DNA) are mixed together and added as an aliquot together to each reaction tube for consistency. This is known as the master mix. The master mix and the samples are kept on ice, and the thermal cycler is preheated to the initial denaturation temperature for a more efficient amplification. Leaving the reaction mixture for 5 minutes at room temperature could have a 1000-fold effect on decreasing the amplification (Rolfs *et al.*, 1992).

2.2.1.2 PCR Controls

PCR protocols should include both negative and positive controls. Negative controls contain all reagents but no template DNA and are designed to detect extraneous DNA contamination (Rolfs *et al.*, 1992). Since no starting template was added to the reaction, no amplification result should be seen; amplification should only be seen in reactions where template DNA was added. If amplification is seen in the negative control, the amplification observed in the samples may not be valid and may be due to contamination of extraneous DNA (contamination control is discussed further in Appendix B). Positive controls contain DNA of interest and verify the occurrence of amplification and should provide an amplification result. If positive controls do not amplify, possible explanations may be that inhibitory activity may be present in the reaction, a reagent was not properly added (omitted or at the wrong concentration), or PCR conditions may not be optimized. Both positive and negative controls must be exposed to the same conditions as the reaction samples, including the preparation and extraction steps, so that the sample results can be compared with controls for the validity of results.

2.2.1.3 Other types of PCR

A multitude of PCR techniques concurrently exist, still based on the three basic steps of denaturation, annealing, and extension. Some of the modifications include the following techniques:

1) Hot-start PCR is a method designed to avoid mispriming by keeping the

master mix components separate from the template prior to denaturation (Chou *et al.*,1992). As the reaction temperature rises to the starting denaturation temperature, primers could inadvertently anneal as the temperature passes through the annealing temperature range. The Hot-start PCR tube contains a wax-barrier that melts when denaturation temperature is reached.

2) Touchdown PCR is the variation of the annealing temperature during the reaction itself (Don *et al.*, 1991). The initial annealing temperature is set above the calculated annealing temperature. It is decreased by 1°C increments every two cycles to below the calculated temperature. This decrease is known as the 'touch-down'. These variations in temperature will increase the stringency of the reaction and decrease the non-specific priming over the range of temperatures. It is used in circumstances with several primers to encompass different annealing temperatures, or used to overcome instances when it is difficult to identify the optimal annealing temperature.

3) Fluorescent PCR uses fluorescently labeled primers that can be detected at a greater sensitivity than conventional PCR primers (Chehab *et al.*, 1992). The amplified product is detected with the assistance of computer software rather than conventional gel electrophoresis.

None of these techniques were used in these experiments, or were tested with little improvements in conditions.

2.2.2 Whole Genome Amplification (WGA)

One of the constraints in performing single cell genetic analysis is the limited quantity of DNA. A single cell contains approximately 10pg of DNA, which can limit the number of diagnostic procedures that can be performed to gather information from the cell. To overcome these limitations, whole genome amplification techniques such as primer extension preamplification (PEP), degenerate oligonucleotide primers (DOP), or multiple displacement amplification (MDA) can be applied to increase the amount of DNA (Zhang *et al.*, 1992; Xu *et al.*, 1993; Kristjansson *et al.*, 1994). These techniques use primers consisting of random nucleotides that have the capability of producing multiple copies of

segments of the genome. An explanation for several whole genome amplification techniques follows.

2.2.2.1 Primer Extension Preamplification (PEP)

PEP is a PCR-based molecular technique that initially was applied to spermatozoa to amplify and study several large fragments of DNA (Zhang *et al.*, 1992). A mixture of 15-base oligonucleotide primers is added, containing random combinations of the four nucleotide bases. The primer mixture is theoretically composed of 4^{15} sequences (4^{15} = approx 1.07 billion). The cell is lysed and the primer mixture is added with buffer, dNTPs, and *Taq* polymerase. The sample is then thermocycled for 50 cycles with a slow rise (ramping) of annealing temperatures in the second step to encompass several annealings during that step. In the initial experiments, Zhang and colleagues (1992) subjected single human sperm to PEP, then analyzed each of 18 sperm for 12 different loci in 30 aliquots by PCR. From these experiments, the researchers concluded that approximately 78% of the genome had been copied at least 30 times. Similarly, several studies were undertaken to demonstrate the ability of blastomeres to amplify multiple loci post-PEP (Xu *et al.*, 1993; Kristjansson *et al.*, 1994).

2.2.2.2 Degenerate Oligonucleotide Primers (DOP)

DOP is another WGA PCR-based technique, which differs from PEP in several ways. The primer mix consists of a random hexamer surrounded by a known oligonucleotide sequence on either side of the random nucleotides (5' – CCGACTCGAGNNNNNNATGTGG- 3', where N is any nucleotide). This partial 'degeneracy' is thought to allow for the priming from multiple dispersed sites along the genome since the ATGTGG sequence should occur once every 4630 base pairs (Telenius *et al.*, 1992). Rather than ramping the annealing temperature as in PEP, there are two series of steps in the DOP process. Initially, the annealing temperature is lowered to around 30°C for eight cycles; as discussed previously, lower annealing temperatures lower the stringency. This allows more random primer binding, including the chance that any of the six bases will anneal at the 3' end. The annealing temperature is subsequently raised to 60°C for more specific annealing.

Wells *et al.* (1999) compared the techniques of DOP and PEP. Ninety one percent (228/250 cells) of the genome amplified post PEP while 89% (223/250) amplified post-DOP. Furthermore, DOP reactions produced larger quantities of DNA than PEP as evidenced by the presence of a DNA smear on a 1% agarose gel. No smear appearance was seen on the gel with the PEP products. Unfortunately, the study did not specify the quantitation of DNA in their results although they were able to conduct 90 separate PCR amplifications from a single cell post DOP. PEP generates approximately 40ng of product while DOP can generate 1-6μg (Lasken and Egholm, 2003).

There are a few disadvantages of these WGA techniques. The process is relatively easy to set-up, but the amplification process requires approximately 10 hours. Also the products tended to be less than 500 base pairs which can introduce bias. Internal regions of previously amplified product would amplify in subsequent cycles so that whole segments of the genome were either under represented or not present at all in the final product. This could lead to allele drop-out (ADO, the amplification of only one allele of the two alleles present) (Paunio *et al.*, 1996; Wells *et al.*, 1999).

2.2.2.3 Multiple Displacement Amplification (MDA)

A newly introduced WGA technique offers great promise as a comprehensive technique with minimal bias (Dean *et al.*, 2002). The technique, MDA, also known as rolling circle amplification, was first proposed by Lizardi *et al.* (1998). This WGA technique differs from DOP or PEP in that it does not require PCR. The technique is isothermal, and amplifications take place entirely at 30°C. The method involves strand displacement in that the template is amplified over and over again; the polymerase amplifies a new copy as it displaces previously synthesized copies. This is in contrast to PCR where the polymerase dissociates from the previously made copy and new polymerase starts the next strand synthesis (Figure 5).

The key to the MDA technique, just as in the PCR, is the type of polymerase used. For MDA, Φ29 (Phi 29) polymerase is used. Φ29 DNA polymerase (from the bacteriophage of the same name) is highly processive and

has the ability to incorporate 70,000 nucleotides in a single binding event (Blanco *et al.*, 1989.) As the Φ29 polymerase encounters a synthesized strand of DNA, the polymerase displaces the strand, thus inducing single stranded DNA that can serve as a template for further primer annealing and strand replication. The products generated from an MDA reaction are generally up to 12 kb in length. The isothermal technique, while eliminating the cycling, avoids sequence-dependent events such as differences in G-C content that favor amplification of certain regions over others (Lasken and Egholm (2003). The error rate for Φ29 is only 1 in 10^6 to 10^7 bases (Esteban *et al.*, 1993).

The three primary advantages of MDA are:

1) it is an easy method to perform (and thus can be automated)

2) large quantities of DNA are generated

3) DNA yields are uniform and independent of initial starting material since the reaction proceeds until reaching a similar quantity endpoint.

Isothermal amplification in human single lymphocytes (n=5) and blastomeres (n=11) has been reported (Handyside *et al.*, 2004; Hellani *et al.*, 2004). Individual cells were subjected to MDA, and an aliquot of the sample post-MDA was then subjected to PCR amplification of 20 different loci. All five lymphocytes and 10 of 11 blastomeres demonstrated successful WGA as evidenced by either DNA yield and/or PCR amplification (100% and 91%, respectively). The lymphocyte aliquots amplified in 92/100 (92%) subsequent PCR attempts. However, ADO was detected in 22/70 reaction (31%). The 10 blastomeres that were successfully amplified by the isothermal technique were subjected to subsequent PCR as well. Eight of the ten blastomeres demonstrated PCR amplification with an Allele drop-out (ADO) rate of 4/25 reactions (16%) (Handyside *et al.*, 2004). Similarly, Hellani *et al.* (2004) subjected 45 single leukocyte cells to the isothermal WGA technique. Successful amplification was determined in all 45 cells as evidenced by the presence of a smear on a 1% agarose gel. Forty of the cells were subsequently subjected to PCR for a known β globin mutation. Thirty-nine of 40 cells amplified by PCR (97.5%). There was evidence of ADO in 4/39 cells (10.3%). In the Handyside studies (2004), the yield

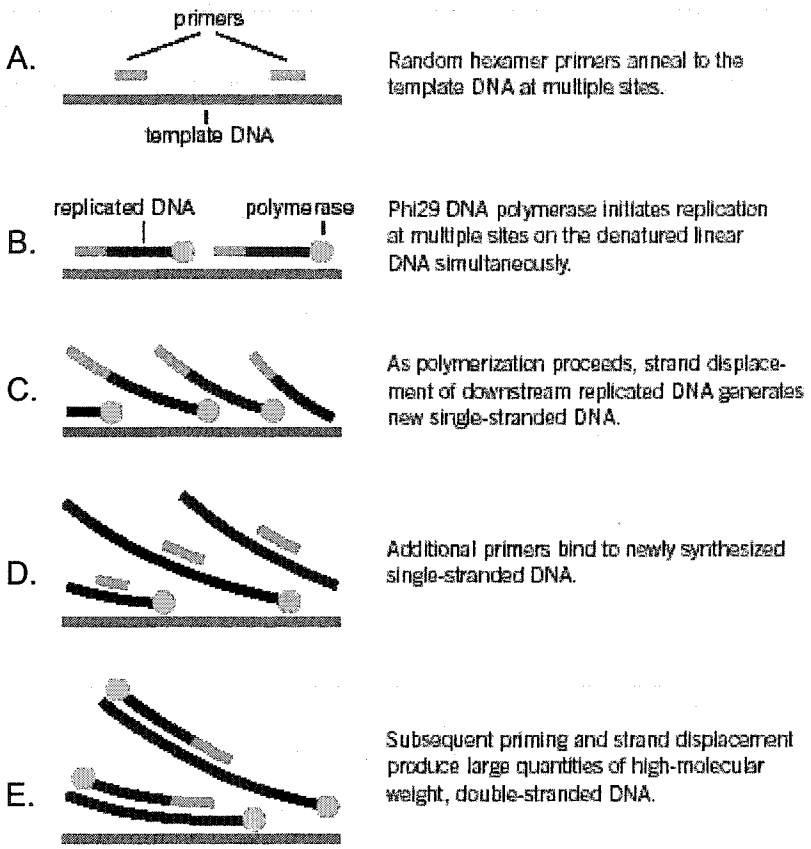

Figure 5. Multiple Displacement Amplification. A. Random hexamer primers anneal to the template DNA at multiple sites. B. Phi 29 DNA polymerase initiates replication at multiple sites on the denatured DNA simultaneously. C. As polymerization proceeds, strand displacement of downstream replicated DNA generates new single stranded DNA. D. Additional primers bind to the newly synthesized single-stranded DNA. E. Subsequent priming and strand displacement produce large quantities of high-molecular weight, double-stranded DNA

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of DNA after MDA ranged from 42-53μg as assessed by Picogreen analysis. Hellani *et al.* (2004) reported 35± 5μg in their MDA products. From these studies, the researchers concluded that these data provide the initial steps to the applications of isothermal WGA techniques in single cell genetic analysis.

2.2.3 Microarray Analysis

Standard microarray analysis involves the simultaneous use of two fluorescent dyes, one for the sample of DNA/RNA to be tested, and one for the normal control. The sample and control are labeled with the separate dyes, then the two are combined and allowed to hybridize to the microarray. The dye intensities are compared to determine gene expression or levels of DNA. The premise is that the measured spot intensity is proportional to the abundance of corresponding gene or message present (Edwards, 2003).

The first microarray paper studied quantitative gene expression in *Arabidopsis thaliana*, a small mustard plant (Schena *et al.*,1995). The technology developed rapidly the following year to included gene expression studies in the yeast, mouse and human (Shalon *et al*, 1996; Schena *et al.*, 1996; Lockhart *et al.*, 1996, respectively). To date, microarray technology has now been used in over 100 organisms (Stears *et al.*, 2003). The technology can be used to determine and monitor the level of thousands of genes simultaneously since the DNA or RNA probes are miniaturized onto a small glass or silica slide. The principles of DNA and RNA detection are that a complementary probe binds or hybridizes to the sequence of interest; in microarray technology the scale has changed dramatically in that thousands of sequences can be detected from a single array. Generally a two-fold change (increase or decrease) is used as the threshold to determine significance. There are three components to microarray technology:

1) The array slides containing the target DNA
2) Labeled samples (probes) which that are hybridized to the array
3) A detection system to quantitate the hybridization signal.

1) The array slides containing the target DNA (Figure 6)

Microarray slides are 'spotted' with DNA oligonucleotides (generally 15-30 bases) or PCR amplified cDNA sequences (Stears *et al.*, 2003). The short oligonucleotide arrays are generally used for SNPs (single nucleotide polymorphisms) that require single mismatch discrimination. They can also be used for gene expression analysis although the short oligos may lack the single gene specificity. cDNAs work well for gene expression analysis. These longer sequences (0.2-1.2kb) tend to produce strong signals that are highly specific (Heller *et al.*, 1997). Either of these elements is deposited (spotted) onto the surface of a glass slide, generally as 0.5-10nL droplets with a concentration of approximately 500ng/μl on spots that are 100-150μm in diameter (Wildsmith and Elcock, 2001). The spots are generally 200-250μm apart, depending on the type of instrument performing the spotting. The spots are cross-linked and dried (Sasik *et al.*, 2004). The spots generally are duplicated elsewhere on the slide to serve as hybridization controls that can aid in determining reproducibility. Additionally, blank spots with no DNA are used as negative controls and can be used to detect background fluorescence since they should not exhibit signal.

2) Labeled samples (probes) that are hybridized to the array

In microarray technology, the labeled sample/control is known as the probe. The sample is labeled with fluorescent dyes as explained below.

2.2.3.1 Dyes

Fluorescence is defined as the molecular absorption of light energy (photon) and its re-emission at another wavelength. Molecules that can both absorb and emit light are known as fluorochromes or fluorophores. Fluorescent dyes are used to label the sample and control. The advantage that fluorescent dyes have is that more than one can be used and detected at a time. The fundamental principle of microarray analysis is that there is a presumed relationship between the signal intensity and the abundance of a gene transcript or target DNA (Cox *et al.*, 2004). Therefore the ratio of the two dyes should be 1:1 in genes that are equally expressed (or in DNA present in the same amount). Furthermore, the signals

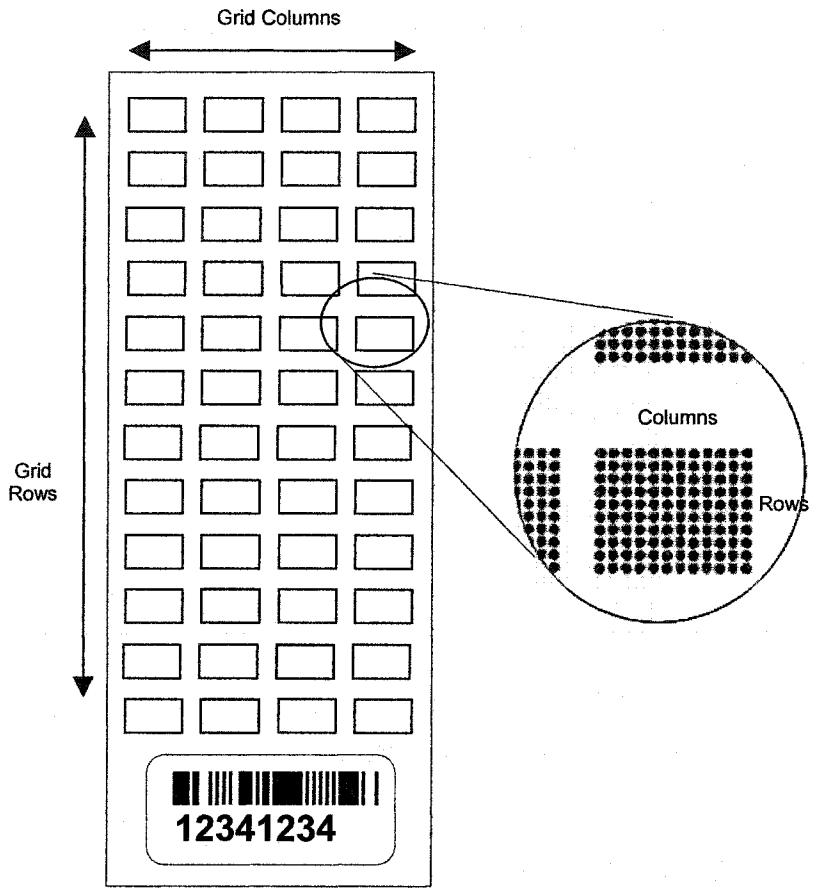

Figure 6. Illustration of microarray slide. The microarrays used in these experiments contained 4 grid columns and 8 rows, each grid with 12 columns and 10 rows. The grid rows begin 1.75 cm from the non-labeled end of the slide.

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should be tightly distributed along a linear regression line. Higher signal correlation between the two dyes provides more confidence in detecting smaller changes in expression levels and allows for greater resolution of the difference between sample and control. The labeling step is critical and can be the primary cause for lack of reproducibility of results (Wildsmith *et al.*, 2001).

Cyanine dyes, Cy3™ and Cy5™ dyes (Amersham Biosciences, Piscataway, NJ) are commonly used in microarray analysis (Schena *et al.*, 1995). They are water-soluble and pH insensitive (Wildsmith *et al.*, 2001). These dyes are incorporated by labeling a dCTP with the Cy dye, then incorporating the dye with a random labeling method. In this method, the Klenow fragment of DNA polymerase incorporates the fluorescently labeled nucleotide in a DNA synthesis reaction, which is primed with random hexamer primers. This method is a practical solution for genomic microarrays although enzymatic reactions do not copy all nucleic acid sequences (Microarray Handbook, 2004)

Alexa Fluor dyes (Alexa 555 and Alexa 647) have emerged as an alternative to the Cy dyes. Alexa Fluor 555 and Cy3™ are structurally distinct dyes that have similar absorption and emission spectra. Their maximum absorption peak is at 550nm and their maximum emission peak is at 570nm. Alexa Fluor 647 and Cy5™ dyes are also structurally distinct dyes that have similar absorption and emission data. Both have a maximum absorption peak at 650nm and maximum emission at 670nm.

The Alexa Fluors are water-soluble dyes as well; this is advantageous since nucleic acids that are labeled with Alexa Fluors will not precipitate even in high salt concentrations of hybridizations. They are pH sensitive to the range of 4.0-10.0 (Wildsmith *et al.*, 2001). They are more resistant to quenching (or photobleaching) than the Cy dyes (Berlier *et al.*, 2003). (Quenching is defined as the diminishing or extinguishing of signal intensity due to intense light exposure). The dyes directly label the nucleic acids without using enzymatic incorporation as the Cy dyes require. This technique is rapid and efficient and provides a reliable method for labeling DNA. The reagents react with the N7 of the guanine residue of the DNA, averaging one dye molecule every 20-30 bases (Figure 7). This is

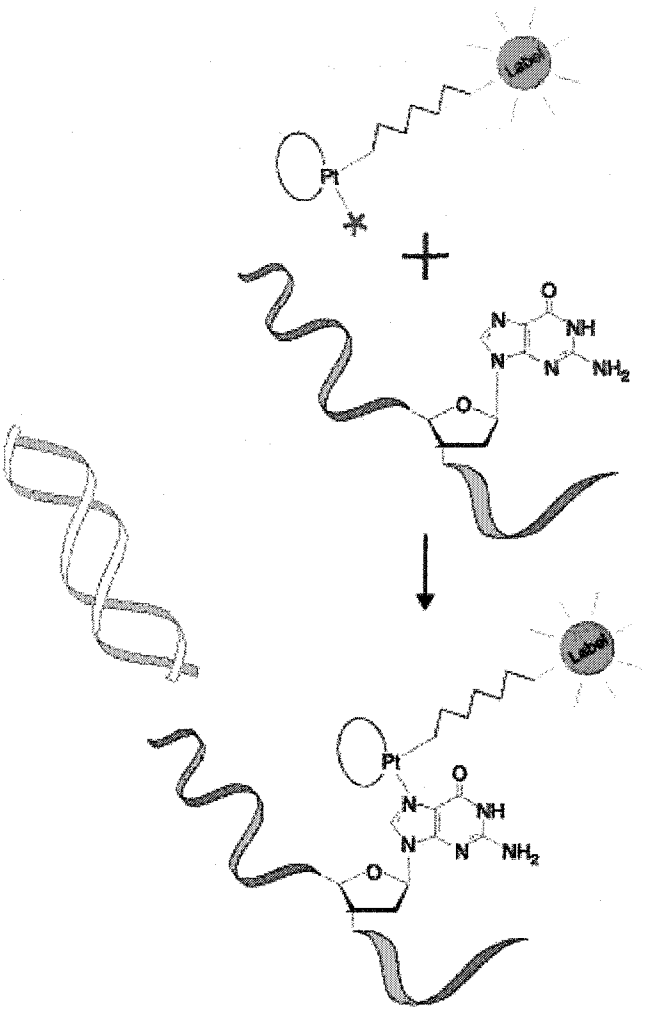

Figure 7. The Alexa Fluor dyes. The Alexa Fluor label reacts with the N-7 position of guanine residue to provide a complex between the nucleotide and the fluorochrome. Reprinted with permission/copyright owned by Molecular Probes, Inc.

much more efficient labeling and showed higher fluorescent intensity than did the Cy dyes. A base:dye ratio can be calculated to determine how well the sample is labeled to ensure that there will be adequate signal prior to array hybridization. A desired level is a base:dye ratio of less than 25. The absorbance (A) at 260nm is measured by UV spectrophotometry and the ratio is calculated (Equation 5).

$$\text{base:dye} = (A_{base} \times \Sigma_{dye}) / (A_{dye} \times \Sigma_{base}) \quad (5)$$

where $A_{base} = A_{260} - (A_{dye} \times CF_{260})$

A_{260} is the absorbance of the dye/nucleic acid conjugate

CF_{260} is the correction factor listed in the product information sheet

Σ_{dye} is the extinction coefficient for the fluorescent dye (found on the product information sheet)

Σ_{base} is the average extinction coefficient for a base in double stranded DNA (dsDNA) or single-stranded DNA (ssDNA) (found in the information sheet). (from Molecular Probes product Information guide, 2004).

Cox *et al.*, (2004) demonstrated that the brightness of the Alexa Fluor dyes showed a linear increase as the amount of dye labeling increased. In contrast, the Cy dyes showed a decrease in brightness as the amount of labeling increased. They concluded that the Cy dyes could be affected by dye-dye interaction that may be responsible for the increased quenching seen with the Cy dyes. From this, the researchers determined that an increased sensitivity may be obtained when using the Alex Fluor dyes, allowing a 95% predictive value to ascertain a difference at 1.3 fold level rather than the standard two-fold level difference.

Dye-Swap normalization can be used to avoid artifacts (Rosenzweig *et al.*, 2004). This involves performing the experiment first with one dye for an aliquot of sample and the other dye in the set for a second aliquot of the same sample to

determine the variability in dye intensity since the ratio in the same sample should be 1:1. Alternatively, the sample can be labeled with one dye and the control labeled with another, then a second experiment performed with the opposite dye for the sample and the opposite for the control. For example, initially the control can be labeled with Cy3™ and the sample with Cy5™. Another experiment 'swaps' the dyes and now the control is labeled with Cy5™ and the sample with Cy3™. These results are then compared to the results when the dyes are reversed and the bias is determined.

2.2.3.2 Hybridization time and temperature

The time and temperature of the hybridization are dependent upon the salt concentration of the hybridization buffer (Wildsmith and Elcock, 2001). Ideally a hybridization buffer contains the following components:

1) A buffering component that acts to stabilize variations in pH

2) A detergent that lowers the surface tension and allow the buffer to flow easily under the coverslip

3) Compounds such as formamide that act as rate enhancers, volume excluders, or to speed up the hybridization and lower the T_m

Formamide is a denaturing reagent that is often used to lower the T_m of the probe and thus the temperature of hybridization. The optimum hybridization temperature for microarrays in aqueous buffers can be as high as 65–75 °C. At such high temperatures, drying of the slide can become problematic. Likewise, the probe is more likely to degrade at higher temperatures. The addition of formamide to a buffer decreases the T_m by 0.65 °C for every 1% concentration (Casey and Davidson, 1977). The addition of 50% formamide to the hybridization lowers the optimum temperature to a more reasonable 42 °C. However, hybridizations carried out in formamide should be left undisturbed for 16 hours unless the probe concentration is increased. Hybridization temperatures can be calculated as 20-25° C less than the melting temperature using the T_m calculation as previously discussed from Sambrook and Russell (1989):

$T_m = 81.5 + 16.6 (\log^{10}[J^+] + 0.41 (\%G+C) - (600/l) - 0.63 (\%FA)$.

Where J^+ = concentration of monovalent ions

L = length of nucleotide

FA = formamide

Hybridizations generally take place for the allotted time in a hybridization chamber submerged in a water bath.

2.2.3.3 Post hybridization washes

After the hybridization time has elapsed, the microarray is exposed to a series of post-hybridization washes. The purpose of these washes is to remove all unattached and/or loosely bound probe molecules. This prevents false positive signals and removes all components of the hybridization buffer, preventing background noise that may appear in the form of smearing and/or speckles when the image is captured. Likewise, it is important not to remove the bound probe. The wash takes place initially under similar salt concentrations and temperatures as the hybridization buffer with decreasing concentrations in a step-wise manner (Wildsmith and Elcock, 2001). The slides remain light sensitive at this stage so the washing steps should be carried out in the dark to minimize signal loss due to quenching of the fluorescent dyes. Once the slides have been washed, they should immediately be dried by centrifugation to prevent smearing that could occur if they were instead air-dried. The slides should then be stored in the dark in a desiccator and scanned as soon as possible. After scanning, if it is determined that the slides have high background or low stringency, rewashing the slide and re-scanning is recommended.

3) A detection system to quantitate the hybridization signal

The detection system used for data analysis involves image acquisition and quantification. Image acquisition refers to the scanning of the microarray while the quantification pertains to the conversion of the image into numerical data stored on a spreadsheet. The array slide is scanned by a laser; the sites that are hybridized with the fluorescently labeled DNA will emit light that is captured and assessed for intensity (Bednar, 2000). Measuring fluorescent light intensity (emitted photons) can be accomplished with any photosensitive device such as a photomultiplier tube (PMT) that can be used for low-light intensity.

This is simply a photoelectric cell with a built-in amplifier. When light of sufficient energy hits the photocathode in the PMT, electrons are emitted, and the resulting current is amplified. The strength of the current is proportional to the intensity of the light detected.

Image acquisition is aided by computer software that can estimate the background surrounding a spot and subtract that from the fluorescent signal. There are several potential sources of error here: The array spot has a small surface area and is therefore representative of a small sample of a background that could fluctuate. Also, the signal can 'leak' from the spot, i.e., the signal does not remain within the ring of the spot or fills only part of the area. This is a frequent cause of expression data seen at low intensity on a scatter plot (Sasik *et al.*, 2004). The image is captured as a TIFF file (tagged image file format) that consists of two-dimensional 16 bit images of the array data.

The general requirements for detecting fluorescent signals in microarray analysis are:

1) It requires high enough resolution to image a large area (24mm x 60mm) in a short period of time.

2) At least two fluorescent spectra must be distinguished to accommodate differential gene expression experiments using two fluorescent dyes.

3) The wide variability of message levels requires an instrument with a sensitive fluorescent detection threshold to allow detection of rare messages while also allowing wide linear dynamic range to measure the more abundant messages.

4) The entire area of the microarray must be scanned uniformly to ensure reproducibility (The Microarray Handbook, 2004).

To determine the reliability of data obtained from microarrays, both inter-assay and intra-assay variation have been calculated. Standard variation on the same microarray can exhibit a 5-10% variation in signal intensity (a form of intra-

assay variation). There can be a 10-30% variation evaluating corresponding components on different microarrays ('inter-assay' variation, Stears *et al.*, 2003).

2.2.3.4 Normalization

Normalization is the process of applying a correction to data that are generated from non-biological causes. This typically is used to account for inherent differences in the intensities of the two different dyes due to their differences in fluorescent properties. The purpose is to ensure uniformity and reproducibility of microarray data (Edwards, 2003). Without normalization, misleading conclusions could be reached. One method of normalization is known as lowess or loess, which is an abbreviation for linear regression (Cleveland 1979). Lowess utilizes the best local linear fit of scatter plot data to detect non-linear points. The application of these methods to oligonucleotide arrays reduces the relative error between replicates by 5-10% compared with a standard global normalization method (Workman *et al.*, 2002)

The information workflow

Microarray experiments are divided into several phases. The experimental design asks the biological question, provides for adequate controls, and determines the goals and pitfalls of the experiment. The experimental process is based on the target preparation, printing the arrays, probe preparation and labeling, hybridization, processing and detection. In order to accomplish these first two steps, the Internet can serve as a valuable resource for sequence databases, electronic manuscripts, protocols, etc. Next, the data are extracted, normalized, quantitated, transformed, then analyzed by scatter plot, expression maps, cluster analysis, etc.

2.3 Preimplantation Genetic Diagnosis (PGD)

PGD offers an opportunity to increase the odds of having a healthy child for couples at risk of having offspring with genetic diseases and/or chromosomal abnormalities. PGD is a combination of two rapidly advancing fields: molecular biology and assisted reproductive technology. The polymerase chain reaction made it possible to gather genetic information from small amounts of starting DNA. Prior to the discovery of PCR, there were no sophisticated methods to be able to

determine a genetic diagnosis from a single cell within a short turn-around time. Southern Blotting techniques and DNA sequencing methods that determine DNA location or sequence were too extensive and not applicable to single cell use due to limitations in the sensitivity at the single cell level. The Human Genome Project provided the ability to know the specific genetic sequences for many devastating heritable conditions. Those scientific advances provided major stepping-stones for the foundation of diagnosing genetic predispositions in the developing embryo.

The first successful clinical application of PGD was reported by Handyside *et al.*, in 1989. The technology was used initially to identify at risk male embryos from carriers of X-linked diseases; in the reported cases, the women carried either X-linked adrenoleukodystrophy or X-linked mental retardation. After embryo biopsy at the 6-8 cell stage, PCR was applied to target a specific repeat region on the Y chromosome in the removed blastomere; no attempt was made to amplify the X chromosome since both males and females have the X chromosome. Although this first report resulted in successful pregnancies in two couples, soon after there were reports of a pregnancy with a PGD misdiagnosis (Hardy and Handyside, 1992). This was the result of attempting to amplify a segment of the Y chromosome only. No amplification of the Y-only segment erroneously meant no Y chromosome; in actuality, the situation was that there was no PCR amplification in that particular cell that came from an embryo that truly had a Y chromosome as evidenced by the implantation and gestation of a male fetus. Even though the controls for that test were normal, the misdiagnosis is indicative of the experimental nature of the procedure and indicated that further optimization of techniques was required to determine the cause.

Subsequent methods were developed using primers designed to simultaneously amplify segments of both the X and Y chromosome to minimize the possibility of a misdiagnosis (Strom *et al.,* 1991). The X chromosome serves as an amplification control in that it should be present in all cells, both male (XY) and female (XX). In previous methods, the researchers found that the Y-specific amplification was only 80% reliable; 20% of known male cells did not yield amplification of the Y chromosome. This prompted the development of a technique

that simultaneously amplified both X and Y sequences by multiplex PCR to reduce the possibility of a misdiagnosis due to amplification failure. In multiplex PCR, more than one set of primers designed to amplify a different gene locus is added to the same reaction since there is not enough template to conduct a separate reaction. Trials of 141 single lymphoblasts with known gender showed no errors in amplification.

Before and after the reported clinical application of PGD, a multitude of studies were undertaken to determine the efficacy of the procedure. Many of the initial studies came from work performed at the Jones Institute by my predecessors. Takeuchi *et al.*, (1992) demonstrated the efficiency of embryo biopsy in the mouse and monitored the subsequent development to the blastocyst stage. More than 80% of biopsied embryos continued development to the blastocyst stage in culture. At the same time, Morsy *et al.*, (1992) demonstrated highly accurate and reproducible amplification when analyzing several replicates of a single lymphoblast for specific gene loci by PCR.

Not only is amplification sensitivity an issue, but accuracy is a concern as well. Generally, sources of errors can come from two causes, extraneous DNA contamination and ADO, the amplification of only one of the two alleles present.

2.3.1 Contamination control

The risk of introducing extraneous DNA is a constant presence even though extraneous DNA contamination has never been a problem in our PGD laboratory and has only been seen in isolated instances. A PCR technique that is sensitive to the level of a single cell would only require the smallest amount of measurable DNA for the original sample to be contaminated. Sources of extraneous DNA that could affect the PCR reaction include carryover of previous PCR products, sample cross-contamination, DNA present in the laboratory, and operator contamination (Kwok and Higuchi, 1989). Several contamination control measures are undertaken in the laboratory to minimize this risk. These measures are listed in Appendix B.

Since DNA particles or amplicons can be aerosolized, it is difficult to keep the laboratory totally DNA-free unless one uses a facility similar to high-level

containment isolation rooms. For most PGD clinics, those facilities are not available, may be cost prohibitive, and are somewhat extreme. One of the strongest allies in controlling surface contamination in the laboratory is the use of UV light. In a little as five minutes, exposed surfaces can be decontaminated (Ou *et al.*, 1991). In our studies, a significant difference was seen in as little as ten minutes although it required 20-30 minutes for complete inhibition of PCR amplification (Gitlin and Gibbons, 1995).

UV rays have shorter wavelengths (10-400nm) than visible light (400-700nm). Exposure to UV irradiation less than 300nm affects the DNA by changing the structure of the template; specifically germicidal lamps emit 254 nm. (Sarkar and Sommer, 1990). The pyrimidine bases (thymine – T and cytosine – C) are particularly sensitive to UV exposure. UV light causes the formation of dimers –stable bonds between identical molecules or units of molecules; in this case, T-T or C-C. Polypyrimidine dimers, T-C, can form as well. The formation of these dimers interferes with the subsequent DNA replication since the pyrimidine dimer cannot fit into the double helix (Stryer, 1988). The structure of the initial DNA template has now been altered and subsequent replication would be blocked as well since the DNA polymerase could not bind.

Successful contamination control using UV light is a function of time and distance of the UV light source to the exposed surface (Sarkar and Sommer, 1993). In order to determine the risk in our laboratory, a study was designed to place DNA-containing PCR reaction tubes in strategic and functional settings in the laboratory, then expose the tubes to ultraviolet light at timed intervals. These assessments allowed us to determine the limits of potential contamination control measures. UV irradiation was shown to be ineffective for products as small as 380 base pairs (Belak and Ballagi-Pordany, 1993) although there are little concurring data available.

UV irradiation is used to minimize the occurrence of contamination. Most reagents used in the PGD laboratory are aliquoted for individual use. After preparation and dispensing in microcentrifuge tubes, those aliquots are exposed

to UV light in the event that inadvertent DNA contamination occurred. The exception to UV exposure is the *Taq* polymerase, the oligonucleotide primers, and the dNTP's. Frothingham *et al.* (1992) studied the effect of UV light to decontaminate the components of the PCR master mix. Their study demonstrated that a longer UV exposure was required when the dNTPs were in the mixture versus when the dNTPs were absent (64 minutes versus 4 minutes, respectively). The researchers concluded that the dNTPs had a higher absorbance of the UV light that effectively decreased the overall efficiency of the PCR decontamination. Another arm of the study determined the sensitivity of the polymerase and the primers to the UV light exposure. Mixtures containing the primers and polymerase ± the dNTPs were exposed to UV light. DNA sample was then added to the UV-exposed tubes and the samples were subjected to PCR. Those tubes containing the dNTPS amplified while those tubes without the irradiated dNTPS did not amplify. This again reflects a protective mechanism of the dNTPs that have absorbed the UV radiation and demonstrates the sensitivity of the polymerase and primers to UV light exposure (Frothingham *et al.*, 1992). Similar findings were reported that a mixture of dNTPS and primers was not decontaminated after UV irradiation even though a stock of the primers in solution by themselves was successfully decontaminated (Ou *et al.*, 1991)

Additionally, the mineral oil used to overlay the PCR reaction was exposed to UV light in our laboratory. Caution is exercised when performing the mineral oil irradiation based on a study performed by Dohner *et al.*, (1995). Those researchers determined that an inhibitory effect on PCR amplification could be achieved in as little time as 30 minutes with a direct exposure of mineral oil in an open container. However, that effect was diminished when the oil was exposed through the PCR tubes since the plastic will absorb much of the radiation. The reason for this was not ascertained; the authors discussed the possibility of either inhibition being due to oxidation of some component in the mineral oil or perhaps due to ozone formation as a result of the UV irradiation of oxygen in the air.

2.3.2 Allele dropout

Of great concern is the occurrence of ADO, defined as the amplification of only one of the two alleles present in the PCR reaction (Findlay *et al.*, 1995; Ray and Handyside,1996; Rechitsky *et al.*, 1998). In the heterozygous condition, both the normal and the abnormal allele are present. If only one of those alleles amplifies, a misdiagnosis for that cell (and thus subsequent embryo) can occur. Since the homozygous normal and homozygous affected conditions have the two copies of the same allele, dropping-out of one of the alleles would not be detected by conventional PCR.

The allele drop-out rate was determined when analyzing polar bodies in a study of over 1000 oocytes by Rechitsky *et al.*, (2001). The ADO was 7.8%. ADO is one of the greatest concerns for a misdiagnosis in PGD, occurring in the heterozygous condition when only one of the two alleles present amplifies. One disadvantage of PB testing is crossing over or genetic recombination. Analyzing both PBI and PBII in a maternal carrier is a means to avoid undetected ADO. Rechitsky *et al.*, (1998, 2001) documented the possible outcomes when performing PGD on polar bodies. The researchers ascertained that the most accurate information occurred when PBI was determined to be a heterozygote, indicating that crossing-over was detected. Results indicating a heterozygous PBI and a normal wild-type PBII infer that the oocyte is carrying the mutant allele; the heterozygous PBI has both alleles, and thus the remaining strands in the oocyte would too. Therefore, upon strand separation, the chromatid in PBII has the opposite result of the chromatid remaining in the oocyte. Conversely, a heterozygous PBI and a mutant PBII infers that the oocyte is normal.

Further examination of ADO is in the discussion section for Specific Aim # 2, which studies the effect on amplification specificity when using different DNA lysis techniques.

2.3.3 Aneuploidy Screening – Assessing chromosome number

From 1923 until 1956, the prevailing opinion was that human cells contained 48 chromosomes, based on studies by the geneticist Thomas Painter (Painter, 1923). The fundamental discovery that normal human somatic cells

contained 46 chromosomes arranged in pairs was made in 1956 (Tjio and Levan, 1956; Ford and Hamerton, 1956). However, those researchers also discovered that cells and even entire individuals could have a deviation in chromosome number from the normal 46. Soon after, the field of cytogenetics began. The oldest technique for enumerating chromosomes (ploidy) and analyzing structural defects (aberrations) is karyotyping, developed in the late 1950's. As time and technology progressed, the resolution and sensitivity of cytogenetic analysis have improved more than 10,000-fold with techniques such as chromosome banding, fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH), multifluor FISH (mFISH), spectral karyotype analysis (SKY), and array CGH as discussed below.

Ten years after the first use of karyotyping, chromosomal banding techniques were applied to detect structural abnormalities; metaphase chromosomes were stained with quinacrine mustard to produce patterns of light and dark bands (Q-bands) along the chromosome (Caspersson *et al.*, 1968). Similarly, the technique of Giemsa staining was used (G-banding); the dark regions tend to be heterochromatic, late-replicating and A-T rich while the bright regions tend to be euchromatic, early-replicating and G-C rich (Bosman and Schaberg, 1973; Comings *et al.*, 1973).

There are two major limitations of karyotyping: the need for cells to be in metaphase and the need for higher resolution to detect smaller aberrations in the chromosome structure. This prompted the development of cytogenetic techniques such as FISH that did not require metaphase chromosomes. (van Prooijen-Knegt *et al.*, 1982). In FISH, fluorescent-labeled probes are designed complementary to specific regions along the length of the studied chromosome. After probe/sample hybridization, the fluorescent signal can easily be detected by microscopy. The technique is limited in that it requires prior knowledge of the location of the aberration. Also, FISH is used for only a limited number of chromosomes due to the ability to detect only a handful of signals. Initially, only three fluorochromes were commercially available to label chromosomes (fluoroscein isothiocyanate - FITC, tetramethylrhodamine isothiocyanate - TRITC,

aminomethylcoumarin - AMCA, respectively emitting in green, red, and blue, Nederlof *et al.*,1990; Dauwerse *et al.*, 1992). To overcome this limitation, combinations of these dyes in distinct ratios allowed for their use in labeling more than three chromosomes. Currently, there are two additional fluorochromes (totaling five) that can be used for labeling. Cells are initially analyzed with one set of five fluorochromes, then can be re-analyzed with a different set of labeled probes (Benadiva *et al.,* 1996; Martini *et al.*, 1997; Bahçe *et al.*, 2000). The utility of FISH in PGD has long been debated. Initial reports had determined that there was no difference in the pregnancy or implantation rate, but newer data suggest that there is an increase in pregnancy (Werlin *et al.*, 2003) and implantation rates (Munné *et al.*, 2002), especially in patients with more than eight pronuclear embryos, and in those with less than 2 failed IVF cycles (Munné *et al.*, 2003)

Conventional CGH can detect and map changes in DNA copy number (Kallioniemi *et al.*, 1992; Thompson and Gray, 1993). Sample DNA and normal reference DNA are labeled with two different fluorochromes. The sample and reference DNA are then hybridized simultaneously to a slide containing normal metaphase chromosome spreads. Images of both fluorescent signals are captured and a ratio of intensities of the two fluorochromes is measured; regions of DNA sequence loss or gain (such as deletions, duplications, or amplifications) are detected by the ratios. CGH is limited in that it cannot detect mosaicism, balanced chromosomal translocations, inversions or whole genome ploidy changes (Kallioniemi *et al.*, 1994). Additionally, CGH applications with limited template DNA such as single cell analysis would require a WGA technique as previously described since most CGH protocols require at least 200ng of DNA (Wells, 2004).

Other techniques such as SKY (McNeil and Ried, 2000) and mFISH have overcome these limitations by allowing visualization of the entire chromosome (as opposed to regions of the chromosome seen in FISH or changes in copy number as seen by CGH). SKY involves the simultaneous hybridization of 24 differently labeled chromosome-painting probes. After a one to three day hybridization, images are visualized under fluorescent microscopy and acquired

via a CCD camera linked to an imaging system. A single image is used to analyze fluorescently labeled chromosomes to produce a full karyotype. M-FISH is a technique to label every chromosome, generating a 24-color composite image (as opposed to the single image in SKY) that can be computer analyzed by the appropriate software to produce a karyotype.

Array CGH uses a combination of comparative genomic hybridization and microarray technology to determine variation in DNA copy number or to determine chromosome aneuploidy (change in chromosome number). The method has the sensitivity to detect single copy changes, deletions and duplications in the genome with high precision/resolution (Pinkel *et al.*, 1998, Pollack *et al.*, 1999; Yu *et al.*, 2003). This technology has been applied to successfully detect aneuploidies in tumor cells (Gray and Collins, 2000; Phillips *et al.*, 2001; Wilhelm *et al.*, 2002; Linn *et al.*, 2003). Array CGH was compared with FISH in 12 patients with known chromosome deletions. One additional deletion was detected by array CGH that had not been detected by FISH, demonstrating the higher sensitivity of the array CGH (Daigo *et al.*, 2001).

Array CGH can overcome one of the major limitations of CGH; CGH requires metaphase chromosomes for hybridization while array CGH utilizes cloned DNA fragments that will allow aberrations to be determined in greater detail (Oostlander *et al.*, 2004). As in conventional CGH, the sample and the normal reference DNA are labeled with two different fluorochromes. The two are then simultaneously hybridized to a glass slide array containing genomic or cDNA. As in conventional microarray analysis previously explained, the ratio of the dye intensities is compared to determine the DNA copy level. One major limitation with array CGH, as in conventional CGH, is its inability to detect balanced chromosome translocations since the number of chromosomes is the same in the balanced state (Daigo *et al.*, 2001)

2.3.3.1 Cytogenetic techniques applied to human oocytes and blastomeres

Advances in cytogenetic techniques have made their use possible in PGD. Less than half of naturally conceived pregnancies reach full-term, with the majority of pregnancy losses occurring at the early embryo or shortly after

implantation stages (Shepard and Fantel, 1979; Byrne *et al.*, 1985). Multiple factors may be involved in these losses although chromosomal error is considered to be one of the most significant factors. These errors could result in altering the regulation of gene expression during the complex process of early embryo development and implantation. Determining the chromosomal status of oocytes and/or embryos during an IVF cycle may be beneficial for several types of patients that are at greater risk for chromosomal abnormalities, including those over the age of 35 (Hassold *et al.*, 1980; Warburton, 1980; Munné *et al.*, 1995; Dailey *et al.*, 1996), women with a history of recurrent miscarriages (Pellicer *et al.*, 1999), women with a history of repeated IVF failure (Gianaroli *et al.*, 1999), and/or couples carrying a known chromosomal structural abnormality (Munné *et al.*,1998a). In addition, detecting chromosome errors may provide insight into the frequency of abnormalities during gametogenesis, embryonic development, and pregnancy (Wells and Levy, 2003).

FISH is the most common technique utilized for detecting an extra or missing copy of select chromosomes in oocytes and embryos (aneuploidy). Currently, the standard for chromosome analysis in embryos is to analyze a single blastomere for nine chromosomes by FISH using two sets of labeled probes (Munné *et al.*, 1998b). These researchers tested for chromosomes 13,15, 16, 17, 18, 21, 22, X, and Y which encompass the majority of chromosomal errors seen in miscarried tissue or in live born. Approximately 85% of the chromosomal abnormalities seen in embryos are detected by 9-color FISH (Voullaire *et al.*, 2002). Abdelhadi *et al.* (2003) performed FISH using probes for 13 chromosomes using three sets of labeled probes with the aforementioned chromosomes as well as a third probe set with chromosomes 2,3,4, and 11. In a comparison study of 426 embryos, 181 (42%) were aneuploid for one or more chromosomes. These researchers detected 97% of the total chromosomal errors utilizing the first two probe sets while only an additional 3% of aneuploidy was detected with the third set. From these studies, the researchers concluded that it may be more efficient to continue with only the first two probe sets and that the

additional 3% aneuploidy detection may not be time efficient or cost effective for the benefit received.

Other cytogenetics techniques have been applied to oocytes/embryos as well. Normally, it is difficult to karyotype the oocyte/embryos since the cell number is limited and the cell must be in metaphase. In an attempt to karyotype the PBII, Verlinsky and Evsikov (1999) removed 38 PBII from normally fertilized zygotes and injected them into individual enucleated oocyte cytoplasm. After forming a haploid pronucleus, 25 metaphase spreads (66%) could then be karyotyped by whole chromosome painting. Although the researchers concluded that this method provided a suitable timetable for chromosome analysis and utilized extra embryonic material (PBII), the method has not been widely used. This is presumed to be due to the labor and time intensity for a method that requires further optimization to achieve greater than 66% success.

Clyde *et al.* (2001) demonstrated the use of mFISH in the oocyte and PBI. Marquez *et al.* (1998) and Sandalinas *et al.* (2002) applied SKY to analyze the chromosomes from polar bodies. That study analyzed 131 fresh oocytes from donors. Of those, only 47 could be used for SKY: of the remaining 84, five were not metaphase, 11 had incomplete metaphase, and 68 had overlapping chromosomes. Twenty-four of the 47 oocytes used were normal or haploid (51.1%), broken down into 64% normal for women under 34 and only 25% normal in women over 35 years. These studies demonstrated the use of the technologies on human oocytes (polar bodies), however further optimization appears to be needed before their practical clinical use.

CGH has been applied to clinical PGD cycles (Voullaire *et al.*, 2002; Wilton *et al.*, 2003). In the former study, 141 embryos were biopsied and analyzed by CGH; 126 (89.3%) had analyzable signals. Seventy-six of 126 (60%) of the single biopsied blastomeres from 20 women with repeated IVF failure were diagnosed as abnormal by CGH. Had those cells been analyzed by standard 9-chromosome FISH instead, at least 18 of those 76 abnormal embryos (23.7%) would have been diagnosed as normal since the aneuploidy detected by CGH was found in a chromosome outside of the standard nine. The researchers

concluded that by using CGH, additional chromosomal abnormalities were diagnosed in this patient population that would not have previously been detected by FISH and may thus help explain their repeated IVF failure.

The use of CGH to analyze a single cell is limited since the technique generally takes over 72 hours to perform; embryos biopsied on day 3 would need to be cryopreserved for transfer in a later cycle since results would not be available during the normal implantation window of 5-6 days after egg retrieval. In a promising study, Wells *et al.* (2002) demonstrated that full chromosome complement information from the PBI could be obtained by CGH. Using an accelerated CGH protocol, the time was reduced in half from 60 hours to 30 hours, enabling embryo transfer on day 4. In that study, 12 oocytes were subjected to PBI biopsy. Of the 11 available for analysis, 10 demonstrated CGH results, yielding only one normal embryo available for transfer. Where possible, results were confirmed by FISH after subsequent single cell embryo biopsy. Interestingly, four of the 9 abnormal embryos demonstrated aneuploidy for chromosomes 2, 5, 14, and 20; these chromosomes are not routinely used in FISH analysis. Had only FISH been used for the aneuploidy screen, those embryos would have been eligible for transfer. This demonstrated the utility of the CGH technique in being able to screen for all chromosomes and detect errors that would have otherwise been undiagnosable with the standard technique.

Array CGH to enumerate chromosomes in human single cells has had limited use thus far. The first published study obtained single lymphoblast cells from normal and trisomic cell lines, then subjected individuals cells to array CGH using DOP as the WGA technique (Hu *et al.*, 2004). Fourteen normal male or female cells were analyzed with the correct ratio 97% (300/308) of the time for the 22 autosomes. While 93% (13/14) of the X chromosomes were detected, only 29% (4/14) of the Y chromosomes were detected. The trisomic cell lines were all diagnosed correctly as falling in the correct ratio range of 0.75-1.25 (as previously discussed, the ratio of dyes should be 1:1. Here the limits of the ratio were 1.0 ±0.25). The overall time for the entire procedure was 30 hours. In a separate study, MDA was used as the WGA prior to array CGH of trisomy 21 single

lymphocytes (n = 3, Hellani *et al.*, 2004). All three samples demonstrated the ability to detect trisomy 21. From these studies, the researchers concluded that array CGH could become a feasible option for aneuploidy studies in human oocytes/embryos although further optimization is required, especially in the ability to detect the Y chromosome.

The studies of this dissertation

The largest collection of PGD data to date is from the ESHRE (European Society for Human Reproduction and Embryology) PGD consortium, collating data from 25 PGD centers worldwide including our institution (ESHRE PGD consortium steering committee, 2002). Even though those data support the usefulness of the technology, a few key points need to be discussed. The overall amplification efficiency in that report was only 83% for all embryos biopsied (n=14,040). This suggests there is much room for improvement of amplification efficiencies. Even more compelling is the misdiagnosis rate for PGD cases utilizing the polymerase chain reaction as the diagnostic tool. Overall, the misdiagnosis rate was 3.4% for the 145 cases reported.

Lewis *et al.,* (2001) reported on a mathematical model designed to aid in determining the accuracy of PGD using single cells. It determines that in any biological system, variability can occur that can affect clinical decisions. The model examines values for the probabilities of amplification occurring, the cell's chromosome content, and the probability for contamination occurring. The authors state that the major sources of error leading to a misdiagnosis are identifiable. This dissertation addresses the three main sources of error that Lewis *et al.* (2001) and Navidi and Arnheim (1991) list:

1) analyzing the anucleate cell

2) contamination

3) non-amplification of alleles

Further, the authors suggest that acceptable levels of accuracy and contamination be established. They established a decision-making model as part of the process to determine which embryos can be transferred and which can be discarded, knowing that the assessed genotype has a high probability of stemming

from an affected embryo. The importance of being able to optimize the conditions for genetic analysis while avoiding the sources of error may translate into the ability to have more embryos available for transfer. The number of embryos is positively correlated with the chance of establishing a successful pregnancy (Vandervost *et al.*,1998).

This dissertation presents studies that were conducted to optimize the conditions involved in the current diagnostic procedure of PGD in addition to developing new technologies. These efforts to maximize the amplification efficiency while minimizing the events that could lead to a misdiagnosis are part of the critical basic science background that lead to the clinical fruition of this technology. This continues as part of an ongoing effort to offer other single cell diagnostic procedures for clinical application. To that effect, the specific aims of these studies are as follows:

SECTION 3
SPECIFIC AIMS

Specific Aim # 1

Inadequate sensitivity of the PCR reaction may result in amplification failure when starting from a one or two copy DNA template. If DNA is truly present in the initial PCR reaction tube yet does not amplify, modification of reaction conditions or techniques might subsequently produce sufficient quantities of DNA to demonstrate PCR amplification after gel electrophoresis.

Aim: To increase amplification efficiency to 'rescue' those cells fail initial PCR amplification.

Hypothesis: Whole genome amplification methods (WGA) can be used to increase the number of target sites of the initial starting template in attempts to increase amplification results.

Experiments: Experiment # 1 will to use a WGA technique consisting of a mixture of random primers sequences (in this case, random 15-mers) in PCR cycling conditions with dNTPS and *Taq* polymerase, producing multiple copies of DNA sequences in the genome. It is estimated that 78% of the genome is copied at least 30 times with the WGA technique, even from a single cell (Zhang *et al.*, 1992). Having multiple copies of initial starting template can aid in the elimination of the variable amplification efficiencies seen when starting with a template from one cell.

Experiment #2 will attempt to provide additional genetic information from previously PCR-processed DNA by amplification of a different gene in that reaction. For example, PGD samples that were previously diagnosed for gender determination will be tested for the specific X-linked mutation that is now known. The initial starting template is still present in the reaction tube although the reaction also contains amplified product, dNTPs, primers, etc. Rather than subjecting embryos to another biopsy procedure, this experiment proposes to recover the previously PCR'ed reaction tubes, add additional specific primers and PCR master mix in an attempt to obtain further genetic information from the initially biopsied cell. Outcomes will be measured by successful PCR

amplification while negative controls will also be tested to ensure that contamination has not occurred.

Specific Aim # 2

Allele drop-out (ADO) has been identified as a cause for misdiagnoses in PGD cycles (Grifo *et al.*, 1998; ESHRE PGD consortium steering committee, 2002). ADO is defined as the amplification of only one of the two alleles present in a cell.

Aim: To increase the amplification efficiency through optimization of DNA lysis conditions and to decrease the incidence of ADO .

Hypothesis: DNA contained in the cell may not be fully accessible by the presently used DNA lysis conditions; alternatively, the template may not be fully denatured. Consequently, the PCR primers and subsequent polymerase cannot attach to amplify that segment of the DNA.

Experiments: In these experiments, individual lymphoblast cells known to be heterozygous at specific DNA loci will be used to compare different methods of DNA lysis with amplification results, analyzing not only the efficiency of amplification (as a percentage), but also the accuracy as determined by the presence of both alleles in these known heterozygous cells. Both heterozygote and compound heterozygote cells will be used to compare the rates of amplification efficiency and ADO. The lysis techniques selected were the most commonly used techniques for DNA lysis in single cell analysis (Handyside *et al.*, 1990; Zhang *et al.*, 1992; Verlinsky and Kuliev, 1992; Gibbons *et al.*, 1995a; Grifo *et al.*, 1998).

Specific Aim # 3

After the above Specific Aims are addressed, there remain instances when PCR amplification may be limited when using a single blastomere from the embryo.

Aim: To identify characteristics of the blastomeres from an embryo before and after embryo biopsy that can aid in determining the success of obtaining PCR amplification.

Hypothesis: Defining optimal characteristics of the blastomere in an embryo before and after biopsy would allow better predictions and determinations of

obtaining successful amplification results. It is important to be able to choose a blastomere to biopsy that has a greater chance of providing a genetic result.
Experiments: These experiments will identify conditions relating to morphology of the removed blastomere and correlate those with amplification efficiencies. The following morphological criteria will be assessed by microscopy: the presence or absence of the blastomere nucleus (which may relate to the state of the DNA), the presence of the cell membrane, the encroachment of the cytoplasm (intact or not). These assessments will be compared with PCR amplification results.

Specific aim # 4

The diagnosis of having missing or extra chromosomes is known as aneuploidy. Aneuploidy is common in human embryos studied after in vitro fertilization (Munné *et al.*, 1998b). Currently, the state of the art to identify chromosomal information in a single cell is limited to primarily using nine-color fluorescent in situ hybridization (FISH) for the most common chromosome abnormalities in either liveborn or miscarried tissues (chromosomes X,Y, 13, 15,16, 17 18, 21, and 22.)

Aim: To identify the chromosomal complement in a single cell.
Hypothesis: Array CGH can be used to study aneuploidy in single cells including blastomeres biopsied from human embryos.
Rationale: Array CGH combines the principles of CGH (comparing a normal control with the unknown sample labeled in a different color fluorochrome), then hybridizing those samples to a microarray. Array CGH has been proposed in the field of tumor biology as a reliable method for assessing chromosome number (Pollack *et al.*, 2002).
Experiment #1: Initial experiments will address the issue of the limited amount of template DNA in a single cell. Generally 5-10μg of DNA is needed for adequate hybridization of a microarray. To increase the amount of DNA available in a single cell, WGA technique known as Multiple Displacement Amplification (MDA) will be used (Lage *et al.*, 2003). In an overnight MDA reaction, over 35μg of DNA can be produced from the initial starting quantity of 10pg in a single cell to achieve quantities adequate for microarray analysis.

Experiment #2: Once the MDA technique is specific and sensitive for use in a single cell, the DNA will be fluorescently labeled and prepared for microarray analysis. The experiments proposed here will determine a labeling technique that is optimal for microarray analysis.

Experiment #3: Third, the labeled DNA will be hybridized to a cDNA microarray and analyzed to determine the chromosome complement. Initially, normal euploid cells will be used a controls until proof of concept is established. Once answered, the feasibility of the use of array CGH for study of aneuploidy in humans will be addressed, an IRB proposal submitted, and the final aspects of the study with regards to sensitivity and specificity of the technique will be determined. This will be accomplished initially by using single cells with known chromosomal abnormalities (e.g. amniocytes, and/or cancer cell lines obtained from the cytogenetics laboratory) prior to assessing aneuploidy rates in human supernumerary embryos donated for research.

3.1 Study models

Three types of cells were used in these experiments as described in the Material and Methods sections of each Specific Aim. Human cells were used exclusively since the ultimate object of these studies was to diagnose human disease. The limited availability of human embryos/blastomeres with the studied disease necessitated another cell type for study. Human lymphoblast cells served as an excellent source for optimization studies in Specific Aims # 1, 2, and 4 since cell lines with and without specific disease mutations were easily obtained. However, different cell types such as lymphoblasts may behave differently due to the chromatin arrangement for the particular cell, therefore supernumerary human embryos that were donated for research were used for final optimization of studies to control for those differences. Finally, blastomeres from actual IVF/PGD patients were obtained and studied for Specific Aims # 1 and 3. All procedures were performed under protocols approved by the Institutional Review Board of Eastern Virginia Medical School as deemed necessary.

SECTION 4
STUDIES FOR SPECIFIC AIM # 1

Specific Aim # 1: To increase amplification efficiency by attempting to 'rescue' those cells that show no initial PCR amplification.

4.1 Material and Methods for Specific Aim # 1

Sources of DNA

DNA was obtained from three sources:

1) Single cells were collected using lymphoblast cultures of known DNA mutations (Coriell Institute, Camden, NJ). Cells were cultured in RPMI-1640 media supplemented with 15% Fetal Bovine Serum (Invitrogen, Carlsbad, CA) in a 37°C incubator with 5% CO_2 and air. Single cells were isolated under a dissecting microscope and serially rinsed through three microdrops of PCR buffer to remove any extraneous cells. The individual cell was then visualized under the dissecting microscope as it was placed in a PCR reaction tube (Gene-Amp, Perkin-Elmer, Norwalk, CT) containing 60 μl sterile DNAse-free water overlaid with sterile mineral oil. Similarly, cells from these cultures were pooled to obtain genomic DNA controls.

2) Individual blastomeres were obtained from growth-arrested abnormally fertilized human embryos. Embryos were placed in an acidified Tyrode's medium to remove the zona pellucida, and then rinsed in culture media. The zona-free embryo was subsequently placed in a calcium-free/magnesium free phosphate buffered saline supplemented with 1% Bovine Serum albumin (Sigma, St. Louis, MO) to dissociate the cells. Individual cells were then collected as described above for the lymphoblasts.

3) Individual blastomeres were obtained from couples undergoing a PGD procedure. Individual cells were removed from the embryo by micromanipulation (embryo biopsy) as described below.

Embryo Biopsy

Embryo biopsy was performed using a Nikon Diaphot Microscope (Nikon, Melville, NY) with Hoffman optics (Modulation Optics, Greenvale, NY). Three sets

of Narishige micromanipulators MN-2 (Narishige USA, Greenvale, NY) were used for the holding pipette, acidified media, and the biopsy pipette. Micropipettes were prepared with a Sutter P-87 pipette puller that initially pulled borosilicate capillary tubes. A Nikon MF-9 microforge was then used to fire-polish the pipettes to their respective diameters listed below.

The embryo was held in place with a 35 micron holding pipette connected to a Narishige IM-6 microinjector. Acidified Tyrode's medium was delivered through a 10 micron I.D. micropipette attached to a similar microinjector. Biopsy was accomplished through a 35-50 micron micropipette attached to a picoinjector.

A dish containing acidified Tyrode's was placed on the microscope stage. The respective pipette was lowered into the acidified media and filled. That dish was then replaced on the stage by a square culture dish containing a droplet of Hepes-buffered Earle's media supplemented with 7.5 % synthetic serum substitute (Irvine Scientific, Santa Ana, CA) layered with washed and equilibrated mineral oil.

Embryos for biopsy were individually removed from their culture dish and placed into the droplet. The embryo was immobilized by suction applied to the aforementioned holding pipette. Care was taken to situate the embryo so that a blastomere would easily be accessible. The acid pipette was then placed next to the zona pellicuda, and acidified Tyrode's was slowly released while the pipette was moved against the zona in an up and down motion. After several seconds, an opening broke through the zona and the acid flow was stopped. The acid pipette was moved away from the embryo, and the biopsy pipette was placed into the field. Embryo biopsy was performed by inserting the biopsy pipette in through the newly-created opening in the zona and placing the mouth of the pipette next to the arc of the desired blastomere. Suction was slowly applied so that the cell could be drawn into the pipette. The pipette was then removed from the embryo, and the aspirated cell was released into the media. The cell was then aspirated into a drawn-out Pasteur pipette and rinsed through a series of three droplets of PCR buffer to remove any potential contaminating extraneous

cells (from sperm or corona cells). The aspirated blastomere was then placed into a 0.5μl GeneAmp PCR reaction tube (Perkin Elmer, Norwalk, CT) containing 60μl sterile DNAse free water and layered with sterile mineral oil. A stereo microscope was used to visualize that the cell was successfully transferred to the PCR tube. The tube was then frozen/thawed twice in liquid nitrogen and heated to 100°C for 10 minutes to lyse the cell. All samples that were not analyzed immediately were stored at -20°C until use.

Initial Polymerase Chain Reaction

For these experiments, the PCR region to be amplified encompassed the 4 base pair (bp) insertion (1278+TATC) of the β-hexosaminidase A gene, the most common mutation causing Tay-Sachs disease. Outer and nested primer sequences are listed in Table II.

Reagents

A standard PCR master mix consisted of a 10X reaction buffer (1.5 mM $MgCl_2$, 10 mM Tris–hydrochloric acid, pH 8.3, 50 mM potassium chloride, and 0.01% [wt/vol] gelatin), 200 μM dNTPs (USB, Cleveland, OH), 0.2 μM primers (Genosys, The Woodlands, Tex.), and 0.5 unit of *Taq* polymerase (Perkin-Elmer). The remainder of the 100μl volume consisted of 2μl sample and 60μl of sterile water overlaid with 60μl oil.

Nested reaction:

A 2 μl aliquot of the initially amplified product was removed and added to a new PCR reaction tube containing 60μl of sterile water overlaid with 60μl oil, along with fresh master mix containing the new primer set in addition to the other master mix components (buffer, dNTPs, *Taq* polymerase).

Thermocycling conditions continued as described in Table III.

Heteroduplex Formation

A four base pair difference in the PCR product is difficult to distinguish between the normal and abnormal condition when applied to a fast-running polyacrylamide gel. Therefore, an artifact known as heteroduplex formation is used to distinguish between small base pair insertions and deletions (Nagamine *et al.*, 1989; Triggs-Raine and Gravel, 1990). The rationale for this comes from

Table II. Oligonucleotide primer sequences

Gene Loci	Outer primers 5' to 3'	Nested primers 5' to 3'
exon 11, 4 bp insertion β-Hexosaminidase A. (Tay-Sachs disease)	F – CAACAACAGTCTGGTGATGG R - GCCAGACACAATCATACAGG	F- AAGGAGCTGGAACTGGTCAC R - CTCTCAACCACCTTCCCAAT
Δ F508 mutation of cystic fibrosis transmembrane regulator gene.	F - GACTTCACTTCTAATGATGAT R- CTCTTCTAGTTGGCATGC	F- TGGGAGAACTGGAGCCTT R -GCTTTGATGACGCTTCTGTAT

F= forward primer, R = reverse primer.
Outer primers used for first 20 cycles of PCR at specified annealing temperatures, Nested primers used for remaining 30 cycles of nested PCR. PCR conditions described in Table III.

Table III. Thermocycling conditions

Initial reaction	Nested
For the 4bp insertion of the β-hexosaminidase A gene:	
94°C Initial denaturation 3 minutes 20 cycles of: Denaturation at 94°C for 1 minute Annealing at 61°C for 1 minute Extension at 72°C for 1 minute +2 second increase after each cycle	94°C Initial denaturation 3 minutes 30 cycles of: Denaturation at 94°C for 1 minute Annealing at 54°C for 1 minute Extension at 72°C for 1 minute +2 second increase after each cycle
For the ΔF508 mutation of cystic fibrosis:	
Initial reaction	Nested
94°C Initial denaturation 3 minutes 20 cycles of: Denaturation at 94°C for 1 minute Annealing at 40°C for 1 minute Extension at 72°C for 1 minute +2 second increase after each cycle	94°C Initial denaturation 3 minutes 30 cycles of: Denaturation at 94°C for 1 minute Annealing at 50°C for 1 minute Extension at 72°C for 1 minute +2 second increase after each cycle

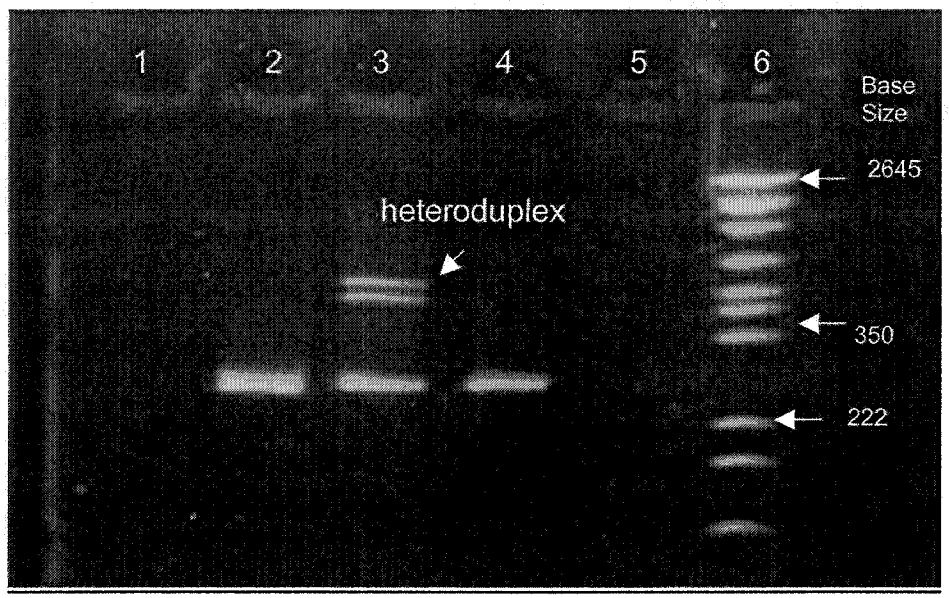

Figure 8. Gel migration for small insertion or deletion. Polyacrylamide gel (7%) photo representing the gel migration pattern for the 4 bp insertion in exon 11 of the β-hexosaminidase A gene (Tay-Sachs, TSD-11).

Lane 1 – negative (no DNA control)
Lane 2 -normal, wild-type
Lane 3 – heterozygous carrier for TSD-11
Lane 4 – affected TSD-11 with the 4 base pair insertion
Lane 5 – negative
Lane 6 – pGEM DNA marker

This gel illustrates the need for further information when attempting to discern the difference in the four base pair insertion. Although the normal (and thus smaller) DNA fragment ran slightly faster on the gel, the information is insufficient to make the critical diagnosis between normal and affected.

the heterozygous condition that has one normal allele and one abnormal allele with the extra four base pairs in this particular case. Those alleles do not exactly match up at the insertion, therefore, an unmatched loop of 4 base pairs is created. That loop causes a resistance in the migration pattern of the DNA when applied to a polyacrylamide gel, resulting in the characteristic heteroduplex or double band formation seen on the gel (Figure 8).

The above example is for the known heterozygous condition. To distinguish the 4-base pair difference between the normal and abnormal alleles in an unknown sample, a mixing experiment is performed. Previously amplified known normal and known abnormal DNA samples is added or mixed with the unknown sample into separate PCR reaction tubes. This is accomplished by adding an equal volume of the known amplified product to the unknown PCR sample in separate aliquots. The mixture is heated to 95°C for 5 minutes to denature the strands, cooled to 65°C for 5 minutes to allow the new mixed strands to anneal, and then cooled to 4°C to stop the reaction. Thus each unknown has 3 lanes on the polyacrylamide gel: the unknown by itself, the unknown plus known amplified normal PCR product, the unknown plus known amplified abnormal PCR product. The pattern of the gel migration and heteroduplex formation will determine the diagnosis (Figure 9).

Post PCR Gel Electrophoresis

Five microliters of a bromophenol blue loading buffer was added to 20μl of PCR amplified or mixed product and applied to a 7% polyacrylamide gel (29:1 acrylamide:bis), run at 175 V using a Fisher Biotech power supply for 90 minutes. The gel was dismantled and stained for 15 minutes in 0.5mg/ml ethidium bromide (Sigma, St. Louis, MO), de-stained with ultrapure water, and photographed under ultraviolet light trans-illumination.

Reamplification Experiments

PEP was used as the WGA method to ascertain if the amplification failure was due to lack of sensitivity. Samples that had previously been subjected to PCR were stored frozen at –20°C in the original PCR tubes containing the cell

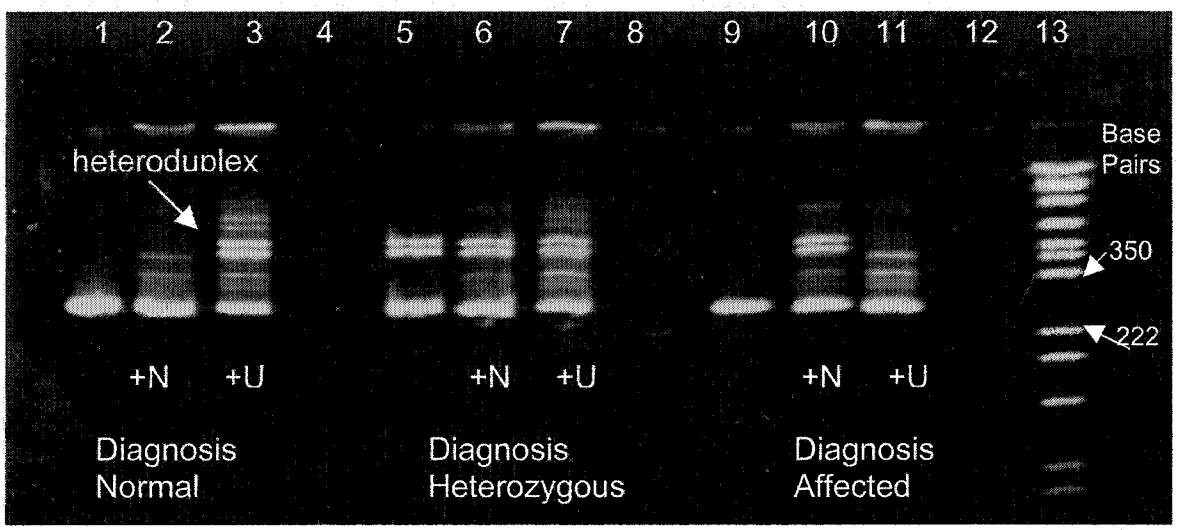

Figure 9. Heteroduplex formation. Polyacrylamide gel (7%) photo representing heteroduplex formation used for diagnosis of TSD-11 (and other deletions/insertions). The artifact known as the heteroduplex is present only when the normal allele and the affected (4bp inserted) allele are in the sample together and do not match up, causing a slower migration pattern on the gel. The diagnosis is made by evaluating the migration pattern of three lanes, knowing that only the normal and affected together will produce the heteroduplex.

Lane 1 - Normal (wild-type) sample
Lane 2 - Normal sample mixed with known normal DNA (+N)
Lane 3 - Normal sample mixed with known affected DNA (+U)
Lane 4 – Negative control
Lane 5 – Heterozygous sample
Lane 6 - Heterozygous sample mixed with known normal DNA (+N)
Lane 7 - Heterozygous sample mixed with known affected DNA (+U)
Lane 8 - Negative control
Lane 9 - Affected sample
Lane 10 - Affected sample mixed with known normal DNA (+N)
Lane 11 - Affected sample mixed with known affected DNA (+U)
Lane 12 – Negative control
Lane 13 – pGEM DNA marker

until further use. The initial PCR reaction volume was 100 μl and the PEP reagents were added directly to the initially tested reaction tube. A published PEP protocol (Zhang *et al.*, 1992) used a smaller reaction volume of 60μl; reagent volumes in the reamplification process were adjusted accordingly to maintain the same concentrations. The PEP reaction consisted of 13.5μl 10x Potassium-free buffer (100mM Tris-HCl, pH 8.3, 25mM $MgCl_2$, 1mg/ml gelatin), 12μl PEP primers (random 15-mers, Operon Technology, Alameda, CA), 12μl mixture of the 4 dNTPs at 2mM each (USB, Cleveland, OH), and 2.5μl *Taq* polymerase (15 units, Perkin-Elmer). Fifty cycles were carried out using a Perkin Elmer Thermocycler (Perkin Elmer Cetus, Norwalk, CT) as follows: 1 minute denaturation at 92°C, 2 minute annealing at 37°C, followed by a timed ramping segment of 10seconds/degree to 55°C, followed by an extension incubation step at 55°C for 4 minutes. A 5μl aliquot of the post PEP product was then removed and subjected to PCR as described above.

Reamplification without PEP using standard PCR

Reamplification was also attempted by simply re-analyzing the sample by PCR with additional master mix components without the PEP procedure. Since the volume of the initial failed reaction was 100μl, the volumes of the master mix components were adjusted accordingly to reflect the same final concentration of reagents in the increased volume. Reamplification was attempted with either primers for the 4 base pair insertion of the β-hexosaminidase A gene or for the 3 base pair deletion of the ΔF508 mutation of the cystic fibrosis transmembrane conductor regulator gene (CFTR). Primer pairs are listed in Table II; thermal cycling conditions are listed in Table III.

Contamination Controls

Negative PCR reaction controls that were previously subjected PCR were simultaneously analyzed in each reamplification reaction to serve as contamination controls for the reamplification process. Negative controls do not contain starting DNA template and showed no initial amplification due to lack of starting template.

Statistical analysis

Fisher's exact statistical testing was used to compare results of amplification for the different genes used (Appendix C). The computer program GRAPHPAD INSTAT (Graphpad Software, San Diego, CA) was used to assist in analysis.

4.2 Results for Specific Aim # 1

Of the 187 single cell lymphoblasts initially evaluated for Tay-Sachs, 97/187 amplified (51.9%). Of the 107 single cell lymphoblasts initially evaluated for cystic fibrosis (CF), 87/107 amplified (81.3%). The amplification efficiency for TSD was significantly lower than for CF ($P<0.0095$). Since the amplification efficiency was so low for TSD as compared with the other loci studied, a sensitivity study was performed. One cell, two cells combined, three, four, five cells were evaluated. Amplification efficiencies were significantly greater when analyzing two cells combined as compared to a single cell (97/187 vs. 16/17, 51.9% vs. 94.1% respectively, $P=0.0006$. Table IV).

Reamplification Studies without Primer Extension Preamplification (PEP)

After the DNA failed to amplify the first time with TSD primers, another attempt with additional TSD primers and fresh master mix did not improve the amplification rate as only three in 19 cells attempted demonstrated PCR amplification. This was in stark contrast to reamplification experiments with CF primers in cells that initially did not amplify for TSD. Of those, 18/25 (72%) amplified for the CF locus after failed TSD amplification. These data demonstrated that DNA was present in at least some of the PCR reaction tubes that initially failed to amplify and that it is technically possible to reamplify DNA in the stored PCR reaction tubes from previous amplification failures (Table V). This could circumvent the need to rebiopsy the embryo to obtain a genetic diagnosis.

Reamplification with PEP

Fifty-nine single lymphoblasts that did not initially amplify using the Tay-Sachs primers were subjected to PEP. An aliquot of the sample after the PEP reaction was then PCR analyzed using TSD primers. Amplification occurred 52.5% of the time (31/59) cells. This was statistically greater than when

Table IV. PCR amplification efficiencies

Number of Cells	Amplification efficiency
One cell	97/187 (51%)[a]
Two cells	16/17 (89%)[a]
Three cells	17/17 (100%)
Four cells	8/9 (89%)
Five cells	7/8 (87.5%)
With 2 cells or greater	32/34 (94%)

[a] P=0.0006.

Table V. Results of reamplification experiments

Experiment	Amplification result
Lymphoblast Reamplification by standard PCR Targeting same gene locus (TSD)	3/19 (16%)[a,b,c]
Lymphoblast Reamplification by standard PCR Targeting a different locus (CF)	18/25 (72%)[a]
Lymphoblast Reamplification by PEP Targeting the same locus (TSD)	31/59 (53%)[b]
Blastomere reamplification by PEP Targeting the same locus	13/24 (54%)[c]
Blastomere reamplification for PGD (initially 4/6 amplified, therefore 6 failed)	4/6 (67%)

[a] P=0.0003.
[b] P = 0.007.
[c] P = 0.0127.

compared with the reamplification attempt not using the PEP procedure at the TSD gene locus (16% versus 53%, *P*=0.007).

Reamplification using PEP for failed blastomere diagnosis

Initially, blastomeres (n=54) from supernumerary human embryos that failed to amplify were reanalyzed by PEP and subsequent PCR testing for TSD. Blastomeres were re-analyzed to ascertain if the procedure could be done for a clinical PGD. Thirteen of 24 amplified (54%) which was not statistically different from the 53% seen in PEP reamplification of the lymphoblasts. With this knowledge, we proceeded to reanalyze the contents of six PCR tubes from failed amplification in a PGD cycle. After the PEP protocol and subsequent PCR for the same TSD mutation, four of the six blastomeres amplified, and a diagnosis was made. Included in this experiment were three negative (no initial DNA) controls that were subjected to the same protocols. All three failed to amplify as expected, thus minimizing the possibility that inadvertent contamination of the PCR tubes with extraneous DNA was the source of the new amplification seen (Table VI).

4.3 Discussion for Specific Aim # 1

For couples where both partners carry a mutation for a recessive disease, the risk of having an affected child is 25% based on Mendelian genetics. When one partner carries the gene for a dominant disease, that risk increases to 50%. For many couples, especially those who have had a pregnancy and perhaps a live birth of an affected child, a 25% risk may be too great. The success with the technology used in PGD must far exceed the anticipated risk in nature, i.e., the PGD success has to exceed the 75% rate that nature statistically would provide. The question then becomes, what risks are acceptable? Since there is never a 100% guarantee of success with a scientific technology, how close to 100% must the test be and still allow a couple to accept that risk? Some of those risk evaluation issues were addressed by the studies presented here.

The initial pre-clinical studies performed in our laboratory demonstrated amplification rates over 90% when using individual lymphoblasts and blastomeres as the source of DNA. However, upon performing a PGD cycle for our first patient, the diagnosis rate was only 4/7 embryos, a disappointing 57%.

Table VI. Reamplification of blastomeres from PGD embryos

Embryo number	Blastomere reamplification result for PGD using PEP
RA Embryo 2	Reamplification failed
RA Embryo 4 blastomere 1	Amplified/normal diagnosis
RA Embryo 4 blastomere 2	Amplified/normal diagnosis
RA Embryo 5 blastomere 2	Amplified/normal diagnosis
RA Embryo 7 blastomere 1	Reamplification failed
RA Embryo 7 blastomere 2	Amplified/normal diagnosis
**********	**********
	Blastomere amplification for a different loci from previously sexed embryos
JB Embryo 1	Male – affected muscular dystrophy
JB Embryo 1A	Male - affected muscular dystrophy
JB Embryo 2	Male - affected muscular dystrophy
JB Embryo 5A	Male – Normal
JB Embryo 5B	Male – Normal
JB Embryo 7	Male – reamplification failed
JB Embryo 8	Male – reamplification failed

The reamplification result of Tay-Sachs disease is given on the right. JB embryos were previously diagnosed for gender only. The amplification result for the muscular dystrophy deletion is given on the right.

This led us to address the issues of amplification failure – what circumstances might cause it to occur and how to alleviate those possibilities. Once the test had been performed and initial amplification failed, was there anything that could be done to try to salvage the genetic diagnosis and provide that couple with the best information and possible opportunity at achieving pregnancy? That question led us to the experiments that were conducted in Specific Aim # 1.

One concern in the pre-clinical studies was the limited quantity of DNA in the starting template from a single cell. Amplification then becomes a sensitivity issue. Nested PCR was implemented to increase the sensitivity to the level of DNA in a single cell. Studies performed in our laboratory supported the hypothesis that 20 cycles of PCR followed by another 30 cycles of nested PCR was much more sensitive at DNA amplification than 50 cycles together (Figure 3). This supports the concept that PCR is a finite reaction; there is a limit to the exponential amplification process. The concentration of the dNTP's and *Taq* polymerase will begin to decline with subsequent cycles of PCR. Removal of an aliquot after several PCR cycles and refreshing the reaction with new primers, buffers, *Taq* polymerase rather than to just continue with additional cycles of PCR yielded a greater sensitivity in the detection of target DNA sequences.

After the results of poor amplification were revealed in the first clinical test, one corrective measure was to determine if having more initial template could resolve that issue. The original DNA remains in the PCR reaction tube, along with the PCR reagents and presumably amplified product. In the first phase of these studies, we used a WGA technique that had been shown to produce a minimum of 30 copies of at least 78% of the genome (Zhang *et al.*, 1992). The WGA technique used in the initial experiments is known as PEP. Primers are designed that randomly contain 15 base sequences of the nucleotides in every possible combination. When added to an initial DNA template, PCR is carried out over 50 cycles of denaturation, annealing of these random sequences, then new strand extension. This technique did not appear to have a practical clinical application in the initial amplification process since it takes approximately 10

hours to perform prior to the standard PCR testing for a PGD cycle. However, after these initial studies were performed, other researchers investigated methods to decrease the time involved in the procedure (Xu *et al.*, 1993; Sermon *et al.*, 1996). Less time could allow for the technique to become more practical for de novo PGD applications.

In the studies by Xu *et al.* (1993), the annealing time was decreased from the initial 150 seconds to 90 seconds and the initial four-minute extension time was decreased by either 10 seconds or 20 seconds. Those two changes resulted in a decrease of their overall reaction time from 8 hours to either 5 hours, 45 minutes or 4 hours, 4 minutes. The shortest time period showed no significant decrease in amplification efficiencies when compared to the longest time period (26/36 versus 48/60, respectively, $P = 0.4543$)

Initially, our study was deemed as proof of concept that the initial template be rescued after having been subjected to PCR amplification. The studies described here in the first experiments demonstrate that indeed the initial template can successfully be accessed and that subsequent PCR amplification can occur.

One of the issues of concern with a small amount of starting template is that reamplification could introduce contamination, especially in an assay designed to be sensitive to the level of a single cell. In these studies, negative controls (no initial starting template) were also subjected to the reamplification procedure. As expected, there was no amplification in those reactions; those results helped to rule out contamination or extraneous DNA as a cause for the amplification seen during the second attempt.

The reason for the inability to achieve successful amplification in the initial PCR attempt is not known. Clearly, there was DNA present in those tubes that subsequently amplified on the second attempt. The DNA may be in an unfavorable conformation and have a certain degree of secondary structure that may have prevented adequate strand denaturation or primer annealing in the first reaction. It is interesting to note that the reamplification attempts with a second set of PCR primers were more successful, even without using the whole genome

amplification step. This may suggest that the gene structure itself for the β-hexosaminidase A gene may then abrogate primer annealing or polymerase extension since amplification was successful 72% of the time when a different gene was studied in the same tubes.

Another reason for the initial amplification failure could be due to sensitivity of the PCR. The rationale for using a WGA technique is to increase the number of copies of the starting template for the PCR reaction. This attempt to reamplify for the same mutation after using PEP resulted in successful amplification in 53% of the cells when analyzing the lymphoblasts. Clinically, the method resulted in obtaining a diagnosis on two of the three embryos that did not previously have a genetic answer for the gene studied. However, the increase in time that the procedure requires has limited its clinical use for our laboratory. Instead, our laboratory chose to focus on other methods that may improve both the sensitivity and specificity while maintaining the ability to provide a genetic diagnosis within a day. That led us to perform the experiments listed in Specific Aim # 2 that follows this discussion.

As stated above, we demonstrated efficient amplification targeting different loci, even without performing the whole genome amplification. Those studies served as a model for a clinical application of diagnosing frozen embryos that had previously been tested for gender determination in the X-linked diseases. For example, a carrier of X-linked Duchenne Muscular Dystrophy (DMD) completed a PGD cycle where only the sex was determined for the embryos. After diagnosis and embryo transfer, the supernumerary embryos were cryopreserved. The amplified PCR tubes were stored at -20°C. Upon developing the technique for diagnosing the specific DMD deletion, the PCR tubes containing the blastomeres from previously-determined male embryos were thawed and subjected to amplification with the primers for that specific deletion. Successful amplification occurred in five of seven embryos, with three embryos being diagnosed as affected with the DMD deletion. This allowed for a subsequent transfer of the two normal male embryos without the need to re-biopsy the cell (Table VI).

4.4 Conclusions for Specific Aim # 1

From these studies we conclude that the process of whole genome amplification and subsequent PCR testing can be performed as a means of rescuing samples that previously failed to amplify in initial tests of PCR. The time involved to perform these tests limits the use of the PEP protocol initially, but the method still can serve as an alternative if amplification fails and the genetic status of the embryo is not known. This could abrogate the need to re-biopsy the embryo since an additional non-invasive technique is used to obtain the genetic answer. In addition, these studies laid the foundation for the clinical application of a reamplification procedure assessing a different genetic locus that resulted in the embryo transfer of male embryos whose previous genetic status was unknown in an at-risk X-linked carrier.

SECTION 5
Studies for Specific Aim # 2

Specific Aim # 2: To increase the amplification efficiency through optimization of DNA lysis conditions and to decrease the incidence of allele drop-out.

5.1 Materials and Methods for Specific Aim # 2

Cell collection

Single and double cells were collected using lymphoblast cultures (#GM03770 Coriell Institute, Camden NJ) known to be heterozygous for the 4 base pair (bp) insertion on exon 11 of the β-hexosaminidase A gene (TSD-11). Heterozygous cells were chosen because a single cell contains copies of both the normal and affected allele. Cells were isolated under a dissecting microscope either singularly or in groups of two, then serially rinsed through three microdrops of PCR buffer to remove any extraneous cells. The cells were then placed in a PCR reaction tube (Gene-Amp, Perkin-Elmer, Norwalk, CT) and 100 samples were evaluated in each of the following methods of DNA preparation:

1). Liquid Nitrogen (LN_2) - cells were placed in 60μL of sterile ultrapure water with a mineral oil layer, frozen-thawed twice in LN_2 then boiled at 100°C for 10 minutes (standard procedure).

2). Potassium Hydroxide (KOH) - cells were placed in 5μL of KOH solution, (200mM potassium hydroxide/50mM dithiothreitol), centrifuged, heated at 65°C for 10 minutes, then 5μL neutralizing solution (900mM Tris-HCl pH 8.3, 300mM potassium chloride, 200mM hydrogen chloride) was added. Fifty microliters of sterile ultrapure water was added along with a mineral oil layer.

3). Boiling (BI) - cells were placed in 60 μL sterile ultrapure water, layered with mineral oil, then boiled at 100°C for 10 minutes.

4). Water (H_20) - cells were placed in 60μL sterile ultrapure water and layered with mineral oil without further preparation prior to PCR and genetic analysis

Cells were analyzed by PCR using nested primers as noted above in the Materials and Methods section for Specific Aim # 1, with the following exception in the PCR buffer for the first round of amplification: For the KOH group, the buffer was 10mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$ chloride, 0.1% (w/v) gelatin. (Due to the potassium ions in the KOH, potassium chloride was not used on the initial buffer). The LN_2, BI, and H_20 groups first round PCR, plus all groups nested round PCR, used the regular PCR buffer (10mM Tris-HCl pH 8.3, 50mM potassium chloride, 1.5mM $MgCl_2$, 0.1% w/v gelatin). Additional PCR experiments were performed using the alternate concentrations to control for the differences in buffers.

PCR products were loaded onto a 7% polyacrylamide gel as described above in Specific Aim # 1. The appearance of a single band rather than the characteristic heteroduplex formation indicated the amplification of only one allele.

Statistical analysis

Amplification rates and accuracy of diagnoses between the different methods of DNA preparation were compared by Chi-Square, using Yates' correction for continuity (Appendix C). The computer program GRAPHPAD INSTAT (Graphpad Software, San Diego, CA) was used to assist in analysis.

5.2 Results for Specific Aim # 2

A representative gel is shown in Figure 10. Results are shown in Table VII for the single cells. Ninety-seven percent of the KOH group samples demonstrated amplification of at least one allele. This was significantly greater than for LN_2, BI, and H_20 group where amplification was detected in 38, 41 (both $P<0.00001$) and 85% ($P<0.006$) of samples, respectively. The sensitivity of the KOH method is even greater when comparing amplification of both alleles in the heterozygous cell, a requirement for a correct diagnosis. Ninety-one of the 97 single cells that amplified using the KOH method showed the presence of both alleles. In the LN_2 group, not only was the amplification rate low (38%), of those cells that did amplify only 11 showed amplification of both alleles. Although the

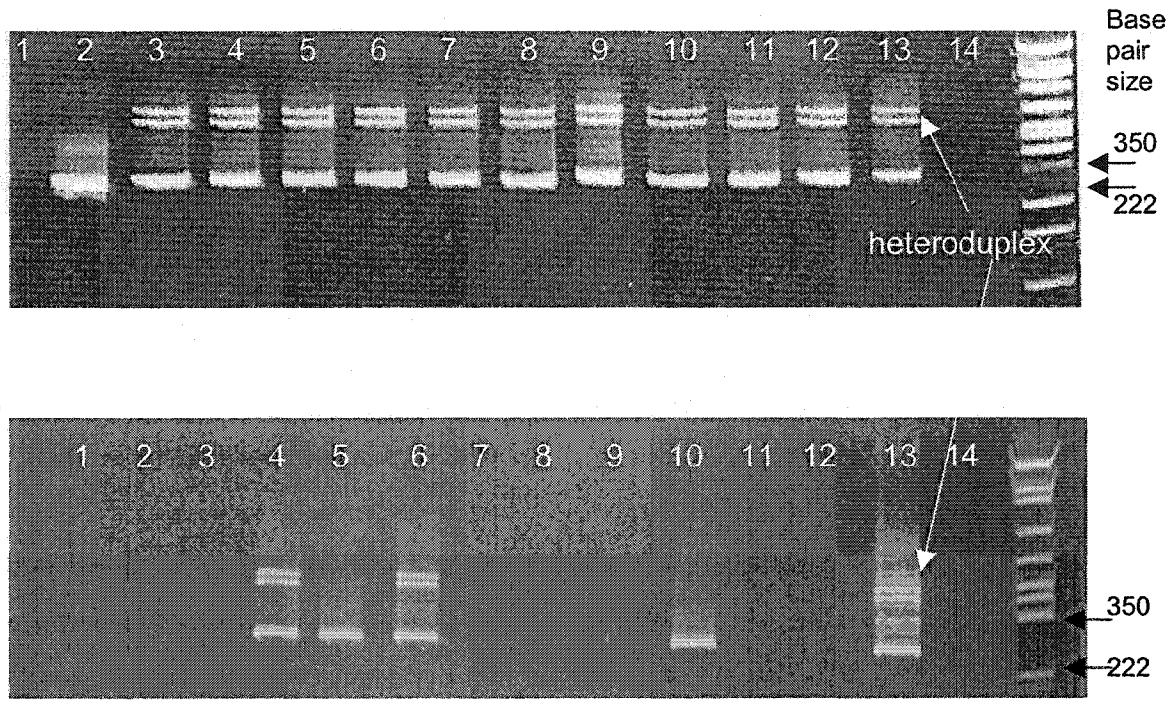

Figure 10. Two of the DNA preparation techniques. Both 15-lane polyacrylamide gels (7%) were set up the same with a negative control in the 1st and 14th lanes and a normal and heterozygous control in the 2nd and 13th lanes, respectively. Lanes 3-12 display the amplification results from the PCR of known heterozygous single cells. This upper gel demonstrates the results of the KOH/DTT lysis method in which all 10 single cells (lanes 3-13) exhibit the characteristic heteroduplex formation and correctly amplified both the normal and affected alleles. The lower gel represents the use of the Liquid Nitrogen method. In this instance only 4 of 10 heterozygous single cells amplified. Of those 4, only 2 (lanes 4,6) correctly displayed the heteroduplex formation, indicating a correct diagnosis.

Table VII. Comparison of different lysis methods used in 400 single cells

Amplification Rate/ Lysis Method	LN_2	KOH	BL	H_2O
Normal allele only	16/100 (16%)	5/100 (5%)	13/100 (13%)	16/100 (16%)
Affected allele only	11/100 (11%)	1/100 (1%)	11/100 (11%)	28/100 (28%)
Both Alleles (expected result)	11/100 (11%)	91/100 (91%)	17/100 (17%)	41/100 (41%)
Total Amplification	38/100[a] (38%)	97/100[a,b] (97%)	41/100[a] (41%)	85/100[b] (85%)

[a] $P<0.00001$.
[b] $P<0.006$.

Table VIII. Comparison of different lysis methods used in 200 two cells

Amplification Rate/ Lysis Method	LN_2	KOH
Normal allele only	14/100 (14%)	1/100 (1%)
Affected allele only	18/100 (18%)	0/100 (0%)
Both Alleles (expected result)	53/100 (53%)	96/100 (96%)
Total Amplification	85/100[a] (85%)	97/100[a] (97%)

[a] $P = 0.006$.

H_20 method had a high amplification rate (85%) for single cells, less than half (n=41) of the cells amplified both alleles.

When amplification of a single band was seen, heteroduplex formation determined if the normal or the affected allele was amplified. There was no significant difference seen in which of the alleles amplified. This also suggests that the single bands are probably not due to contamination since the laboratory workers are homozygous normal for TSD-11 DNA. No evidence of contamination was observed in any of the negatives or media controls for the experiments used in this study.

Using two cells per reaction did not increase the amplification rate for the KOH method (97% vs. 97%), but dramatically increased the rate for the LN_2 method (85 vs. 38%; P <0.00001). However, only 53 of those cells correctly amplified both alleles (Table VIII).

5.3 Discussion for Specific Aim # 2

Maximizing the benefits of a PGD cycle relies upon the success rate of its implementation. Specific Aim # 1 focused on the sensitivity of obtaining a genetic answer; this study tested both sensitivity and specificity - not only obtaining amplification, but also ensuring that the information obtained is correct. One of the greatest concerns is that a misdiagnosis after genetic testing would actually supersede the PGD in the first place. Most of the couples pursuing PGD cycles are fertile and are opting for PGD not only to have a child, but to have an *unaffected* child.

When studying a heterozygote in the autosomal recessive condition with a single mutation, amplification of only the normal allele and not the affected allele would result in making eligible for embryo transfer a true phenotypically normal embryo (genotypically heterozygous), an event that would not be detrimental for the resulting offspring. However, amplification of only the abnormal allele would then remove a true phenotypically normal embryo from the pool of embryos eligible for transfer, thus decreasing the chances for pregnancy. (Table IX – Condition A). One cause of misdiagnoses reported in the literature (Grifo *et al.*, 1998; ESHRE

Table IX. The effect of Allele Drop-out (ADO)

Condition A – autosomal recessive disease such as Tay-Sachs (T and t alleles)

True Genotype	Observed Genotype	Eligible for Transfer?	Impact of Error
Tt (normal phenotype)	T (normal)	Yes	Tolerable
	t (affected)	No	Healthy embryo not transferred

Condition B – ADO in an autosomal dominant disease such as Garner Syndrome (G and g alleles)

True Genotype	Observed Genotype	Eligible for Transfer?	Impact of Error
Gg (affected)	G (affected)	No	Acceptable
	g (normal)	Yes	Transfer of affected embryo

Condition C – ADO in an autosomal recessive disease such as Cystic Fibrosis with two different mutations (C, c, F, f alleles)

True Genotype	Observed Genotype	Eligible for Transfer?	Impact of Error
CcFf (affected)	CFf (normal)	Yes	Transfer of affected embryo
	cFf (affected)	No	Acceptable
	CcF (normal)	Yes	Transfer of affected embryo
	CF (normal)	Yes	Transfer of affected embryo
	cF (normal)	Yes	Transfer of affected embryo
	Cf (normal)	Yes	Transfer of affected embryo
	cf (affected)	No	Acceptable

Table modified from Navidi and Arnheim, 1991. Capital letters (T,G,C,F) represent the dominant allele while lower case letters (t,g,c,f) represent the recessive allele.

2002) has been attributed to ADO or the random amplification of only one of the two alleles present in a single cell. The two main considerations regarding ADO are with either: 1) An autosomal dominant condition where the heterozygous condition is phenotypically affected. Amplification of only the normal allele in a heterozygous cell with a dominant condition could result in the transfer of an affected embryo (Table IX – Condition B), or 2) An autosomal recessive condition involving two different mutations in the same gene (one from each parent) where the affected condition actually carries both mutations. This is known as a compound heterozygote since it is heterozygous for two different mutations in the same gene. In the case of a compound heterozygote, there are actually four alleles to amplify – the normal and abnormal at each of the two mutation sites. Amplification of only a normal allele at either of those sites could also result in a misdiagnosis and subsequent transfer of a truly affected embryo. (Table IX – Condition C)

The first trials of PGD reported in the literature used a lysis method where the biopsied cell was placed into a tube of sterile water to break the cell membrane (Strom *et al.*, 1991; Morsy *et al.*, 1992; Liu *et al.*, 1993). The difference in the osmotic pressure was thought to be enough to break the cell wall and make the DNA more accessible. Trials of amplification efficiencies produced results with amplification rates over 90% so the method was accepted as quick and easy.

At the onset of the initial clinical studies performed in our laboratories, the state of the art for PGD was to lyse the cell by the freeze thaw method (Verlinsky and Kuliev, 1992) or liquid nitrogen (Handyside *et al.*, 1992), similar to the protocols outlined in the Materials and Methods section. This method also appeared to be quick and easy to break open the cell membrane by changes in temperatures and to allow access to the DNA. However, after several other PGD clinics reported poor amplification results and misdiagnoses, other methods for DNA lysis were explored.

Earlier reports of the PEP technique also used the KOH method for lysing the cells. Zhang *et al.* (1992) demonstrated the method for individual sperm cells,

which are known to have highly condensed DNA. The KOH/DTT method was similar to a sperm lysis technique reported by Cui *et al.* (1989). It was thought that that method could be used for the lysis of the blastomere as well. This concept led to the second phase of our optimization studies and the evaluation of different lysis methods.

As our results demonstrated, the KOH lysis method proved superior to the other methods that at the time of the studies were considered the status quo. The exact mechanism for the superiority of the KOH lysis technique is not known. Alkaline buffers are known to hydrolyze the cell membrane, thus making the DNA more accessible. Other DNA protocols use an alkaline lysis buffer to increase DNA isolation (Sambrook and Russell, 1989). DTT is added to the lysis buffer as well. DTT reduces disulfide bonds (Cleland, 1964). Reduced sulfide bonds minimizes the formation of protein aggregates. As stated above, the KOH/DTT protocol was first used to access the DNA in tightly condensed sperm cells. It stands to reason then that the use of DTT may aid in the decondensation of the blastomere DNA as well, thus providing more accessible DNA that can be used for amplification.

The initial step in PCR is elevating the temperature to just below the boiling point of water so that the hydrogen bonds of the double stranded DNA will dissociate and become single stranded DNA. The process is reversible; upon lowering the temperature, the hydrogen bonds will re-form. In the case of PCR, before the bonds re-form, oligonucleotide primers will quickly bind to their complementary sites and extend a newly formed strand in the presence of *Taq* polymerase and dNTPs. One explanation for the occurrence of ADO is that there is insufficient strand separation during the denaturation step. By increasing the temperature during the initial denaturation steps, it is thought to aid in more efficient strand separation (Ray and Handyside, 1996).

ADO could be due to DNA degradation (Piyamongkol *et al.*, 2003). Random breaks in the DNA strands are expected to occur. This may jeopardize the amplification of larger segments of DNA since there is a greater risk of strand breakage between the primers. In a study of seven different loci, Piyamongkol *et*

al. (2003) compared ADO rates with the size of the PCR product and noted that ADO rates increased with the size of the amplicon. These researchers found a linear correlation between the product size and ADO; the greater the size of the final PCR product, the greater the ADO rate, even in differences of 100 base pairs. Sermon *et al.* (1996) and Vrettou *et al.* (1999) have anecdotally reported increased rates of ADO and amplification failure with longer amplified products as well. Vrettou *et al.*, (1999) also concluded from their studies that the DNA sequence, denaturation temperature (contrary to the findings of Ray and Handyside, 1996), and cell type had little affect on the ADO rates.

In the study presented here, testing one cell versus two cells had an inverse effect on the ADO rate. With the superior KOH lysis method, testing two cells showed a decrease in the ADO rate from 6% to 1%, but that difference was not statistically different ($P = 0.1182$) since the ADO rates were low to begin with. However, when testing two cells rather than one cell using the LN_2 method, there was a significant improvement in amplification rates (85% versus 38%, respectively, $P = 0.008$) and decreased ADO rates (71% versus 38%, respectively, $P < 0.0001$). Although the ADO rate was still unacceptably high for this method, these results demonstrated that the ADO rate changes with the amount of starting DNA. Piyamongkol *et al.* (2003) corroborated the effect of the amount of DNA on ADO rates by testing one, two, three cells, and determining the ADO rate decreased significantly between one and two cells (from 24% for a single cell to 4% for two cells, 3% for three cells, $P < 0.0001$ using a proteinase K lysis technique).

Ray and Handyside (1996) demonstrated that, once the ratio of one allele versus the other is over 1:4, the reaction would likely not recover from the altered ratio due to the exponential amplification of both products. These researchers mixed known quantities of starting template of normal versus affected allele for the ΔF508 mutation of CFTR at 1:1, 1:2, 1:4, 1:16, and 1:256 ratios. The 1:1, 1:2, and 1:4 all showed adequate heteroduplex formation, demonstrating the amplification of both alleles. However, the samples containing 1:16 ratio or greater displayed only weak formation of the characteristic heteroduplex bands,

indicating the amplification of only one allele even though the starting template contained both alleles. This suggests that once the amplification of one allele over the other has occurred after as early as three cycles of PCR (4 copies), the eventual outcome of ADO cannot be altered.

More sensitive techniques such as Fluorescent PCR (F-PCR) may aid in detecting the occurrence of ADO (Findlay *et al.*, 1995; Piyamongkol *et al.*, 2001). Fluorescently-labeled primers are added to the PCR master mix. Rather than using standard gel electrophoresis, the resulting PCR product is quantitated with the aid of computer-assisted software to determine the presence or absence of the studied locus. In the instance of ADO, a lower value may be assigned at that particular locus, but the allele is still present nonetheless.

As demonstrated by Table IX, the impact of ADO can result in a misdiagnosis and transfer of an affected embryo, the very outcome that couples who undergo PGD are trying to avoid. ADO and other issues of amplification efficiency, diagnosis, and outcome were studied by varying for this dissertation by varying DNA lysis methods in hopes of optimizing the sensitivity and accuracy of the PGD procedure while minimizing the risk to the patient. Achieving a greater diagnostic sensitivity and specificity could result in having more embryos available for transfer which in turn could increase the overall pregnancy rate and giving more couples the opportunity of having a healthy child.

5.4 Conclusions for Specific Aim # 2

In Specific Aim #2, we concluded that different lysis techniques can affect both the sensitivity and specificity of single cell diagnosis. The KOH lysis method produced a significantly higher rate of amplification and higher accuracy when compared with the other methods tested. Using this method, there was no statistical difference in amplification rate and diagnostic accuracy when testing one versus two cells. These studies also demonstrated the importance of optimizing the PCR conditions by evaluating heterozygous cells that contain both alleles so that the incidence of ADO can be assessed.

SECTION 6
STUDIES FOR SPECIFIC AIM # 3

Specific Aim # 3: To identify characteristics of the blastomeres from an embryo before and after embryo biopsy that can aid in determining the success of obtaining PCR amplification.

6.1 Materials and Methods for Specific Aim # 3

Individual blastomeres were evaluated from embryos from couples undergoing a PGD procedure. Individual cells were removed from the embryo by micromanipulation (embryo biopsy) as described above in the Materials and Methods section of Specific Aim # 1.

Evaluation of the nuclear and cytoplasm status

The characteristics of the cytoplasm and the nucleus were evaluated using both a SMZ-10 Nikon stereo microscope and a Nikon diaphot inverted microscope with Hoffman optics. Nuclei from blastomeres were classified as present or absent, intact or ruptured. The cytoplasm was classified as either intact, or ruptured/degenerating (Figure 11).

Polymerase Chain Reaction analysis

The individual blastomeres were analyzed by nested PCR for the patient specific disease. An example of the protocol for that is listed above in the Materials and Methods section of Specific Aim # 1.

Statistical analysis

Fisher's exact statistical testing was used to compare results of amplification for the different nuclear/cytoplasmic characteristics (Appendix C). The computer program GRAPHPAD INSTAT (Graphpad Software, San Diego, CA) was used to assist in analysis.

6.2 Results for Specific Aim # 3

Table X illustrates the amplification results when a nucleus is present compared with when no nucleus is visible. Of the 89 total blastomeres, 59 (66.3%) had a visible nucleus while 30 (33.7%) did not have a visible nucleus. All 59 of those blastomeres with a visible nucleus amplified in the subsequent PCR

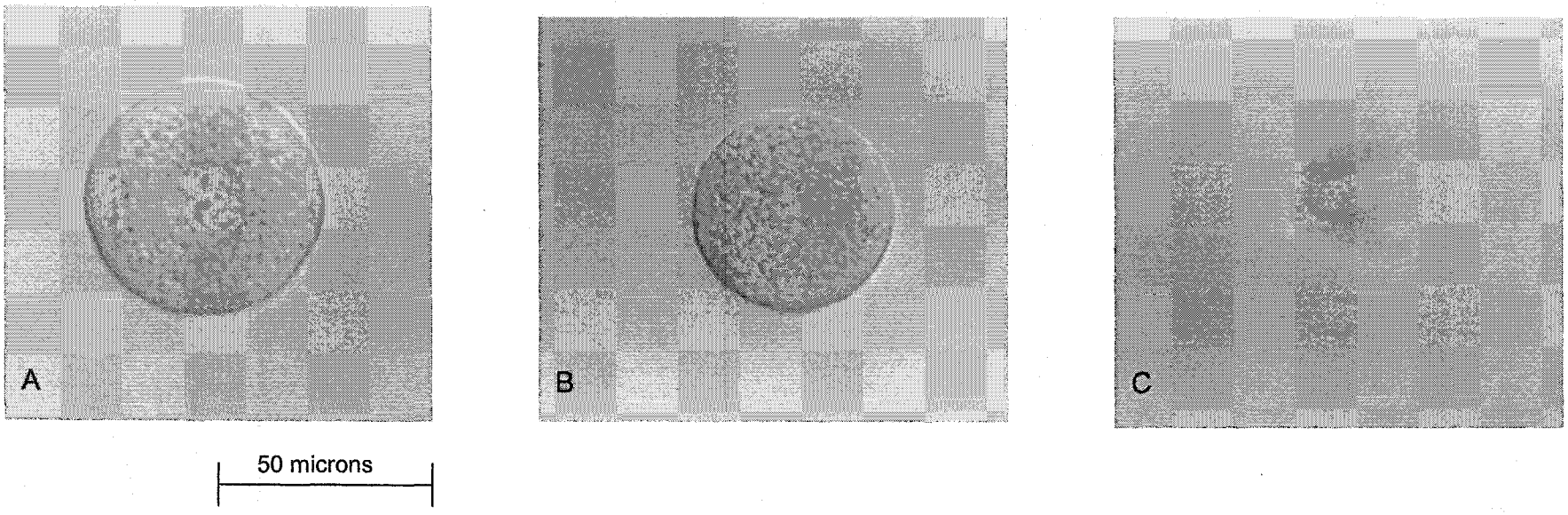

Figure 11. Representation of nuclear and cytoplasmic integrity in human blastomeres. Panel A illustrates a biopsied balstomere with no visible nucleus. The overall amplification rate of cells in this category was 11/30 (36.7%) Panel B illustrates a biopsied blastomere with a visible nucleus. Note the defined nuclear membrane. Nucleoli can be seen in the nucleus as well. Cells in this category demonstrated 100% amplification rates (59/59). Panel C shows the cell membrane of a biopsied blastomere that has ruptured and the cytoplasm is degenerating. Of the 14 cells that lost cytoplasmic integrity, the amplification rate followed the pattern of their nuclear status – if the nucleus was intact, the cell amplified (11/11 = 100%); if the nucleus was not intact, then the amplification rate was poor (0/3 = 0%).

Table X. Evaluation of blastomere morphology and amplification rate

Nuclear status	Amplification rate (%)
Visible Nucleus present	59/59 (100%)[a]
No nucleus seen	11/30 (36.7%)[a]
Cytoplasm/membrane intact	59/75 (78.7%)
With visible nucleus	48/48 (100%)[b]
No nucleus seen	11/27 (40.7%)[b]
Cytoplasm /membrane disrupted	11/14 (78.6%)
With visible nucleus	11/11 (100%)
No nucleus seen	0/3(0%)

[a,b] $P < 0.0001$.

reaction while only 11/30 (36.7%) without a visible nucleus subsequently amplified in the PCR. This difference was statistically significant ($P<0.0001$). Of the 30 blastomeres without a visible nucleus, one blastomere initially had a nucleus seen, but upon biopsy, the cell lost the nuclear integrity and did not subsequently amplify.

The cytoplasm of nine blastomeres degenerated during or after the biopsy process and the blastomere membrane ruptured in an additional five cells. These breaches did not have an effect on the amplification rate; the amplification rates in the intact versus disrupted cytoplasm groups were equal at almost 79%. Those categories were further split into the cells that had a visible nucleus versus those that did not. Of those14 blastomeres that showed loss of cytoplasmic/membrane integrity, 11 had a visible nucleus (i.e. nuclear membrane remained intact in spite of the cytoplasmic integrity). All 11 with a visible nucleus subsequently amplified while the three with no visible nucleus did not amplify. Again, the amplification rates followed the pattern of nuclear morphology; regardless of the cytoplasmic integrity, cells with a visible nucleus amplified 100% of the time.

A total of 89 blastomeres were biopsied from 51 embryos obtained from couples seeking PGD. Overall, the amplification rate per embryos analyzed was 47/51 (92.2%). The four embryos that showed no PCR amplification had no visible nucleated cells at the time of the biopsy (One, one, two, and three blastomeres removed from those four embryos). No other form of nuclear assessment such as the use of Hoechst dye was performed since these cells were evaluated immediately after biopsy for clinical PGD.

6.3 Discussion for Specific Aim # 3

Theoretically, it should come as no surprise that blastomeres with visible nuclei should have greater amplification efficiency than those that do not. In simplistic terms, the DNA in interphase nuclei (when the nucleus is visible) is more accessible than the tightly condensed DNA in cells where the nuclear envelope has broken down as the cell prepares for cytokinesis. Interphase chromosomes are thin and extended; the chromosomes themselves are not readily visible under the microscope, but rather the chromatin appears as well

dispersed fibers and filaments throughout the nucleus. (Comings, 1980) This dispersion allows the DNA to be more accessible for the regions of the chromatin in the nucleus that are transcriptionally active. Dispersed DNA strands provide an accessible template for PCR amplification.

In contrast, metaphase chromosomes are tightly coiled, reducing a 5cm length of DNA to approximately 5μm. This coiling, mediated by H1 histone phosphorylation, protects the chromosomes from breakage during segregation. Mitotic chromosomes are transcriptionally inactive and RNA synthesis is temporarily halted while the chromosomes are condensed. (Alberts *et al.*, 1994). The tightly-bound DNA template may consequentially be difficult to access during the PCR process. Under normal PCR conditions using large quantities of DNA, this does not appear to be an issue of concern since there will be asynchronous cell cycles and interphase cells should be present at any given time. However, in PGD when only one cell is involved, the ability to amplify that single cell is crucial.

The visible nuclear envelope is composed of two membranes. The inner surface is associated with the nuclear chromatin. It was first described by Fleming in 1882 as " the existence of a special achromatinous lamella, that is real - though in most types of nuclei very thin – layer of substance which is not merely the expression of the region of contact between nuclear substance and cytoplasmic structure" (republished as historical work years later, Fleming, 1965). Thus, the envelope serves as a 'barrier' between the nucleus and the organelle-containing cytoplasm.

Nuclear envelope breakdown is part of the normal cell cycle to allow the chromosome pairs to migrate to the cell periphery in anticipation of cytokinesis. Even though it is driven by the mitotic spindle, chromosome segregation is mediated by nuclear envelope components. The kinetochore is functional once the envelope has broken down; the pores have disassembled and nuclear pore components have been transferred to the kinetechore. This may be a fail-safe mechanism that determines the mitotic status of the cell (Salina *et al.*, 2003)

When the nuclear envelope is exposed to mechanical stress, the membrane proper may no longer be visible. (Franke *et al.*, 1981). The basic structural elements of the pore complex can remain intact under stress conditions. Lack of a visible nucleus may then reflect some degree of DNA degradation. DNA degradation can also occur due to changes in temperature, pH, and DNA secondary structure (Lindahl, 1993). In normal metabolically active cells, there is some ensuing degree of DNA repair. Although there is evidence for DNA repair in embryos, those mechanisms are poorly understood (Harrouk *et al.*, 2000; Menisser-de Murcia *et al.*, 2001; Makarevich and Markkula, 2002; Zeng *et al.*, 2004). This DNA degradation could lead to a reduced or no PCR amplification.

How does partial DNA degradation affect the PCR signal? If small parts of the nitrogenous bases are involved in the degradation, the *Taq* polymerase can still incorporate the DNA, but the signal will be reduced. This would consequentially result in a weak signal (Cui and Matthews, 1996). However, if there is more serious damage to the bases or the base-sugar bonds, phosphodiester bonds are broken; subsequently the *Taq* will not incorporate the bases and extension will stop. This was well illustrated by Cui and Matthews (1996) who demonstrated that in normal DNA, the *Taq* polymerase works efficiently as it incorporates the bases and extends new DNA strands. However, if there are bases that are degraded (due to hydrolysis, oxidation and/or methylation, Lindahl, 1993), the incorporation rate is very slow. If the degradation is severe and the phosphodiester bonds are broken, the Taq will not recognize the DNA strand to attach to, and extension will cease. These observations were made in blastomeres with nuclei that were severely abnormal or absent (Cui and Matthews, 1996).

The ability to perform this study was made possible by first optimizing the amplification efficiency. Knowing what conditions work well in single cell amplification provides a strong foundation to try to assess the differences when optimal conditions are altered. Examples are instances when new reagents or reaction temperatures are altered to determine the effect of that particular change. Initial studies in our laboratory to optimize single cell diagnosis set the baseline for

the ability to discern these differences as clinical studies came to fruition (Morsey *et al.*, 1992). As in the previous study for Specific Aim # 2 demonstrating lysis conditions that improved amplification efficiencies, this study provides objective criteria to select optimal cells for PGD.

6.4 Conclusions for Specific Aim # 3

This set of studies demonstrated the importance of biopsying a blastomere with a visible nucleus. The integrity of the blastomere membrane or the integrity of the cytoplasm itself does not affect the amplification results if the nucleus and nuclear membrane remains intact.

SECTION 7

Studies for Specific Aim # 4

Specific Aim # 4: To identify the chromosomal complement in a single cell.

7.1 Materials and Methods for Specific Aim # 4

Cell collection

A male lymphoblast cell line normal for the studied genes and a male cell line heterozygous for the ΔF508 mutation of CFTR were obtained from Coriell Institute for Medical Research, Camden NJ (lines GM 03070 and GM07828, respectively). Cells were cultured as described in Specific Aim # 1. Lymphoblast cells were isolated, counted, and collected under a dissecting microscope using a drawn out sterile Pasteur pipette. Replicates of 1,2,3,4,5,6,8,10, and 20 cells were placed in individual PCR reaction tubes (USA Scientific, Ocala FL) containing 0.5μl of the lysis buffer (400mM KOH, 100mM DTT, 10mM EDTA). The tubes containing the cells were incubated on ice for 10 minutes. Subsequently 0.5μl of a neutralizing buffer consisting of 400mM HCl, 600mM Tris-HCl (pH 0.6) was added. The samples were stored on ice if being prepared for immediate assay or stored long-term at -20°C.

Whole Genome Amplification – Multiple Displacement Amplification (MDA)

The WGA technique of MDA was used to generate sufficient quantities of DNA for microarray analysis. MDA was accomplished through the use of a GenomiPhi™ DNA amplification kit (Amersham Biosciences, Piscataway, NJ). The kit supplied sample buffer, reaction buffer containing the random hexamers and dNTPs, along with the enzyme mix containing Φ29 polymerase. Other components and concentrations are unknown due to the proprietary nature of the kit. The kit supplied a 10ng/μl control (lambda DNA) to use as a positive MDA control. Negative controls were used as well that contained the kit components and were exposed to the same conditions as the samples, but did not contain template DNA.

Nine microliters of sample buffer were added to the template. The sample was denatured at 95°C for 3 minutes, then cooled on ice. Nine microliters of

reaction buffer and 1μl of enzyme mix were added to each cooled sample. The sample was then incubated at 30°C for 16-18 hours. After that time, the sample was heated to 65°C for 10 minutes to inactivate the enzyme, then cooled to 4°C until subsequent use.

Agarose gel verification

To confirm that amplified product had been generated, a representative 1μl sample was run on a 1% agarose gel and the gel stained with ethidium bromide. The gel was analyzed and photographed under UV light for evidence of a streak of DNA to confirm the presence of amplified DNA (see Figure 12). The streak is represented of the multiple copies of varying sizes of DNA products generated from the MDA.

Purification of amplified sample

Post-amplification purification or clean-up is recommended to remove any remaining reagents, enzymes, and oligonucleotides from the MDA process. This was accomplished by the use of a QiaQuick PCR purification kit (Qiagen, Valencia, CA) that is 90-95% successful at recovering 100bp-10 kb products while removing oligonucleotides. The sample was diluted with five times the volume of supplied buffer, the diluted sample was applied to the silica membrane of the QiaQuick spin column, and centrifuged to bind the DNA. The column was then washed with ethanol. Lastly, the DNA was eluted from the column with sterile water and was ready for subsequent use.

Quantification

Representative samples were quantitated by fluorometry and PicoGreen® dsDNA reagent kit (Molecular Probes, Eugene, OR) after purification. This method is preferred over standard UV spectrophotometry (absorbance at 260 nm) that may provide inaccurate readings due to contaminants commonly found in preparation methods. The PicoGreen is sensitive to the level of 25 pg/ml. A working stock of control DNA was used to generate a standard curve to extrapolate the quantitative value of the sample DNA. PicoGreen reagent was added to all samples and the standard curve stock samples. All were read using

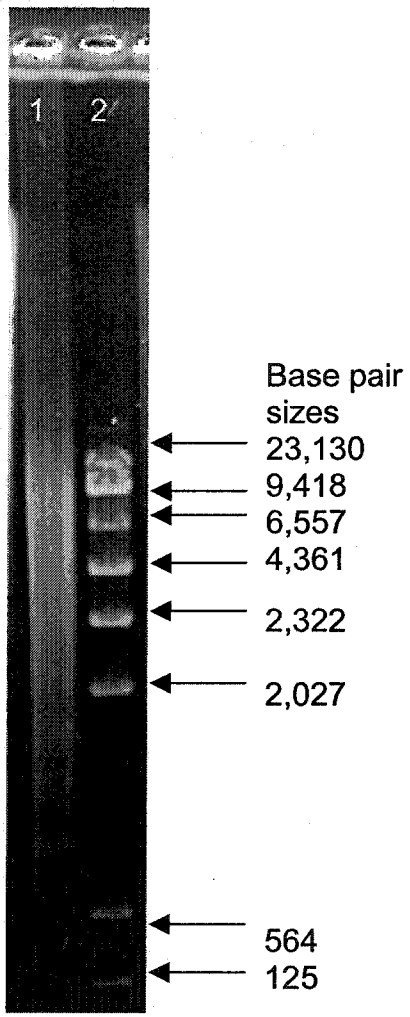

Figure 12. DNA products after multiple displacement amplification (MDA). Agarose gel (1%) image demonstrating the 'smear' of DNA in lane 1 in the product after successful MDA. Lane 2 is a Lambda DNA/*Hind* III size ladder (indicating the base pair sizes). The presence of a smear illustrates that large quantities of DNA in various sizes were produced after the amplification technique.

a Versa Fluor fluorometer (Biorad, Hercules, CA) with a 480/20(nm) excitation filter and a 520/10 (nm) emission filter.

PCR

A 1μl aliquot from each sample was subsequently analyzed by PCR for one to seven different loci:

1) APC = Adenomatous polyposis coli gene on chromosome 5 (5q21-q22)
2) CF = Cystic fibrosis transmembrane conductance regulator gene on chromosome 7 (7q 31.2)
3) DYT = Dystonia gene on chromosome 9 (9q34)
4) SC = Sickle cell, ß-globin gene on chromosome 11 (11p15.5)
5) TSD = Tay-Sachs disease, ß-hexosaminidase A gene on chromosome 15 (15q23-24)
6) ZFX = Zinc finger region of the X chromosome (Xp22.2-p21.3)
7) ZFY = Zinc finger region of the Y chromosome (Yp11.3)

These loci were chosen since they involve seven different chromosomes that had primers readily available in our laboratory and were known to efficiently amplify; this served as a means to determine that separate regions of the genome were amplified. PCR using nested primers was performed as previously described in the Material and Methods section of Specific Aim # 1 with the exception of the different primers and annealing temperatures as listed in Table XI. The PCR experiments confirmed the presence of DNA on several different chromosomes and illustrated that multiple loci were amplified by the MDA.

An additional buffer was added during the initial PCR process for single cells only. This buffer contained 10mM Tris (pH 8.0), 0.1% Tween 20 and 37.5μg/ml BSA.

Restriction Enzyme Digestion

MDA generates DNA fragments ranging from 100bp-10kb in length. In order to optimize the hybridization of the DNA to the microarray, the fragments

Table XI. Geni Loci and Primers. Below lists the gene loci and the primers used for the PCR experiments performed after whole genome amplification by multiple displacement amplification.

Gene Loci	Outer primers 5' to 3' (annealing temperature)	Nested primers 5' to 3' (annealing temperature)
Adenomatous polyposis coli chromosome 5q21-q22	F-ACAGAAAGATGTGGAATTAA R-TTCTCCAGCAGCTAACTCAT (55°C)	F-ACAGAAAGATGTGGAATTAA R-ATGGCAGAAATAATACATTC (54°C)
Δ F508 mutation of cystic fibrosis transmembrane regulator gene. Chromosome 7q31.2	F - GACTTCACTTCTAATGATGAT R- CTCTTCTAGTTGGCATGC (40°)	F- TGGGAGAACTGGAGCCTT R -GCTTTGATGACGCTTCTGTAT (50°)
Dystonia Chromosome 9q34	F-CCTGGAATACAAACACCTA R-GTGGAAGGACTGAGTGTTG (61°C)	F-GGCTGCCAATCATGACTGTC R-GTGGAAGGACTGAGTGTTG (58°C)
Sickle cell Beta globin gene Chromosome 11p15.5	F- GGCTGAGGGTTTGAAGTCCA R – CCATAGAAAAGAAGGGGAAA (49°C)	F- ACTCCTAAGCCAGTGCCAGA R- ATCAAGGGTCCCATAGACTC (58°C)
Tay-Sachs β-hexosaminidase A chromosome 15q23-24	F – CAACAACAGTCTGGTGATGG R – GCCAGACACAATCATACAGG (61°C)	F- AAGGAGCTGGAACTGGTCAC R – CTCTCAACCACCTTCCCAAT (54°C)
Zinc Finger region X chromosome Xp22.2-21.3 Y chromosome Yp11.3	F-ACCRCTGTACTGACTGTGATTACAC R-GCACYTCTTTGGTATCYGAGAAAGT (49°C)	F-AYAACCACCTGGAGAGCCACAAGCT R- TGCAGACCTATATTCRCAGTACTGGCA (58°C)

should be reduced in size to bind to the less than 1kb size of the microarray spotted DNA. In initial experiments, the post-MDA products were incubated at 37°C for 90 minutes with 20 units of the restriction enzyme DpnI (New England Biolabs, Beverly, MA) that recognized and cleaved (cut) the sequence GA'TC. In later experiments, 20 units of DpnI and 20 units of EcoRI (New England Biolabs) that recognized and cut the sequence G'AATTC were used.

Fluorescent labeling of DNA for microarray analysis

The protocol was followed in low-light or in darkness where applicable to minimize the photobleaching of the dyes. In the initial phase of microarray analysis, samples were labeled either using Cy 3 and Cy 5 dyes. For the proof of concept phase of the experiments, the samples were equally divided, and half was separately labeled with each dye. (Theoretically, the sample of interest would be labeled with one dye and the normal control labeled with the other dye). The Cy dyes were incorporated by random-priming using the BioPrime® DNA labeling system. The kit supplied several of the reagents needed, including the random primer solution, the Klenow fragment of DNA polymerase and the stop buffer. The kit dNTP mixture was not used, but rather Cy3 CTP and Cy5 CTP (Amersham, Piscataway, NJ) were used along with standard dNTPs (USB, Cleveland OH). The sample volume was brought up to 20μl after purification and the sample was placed on ice. Twenty microliters of the supplied 2.5X random primers solution was added, the sample mixture was denatured for 5 minutes at 100°C, then the mixture was cooled on ice. Two and a half microliters each of dATP, dGTP, and dTTP(1.2 mM) was added along with 1.25μl (0.6 mM) dCTP. Three microliters of Cy5-dCTP or Cy3-dCTP (1 mM stocks) was added, along with 1μl of the supplied Klenow polymerase. The sample was mixed gently and briefly centrifuged. The reaction was incubated in the dark at 37°C for 1 to 2 hours. At the end of that time, 5μl of the supplied stop buffer was added to each sample. The labeled probe was individually purified using the QiaQuick DNA purification kit as described above. The final volume for each sample was adjusted to 30μl with sterile water. The two samples were then combined in a new microcentrifuge tube.

Alexa Fluor Dyes

After the initial microarray experiments, Alexa Fluor dyes 546 and 647 were substituted for the Cy dyes and used to directly label the DNA with the Ulysis® nucleic acid labeling kit (Molecular Probes, Eugene, OR). Rather than using the random primers for labeling, the following protocol was used. After Qiaquick clean-up, the sample volume was adjusted to 21μl and was denatured at 95°C for 5 minutes, briefly centrifuged and then placed on ice. Four microliters of the respective Alexa Fluor dye was added for a final volume of 25μl. The mixture was heated in the dark at 80°C for 15 minutes, briefly centrifuged, then placed on ice. QiaQuick purification was used individually on each sample as described above. The final volume for each sample was adjusted to 30μl. The two samples were then combined in a new microcentrifuge tube.

After the respective labeling method and the combination of the samples, 30-50μg of human Cot-1 DNA (Invitrogen, Carlsbad, CA) was added (to block hybridization to repetitive DNAs if present on array). One hundred micrograms yeast tRNA (Invitrogen) was added (to block non-specific DNA hybridization). Twenty micrograms of poly(dA)-poly(dT) (Sigma, St. Louis, MO) was added (to block hybridization to polyA tails of cDNA array elements).

The mixture was dried in a Savant Speed Vac Concentrator (Thermo Electron, Woburn, MA) to a volume of 24μl or less. If less, the volume was brought up to 24μl total with sterile water. A mixture of 5.1μl 20X SSC (Fisher Chemical, Fairlawn NJ) and 0.9μl 10% SDS (Fisher chemical) was added (for a final conc. of 3.4%SSC and a final conc. of 0.3% SDS).

The hybridization mixture was denatured at 100° C for 1.5 minutes, then incubated for 30 minutes at 37°C (as a Cot-1 preannealing step). During the 30 minutes, the microarray slide (University Health Network, Toronto, Canada) and the hybridization chamber were prewarmed to 65°C or 37°C, corresponding to the eventual hybridization temperature. The microarray slide (GAPII, Corning, NY) was shipped ready for use, spotted with double stranded DNA as 1718 different ESTs (Expressed Sequence Tags) derived from PCR amplification of the EST clones which are potential mRNAs. The slide appears as 8 grid rows

and 4 columns similar to the diagram (Figure 6). Each grid has 10 rows and 12 columns of spots.

The hybridization chamber has a small well in the edge of the lower slide holder. At the end of the 30 minutes, a 10 μl drop of sterile water was added to the well to maintain humidity in the chamber. The microarray slide was placed on the bottom half of the chamber. The probe solution was then added to the center of the array where the slide is spotted. The final volume of hybridization was 30ul. This volume was appropriate for hybridization under a 30x50 mm^2 coverslip. The coverslip was polished with ethanol and dried. The edge of the coverslip was held at the edge of the drop of the probe mixture and gently dragged back and laid in place over the microarray with minimal air bubbles. The top portion of the hybridization chamber was assembled over the slide. The entire chamber was submerged into a water bath and hybridized overnight (16-20 hrs) at either 65°C (following the protocol of Brown, http://cmgm.stanford.edu/pbrown/protocols/4_genomic.html) or 37°C at the manufacturer's recommendation. At the end of the hybridization time, the chamber was removed from the water bath, the chamber's edges were dried, and the chamber was carefully disassembled so water did not enter the chamber. The slide was removed and passed through a series of washes in Coplin jars as outlined in Table XII below. In the first wash (1A) the slide was placed in the Coplin jar and left undisturbed until the coverslip fell. After the coverslip was safely removed, a gentle agitation was performed for the allotted time.
It is important that the slides are rinsed well; detergents can form aggregates that can trap unbound dye. These aggregates can dry on the side and interfere with interpretation of results. After the series of washes, the slide was spun dry for one minute in a microcentrifuge equipped with a slide holder. The slide was either scanned immediately or held in a dark slide box in a desiccator for up to three days.

Scanning

The microarray slides were scanned using ScanArray® Express microarray scanner (Perkin Elmer Life Sciences, Boston, MA). Slides were scanned at a

Table XII. Post-hybridization wash steps

Wash	Description	Vol (ml)	SSC	SDS (10%)	Time/ Temperature
1A	2x SSC, 0.03% SDS	200	200 ml 2x	0.6 ml	2 min 37° or 65°C
1B	2x SSC	200	200 ml 2x	--	rinse
2	1x SSC	200	200 ml 1x	--	2 min 37°
3	0.2x SSC	200	200 ml 0.2x	--	2 min 37°

10μm resolution at the appropriate wavelength for the dyes used: Cy3 or Alexa Fluor 546 versus Cy5 or Alexa Fluor 647. The software package has preset wavelengths for various fluorophores that are highlighted appropriately. PMT Gain settings were used that will measure the signal to noise ratio. A good general starting value is PMT gain of 70-80%. Several settings

were measured and saved so the intensities were available during the analysis. The spotted portion of the array was defined by moving the mouse to the edges of the dimensions of the array area.

Quantitation

The software package with Scanarray express assisted with quantitation. A GAL file (Gene Array List) was supplied with the microarray slide detailing the contents at each spot, the gene, the corresponding loci, and the accession number for identification in Genbank (collection of sequences from NCBI – the National Center for Biotechnology Information). The software package allows for imaging of each individual spot that can be highlighted in a subarray grid. An adaptive circle method was used for the software to measure the intensity of each pixel within a defined spot that is manually circled. In the adaptive method, the initial spot mask and background mask are constructed as with the fixed circle method. This method was refined using a modified statistical testing process (Mann Whitney test) on a pixel by pixel basis. This method compensated for changes in the spot morphology since not every spot will hybridize as a perfect circle. Once all spots are highlighted, the software package found the spots and diagramed each spot on a scatter plot. Normal samples or samples of equal value should have a 1:1 ratio of each dye. A different PMT gain setting may be chosen to adjust that ratio as close to 1:1 as possible.

Degenerate Oligonucleotide Primer (DOP)

Replicates of 1,5, and10 lymphoblast cells were collected as decribed above. The cells were placed into a 0.5ml PCR tube containing the lysis buffer and neutralized as described above. The reaction contained 2μM DOP primer (5' – CCGACTCGAGNNNNNNATGTGG- 3', Sigma Genosys, The Woodlands, TX), 200μM dNTP mixture, PCR buffer (10mM Tris –HCl pH 9.0, 50mM KCl, 1.5mM

Table XIII. PCR amplification after 20 cells were subjected to MDA

MDA	APC(5)	CF(7	DYT(9)	SC(11)	TSD(15)	ZFX	ZFY
20 cells	+	+	+	+	+	+	+
20 cells	+	+	+	+	+	+	+
20 cells	+	+	+	+	+	+	+
20 cells	+	+	+	+	+	+	+
20 cells	+	+	+	+	+	+	+
20 cells	+	+	+	+	+	+	+
20 cells	+	+	+	+	+	+	+
20 cells	+	+	+	+	+	+	+
20 cells	+	+	+	+	--	+	+
Control	faint	+	+	+	+	+	+
Control	faint	+	+	+	--	+	+
Blank	--	--	--	--	--	--	--

APC = Adenomatous polyposis coli gene on chromosome 5 (5q21-q22).
CF = Cystic fibrosis transmembrane conductance regulator gene on chromosome 7 (7q 31.2).
DYT = Dystonia gene on chromosome 9 (9q34).
SC = Sickle cell, ßglobin gene on chromosome 11 (11p15.5).
TSD = Tay-Sachs disease, ß-hexosaminidase A gene on chromosome 15 (15q23-24).
ZFX = Zinc finger region of the X chromosome (Xp22.2-p21.3).
ZFY = Zinc finger region of the Y chromosome (Y11.3).

Table XIV. Results of PCR amplification after MDA

MDA # of cells	APC(5)	CF(7)	DYT(9)	SC(11)	TSD(15)	ZFX	ZFY
10 cells	+	+	+	+	+	+	+
10 cells		+				+	+
10 cells		+				+	+
8 cells		ADO				+	+
6 cells		+				+	+
4 cells		ADO				+	+
1 cells*		-				-	-
1 cells*		-				-	-
2 cells		ADO				-	-
2 cells		+				+	+
3 cells		-				-	-
4 cells					+		
3 cells					+		
1 cell	+	ADO	+	+	+	+	+
1 cell		ADO				+	+
1 cell		+				+	-
1 cell		-				+	-
1 cell		+				+	+
1 cell		+				+	+
2 cell		ADO				+	+
2 cell		ADO				+	+
3 cells		+				+	+
1 cell		+				+	+
1 cell		-				-	-
1 cell		ADO				+	+
1 cell		ADO				+	+
2 cell		-				-	-
2 cell		-				-	-
3 cell		ADO				+	+
1 cell		ADO				+	+
1 cell		-				+	+
1 cell		-				+	+
1 cell		+				+	+
1 cell		-				-	-
1 cell		ADO				+	+

ADO = allele dropout, only one of the two alleles amplified. * - did not use additive buffer. (+) represents PCR positive amplification, (-) indicates no PCR amplification. Blank spaces indicate that those loci were not tested on that cell.

Mg^{2+}, 0.1% (v/v) Triton X-100 and 0.01% (w/v) gelatin, 2.5 units Amplitaq polymerase. After an initial denaturation of 5 minutes at 96°C, cycling ensued at 94°C for 1 minute, 30°C for 1.5 minute, then 72°C for three minutes for 8 cycles. This was followed by 50 cycles of 94°C for 1 minute, 62°C for 1 minute, then 72°C for three minutes. Samples were then cooled to 4°C and held at that temperature if assayed immediately or stored long-term at 20°C.

7.2 Results for Specific Aim # 4

Initially, 20 cells per reaction tube (n=10 replicates) were subjected to MDA to determine the reproducibility and reliability of the technique. The samples were subsequently analyzed by PCR for the seven different loci. Those results are listed in Table XIII. All 10/10 (100%) showed PCR amplification after MDA, demonstrating that the MDA reaction was successful at the 20-cell level of sensitivity. Additionally, seven loci amplified for each of the 10 replicates (100%), demonstrating that multiple areas of the genome were successfully amplified (Figure 13).

Three replicates of 10 cells per reaction tube were subjected to MDA and analyzed by PCR. All samples successfully amplified at all loci tested as indicated in Table XIV. Subsequently 8,6, or 4 cells respectively were subjected to MDA, and those demonstrated PCR amplification as well. Results of single cell amplification were sporadic and only occurred in 11/16 cells (69%). Allele drop-out was seen in 6/11 of the amplified single cells.

DNA Quantitation

Representative samples post amplification were quantified using the Pico Green Kit. Values for single cells ranged from 2-3.4μg.

Microarrays

Microarray experiments are listed in Table XV with comments on the hybridization results (Figure 14). After poor hybridization results following the initial hybridization temperature of 65°C (Microarrays 1 and 2), DNA from an outside source (MCF7 breast cancer cell line from Dr. Tim Bos of Eastern Virginia Medical School) was used to confirm the labeling and hybridization technique. After successful amplification at 37°C, a comparison study was

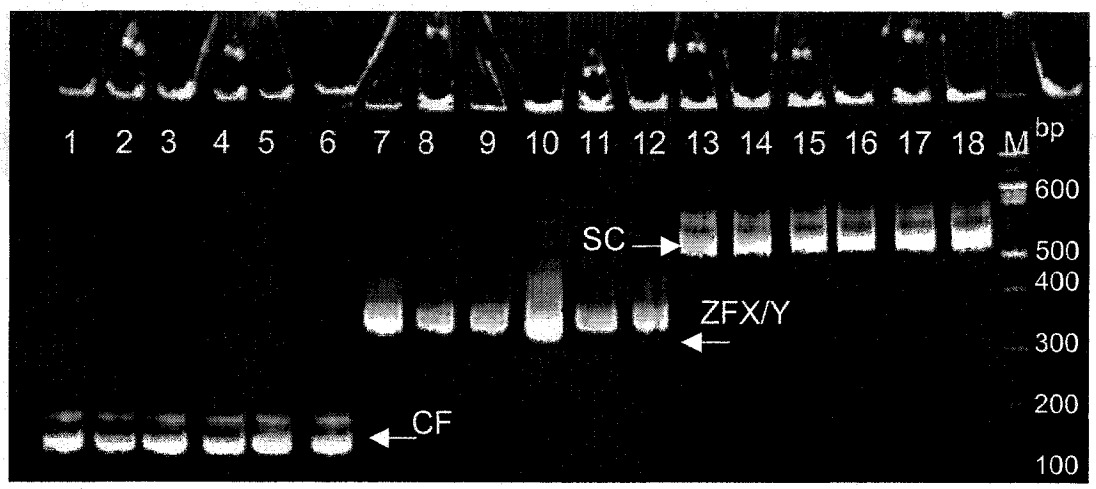

Figure 13. Representative gel of PCR amplification after multiple displacement amplification (MDA). Six single cell replicates for four different loci from each cell are represented (cystic fibrosis, chromosome 7, zinc finger region of X/Y chromosome, sickle cell, ß-globin of chromosome 11). An additional restriction enzyme digest of the ZFX/Y product (not shown here) will determine the presence of the Y chromosome.

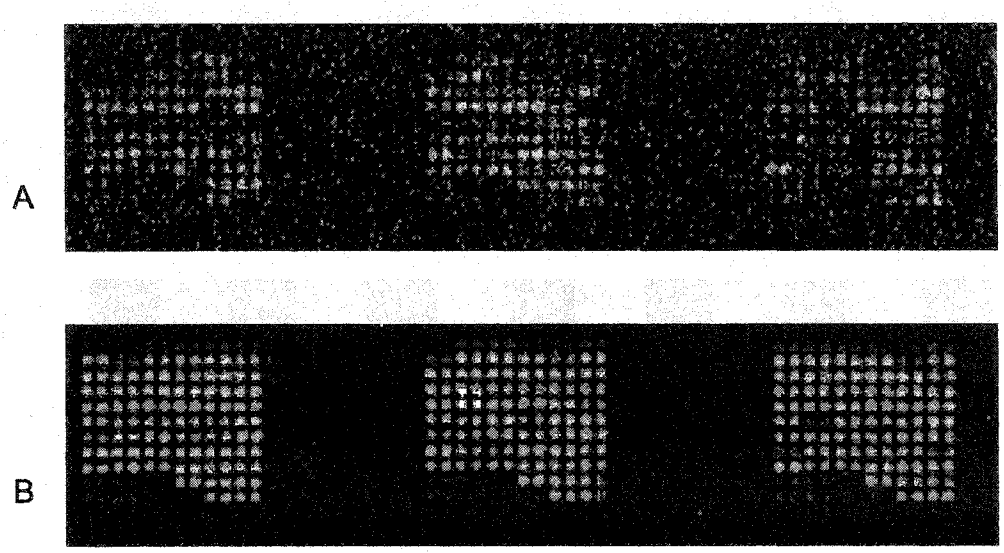

Figure 14. Microarray results. Portions of microarray images illustrating poor quality (A) versus good quality (B) hybridization. Both image A and B are composite images of both dyes. Both images illustrate three grids – 10 rows and 12 columns. In poor quality hybridizations, few spots are recognizable and there is higher background outside the spots. In good quality hybridizations, the majority of spots are recognized. Some spots are intentionally blank.

Table XV. Summary of microarray experiments performed

Array #	Hyb Temp[a]	Dyes[b]	DNA Type/ Quantity	Restriction Enzyme	Purpose of Expts and comments	Hybridization[d]
1	65°	Cy3/5	5 cells s/p MDA	DpnI 20units	Proof of concept	Poor
2	65°	Cy3/5	5 cells s/p MDA	DpnI 40units	Increase enzyme for smaller DNA fragments	Poor
3	37°	Cy3/5	genomic s/p MDA	DpnI/EcoR1 20units	Increase enzyme;change hyb temp,	Some
4	65°	Cy 5	MCF7 DNA 5 ug	DpnI/EcoR1 20units	use of 'proven' DNA, no WGA, compare hyb temp	Poor
5	37°	Cy 5	MCF7 DNA 5 ug	DpnI/EcoR1 20units	use of 'proven' DNA, no WGA, compare hyb temp	Good
6	37°	Cy 3/5	Bos DNA 5 ug	DpnI/EcoR1 20units	optimized conditions, 2 dyes, proven DNA	Good
7	37°	Cy 3	10 cells s/p MDA	DpnI/EcoR1 20units	optimized conditions with 10 cells, MDA, 1 dye	Weak
8	37°	Cy 3	1 cell MDA 2ug	DpnI/EcoR1 20units	optimized conditions, 1 cell, MDA, 1 dye	Weak
9	37°	Cy3/5	1 cell each 3ug	DpnI/EcoR1 20units	optimized conditions,1 cell, MDA ,2 dyes, cells combined/split	Some
10	37°	Cy 3/5	1 cell each 2.4ug	DpnI/EcoR1 20units	optimized conditions,1 cell, MDA ,2 dyes, cells combined/split	Poor
11	37°	546/647	1 cell each 3.1 ug	DpnI/EcoR1 20units	Optimized condition, Alexa Fluors	Strong

Table XV (continued)

Array #	Hyb Temp[a]	Dyes[b]	DNA Type/ Quantity	Restriction Enzyme	Purpose of Expts and comments	Hybridization[d]
12	37°	546/647	1 cell each 3.4μg	DpnI/EcoR1 20units	Repeat above – strong hybridization	Strong
13	37°	546/647	1 cell 3.4 ug/ MDA neg	DpnI/EcoR1 20units	Test 'neg' MDA	Sample- strong Negative – strong
14	37°	546/647	DOP 10 cell/ DOP neg	DpnI/EcoR1 20units	Compare DOP with MDA	DOP-strong Negative –none
15	37°	546/647	PEP 5 cell each - 1ug	DpnI/EcoR1 20units	Test PEP cells no hybridization	None

[a]Hyb temp = Hybridization temperature, [b]Dyes = dyes in labeling Cy 3/Cy5 or Alexa Fluor 546/Alexa Fluor647, [c]DNA type/quant – cells were lymphocytes at the quantity indicated except for the MCF7 DNA, which was proven hybridizable DNA from a cancer line, [d]Examples of 'poor' and 'good' quality hybridizations are shown in Figure 17.

performed at 37°C and 65°C. The 65°C array was initially chosen based on other Array-CGH protocols. However, no hybridization occurred at 65°C in our studies so the lower stringency of the 37°C was used, resulting in successful hybridization. Subsequent experiments were performed at 37°C.

Concurrently, the amount of restriction enzyme used to cut the post-WGA fragments was increased. Poor hybridization was seen after the initial use of 20 units of DpnI; that amount was doubled with no improvement of results. Subsequently, an additional restriction enzyme, 20 units of EcoRI was added at the time when other variables were changed as well. With successful hybridization results, both enzymes were used for the remaining experiments.

After these initial changes in experimental conditions, the use of MDA cells continued as in Microarray 7. Two samples were combined and equally split so that there would not be variation from sample to sample. For example, a single cell sample and a single cell sample were combined, the DNA measured, then the sample was equally split and labeled separately. Those arrays still exhibited weak or minimal hybridization.

Next, the Alexa Fluor dyes were used for the labeling process instead of the Cy dyes. Microarrays 11 and 12 exhibited strong hybridization signals.

Non-specific DNA is generated after MDA that had minimal amounts of starting template due to the random primers that are available in the reaction to amplify fragments of DNA. To test the impact of the non-specific amplification, a reaction tube that contained only reagents and no template DNA was subjected to MDA. This 'negative' sample was subsequently assayed by PCR and yielded no PCR amplification in the seven loci tested. However, due to the nature of DNA fragments on the microarray, a test was designed to determine if the non-specific DNA interfered with hybridization. Microarray 13 (Figure 15) shows the results when a single cell post-MDA was labeled with Alexa 546 and the MDA negative control were labeled with Alexa 647. Both the sample and the negative control showed hybridization, indicating that the negative DNA samples did indeed hybridize to the microarray slide.

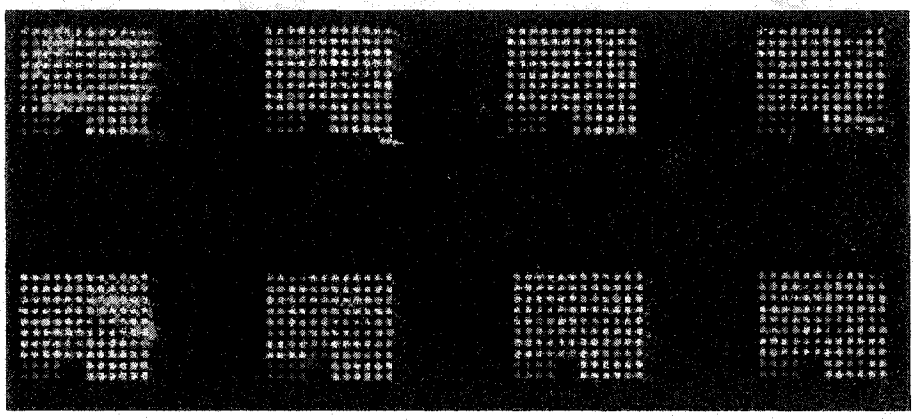

Figure 15. Microarray experiment with Alexa Fluor Dyes. Composite photo of the microarray results in experiment 13 after multiple displacement amplification (MDA). The single cell sample post- MDA is labeled with Alexa Fluor 546 while the sample subjected MDA without added DNA (MDA negative) is labeled with Alexa Fluor 647. There is a composite from both dyes even though the MDA negative contained no template DNA. This demonstrates the hybridization of non-specific DNA generated from the MDA process.

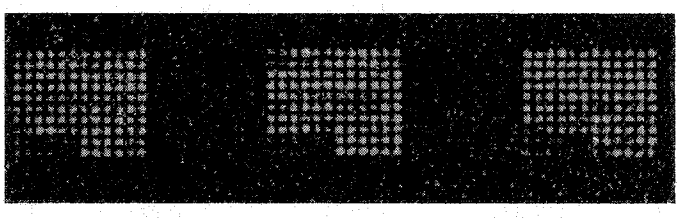

Figure 16. Composite of Microarray 14 with samples from Degenerate Oligonucleotide Primers (DOP). A ten-cell sample was labeled with Alexa Fluor 546 while a DOP negative containing no template DNA was labeled with Alexa Fluor 647. In contrast to the sample without starting template (negative) after MDA in Figure 15, no hybridization was seen with the DOP negative and the composite to be of uniform color. These results suggest that DOP-generated products may have a use for future microarray experiments.

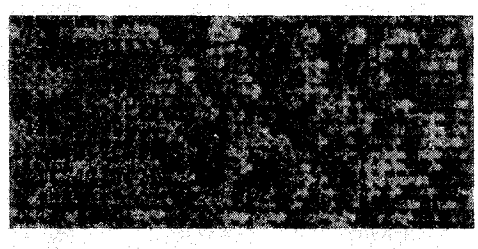

Figure 17. A composite of microarray 15 after single cells were exposed to primer extension preamplification (PEP). It is difficult to determine where the spots of the array begin due to the increased background intensity. These results suggest that PEP by itself is insufficient to generate enough DNA for hybridization.

Table XVI. Results of PEP/MDA experiments using single cells

PEP duration (cycles)	MDA Duration (hours)	PEP volume used for MDA	Final PEP reaction volume	PEP/PCR amplification result of remaining sample	PEP/MDA PCR amplification result
50 cycles (PEP control)	N/A	N/A	60μl	5/10 = 50%	N/A
4 cycles	10 hours	5μl	60μl	N/A	0/5 = 0%
4 cycles	10 hours	5μl	30μl	N/A	0/5 = 0%
4 cycles	12 hours	5μl	60μl	1/3 = 33%	0/3 = 0%
4 cycles	12 hours	5μl	30μl	0/3=0%	0/3=0%
10 cycles	10 hours	1μl	60μl	3/5 = 60%	0/5 = 0%
50 cycles	10 hours	5μl	60μl	3/3 = 100%	0/3=0%

N/A = not applicable.

To determine if the negative sample hybridization was an artifact of the actual MDA process, a similar experiment was conducted with a sample using DOP-generated DNA and a DOP negative. Microarray 14 (Figure 16) demonstrates that the DOP sample hybridized with strong signals while the DOP negative did not, indicating that the artifact was probably generated from the MDA process.

Non-specific DNA presumably does not interfere with larger quantities of starting template in the MDA process since the majority of the reagents will have template to react with. To compensate for the effect of the non-specific DNA, experiments were designed that involved the use of PEP to initially produce several starting copies of the single cell template prior to the initiation of MDA.

Previous experiments demonstrated the effective use of PEP. Microarray 15 (Figure 17) shows the results of using cells after PEP for the microarray hybridization. No hybridization was seen. The results of those experiments are in Table XVI. No subsequent PCR amplification was seen in these combination experiments.

7.3 Discussion for Specific Aim # 4

Cytogenetic techniques allow for the assessment of chromosome numbers. Classical techniques of karyotyping have given way to newer techniques such as FISH and CGH. These techniques have been used successfully to diagnose certain chromosomal aneuploidies in oocytes and embryos (Delhanty *et al.*, 1993; Munné *et al.*, 1998b; Verlinsky *et al.*, 1998; Wilton *et al.*, 2001, Wells and Delhanty, 2000). FISH is limited to analysis of a small number of chromosomes (Delhanty *et al.*, 1993; Munné *et al.*, 1998b; Verlinsky *et al.*, 1998). Interpretation of results can be difficult due to signal overlap or loss of hybridization signals (Wells and Delhanty, 2000). A major limitation of CGH is that the technique takes almost 72 hours to complete and requires the use of metaphase chromosomes. (Wilton *et al.*,2001; Wells and Delhanty, 2000). Microarray technology (array CGH) is emerging as another molecular technique that can be used to assess chromosome number. Array CGH can detect all chromosomes, is easier to perform than conventional CGH,

and does not require metaphase cells (Oostlander *et al.*, 2004). The purpose of this study was to determine if array CGH can be used to assess chromosome number in single cells. The work is ongoing as techniques are continually evaluated.

Initially, the focus of these experiments was to identify a WGA strategy that would provide an adequate amount of DNA for hybridization in array CGH. MDA appeared to be an attractive WGA technique because it is a simple isothermal reaction and reported to amplify up to 35μg quantities of the genome from a single cell (Handyside *et al.*, 2004; Hellani *et al.*, 2004). Our quantitation results from a single cell were approximately ten times less than those reports although still ample amounts to be used in array CGH. Additionally, our results in single cells were inconsistent, and amplification was seen less than 70% of the time with half of the cells showing absence of one allele. The reasons that these results differ from those of other investigators are unclear. One possible explanation is that there could be DNA degradation in the lymphoblast samples that were used in these experiments. Another possible explanation is that, although our protocols and those of Handyside *et al.*(2004) and Hellani *et al.* (2004) all use the MDA technique with Φ29 polymerase, our laboratory used a different commercially available kit (GenomiPhi™, Amersham Biosciences) than did other researchers (Repli-G™, Molecular Staging). The differences between these kits are not known since information regarding reagent concentrations and quantities is proprietary. The GenomiPhi™ kit was the only kit available when these studies were initiated, and Repli-G™ was primarily available overseas where the other studies were conducted. Repli-G™ is now widely available in the United States, and trials in our laboratory using that kit for MDA are in progress. Additionally, Φ29 polymerase is now commercially available without purchasing the corresponding reagents. This would allow for more flexibility in optimization trials. However, the essential components of the reaction will need to be determined by trial and error; there is little information in the literature to determine the foundation for MDA protocols outside the use of supplied kit reagents.

Nonetheless, even with our WGA results, many of the initial experiments were deemed proof-of-concept that there were adequate quantities of DNA in samples that did amplify post-MDA to continue to the array CGH phase. There were no published protocols for the use of array CGH in single cells at the time these experiments were initiated so the knowledge of optimal conditions was limited. Microarrays can cost hundreds of dollars and contain hundreds to thousands of gene sequences. In addition to cost, the choice of arrays to use in initial experiments was made based on the reputation and results of the providing institution. Literature searches provide array CGH protocols with 65°C hybridization temperatures. That appeared to be a foundation for the protocol here since higher temperatures translated into higher stringencies; single cell analysis most certainly requires higher stringencies. However, after two experiments with poor hybridization results, the hybridization temperature was changed to 37°C, in accordance with the manufacturer's protocols that were for standard microarray and not array CGH. Some hybridization signals were seen at the lower temperature; this prompted a side-by-side temperature comparison at 37°C and 65°C of two arrays that contained aliquots of the same labeled sample. In those results, the 37°C exhibited stronger hybridization signals and was used from that point forward. The 37°C temperature theoretically decreases the stringency of the reaction; further optimization experiments will determine if this change has compromised the results and could be the cause of non-specific binding.

Concurrent with the hybridization temperature change was the addition of another restriction enzyme to cut the post-MDA product. MDA products can be as large as 10 kb while the microarray slides are spotted with cDNA fragments that are generally less than 1kb. A restriction enzyme is used after MDA to randomly reduce the size of the fragments so that the MDA products are theoretically not larger than the target DNA on the slide. Initially, DpnI was chosen as the restriction enzyme for these experiments. DpnI recognizes the sequence GATC and is thus called a '4-cutter', that is, there is a one in 4 chance that a particular nucleotide will be in a specific position in a DNA sequence and

this enzyme recognized 4 nucleotides. Theoretically, this sequence should appear once every 4^4 or every 256 bases. Four-cutters are used to produce many small fragments of DNA (Lucito *et al.*, 1998). The first microarray in our experiments did not hybridize. It was not certain if enough restriction enzyme was used and the DNA fragments were still too large. Therefore, the amount was doubled for the next experiment. After another poor hybridization, the hybridization temperature was changed; concurrently another restriction enzyme, EcoRI, was added. EcoRI recognizes the sequence GAATTC and is therefore a 6-cutter. Theoretically, the recognized sequence should be present every 4^6 times or every 4096 bases and would produce larger fragments than the DpnI. However, it is possible the DpnI may actually 'over' cut the sample and cut essential sequences that recognize the target DNA on the array. Since the addition of the EcoRI enzyme occurred in the same successful hybridization experiment as the temperature change, it is not known if the effect was a result of the temperature change or the new enzyme. This could easily be ascertained by reverting to one and then subsequently the other previously used method of enzyme and temperature; these experiments should be performed in the future.

There are many variables in experimental conditions that could also affect the results including the labeling procedure. The next set of experiments used DNA from a MCF7 cancer cell that has been successfully hybridized to other microarrays containing suspected cancer genes. This experiment was conducted to ascertain if the labeling and hybridization conditions were sufficient to produce strong hybridization signals and demonstrated that the techniques were successful for at least 5μg DNA. However, less than 5μg DNA was obtained from our single cells. The next several attempts at array analysis with similar conditions using the known cell line did not have consistent results and hybridizations were weak for the most part. This prompted the switch from the Cy dyes to the Alexa Fluor dyes since the Alexa Fluor dyes are much more efficient at labeling and showed higher fluorescent intensity than the Cy dyes (Berlier *et al.*, 2003). From that point on, strong hybridization signals were seen. This may be due to the lower resistance to photobleaching seen in the Alexa Fluors. This is

the first report of the use of Alexa Fluors in single cell analysis. Further optimization could determine if stronger hybridizations will contribute to their future use as a preferred dye for labeling.

Phi-29 polymerase is a robust enzyme that can yield higher quantities of DNA than the standard WGA techniques of PEP or DOP and can provide reliable representation of the genome (Hosono *et al.*, 2003). Our results support those findings as demonstrated by the microgram quantities recovered and by the ability to amplify at least seven loci from a single sample. However, one limitation of the robust Φ29 is that the enzymatic reaction can create non-specific DNA products. These are products that randomly amplify segments of bases, even if no starting template is added to the reaction, due to excess polymerase and dNTPs in the reaction. Short sequences of DNA can be created that are not complementary to any specific gene sequence. Low quantities of starting template could theoretically promote the generation of the non-specific sequences since there is little template for the reagents to bind to in the initial phase of the amplification. This does not interfere with subsequent PCR reactions as evidenced by the lack of PCR amplification. However, there was a possibility that these sequences would hybridize to the microarray, especially after restriction enzyme digest into smaller fragments that could anneal to the DNA targets on the array. An experiment was conducted that demonstrated these samples could in fact hybridize to the arrays and thus could invalidate the microarray results by not being able to differentiate between hybridization of true DNA versus non-specific DNA.

To answer the question if the non-specific hybridization was an artifact of the MDA technique versus other WGA techniques, samples that were amplified with either PEP or DOP (as the sole WGA) were labeled and hybridized to the array along with a negative control exposed to the respective WGA procedure that showed no PCR amplification (Figure 16). The PEP samples showed poor hybridization results; this was thought to be due to the low overall quantity of DNA that was less than 1μg. The DOP-amplified sample did show strong hybridization signals while the DOP-negative (no template DNA) did not. These

results suggest that the hybridization of non-specific DNA seen after MDA may indeed be an artifact of the MDA technique and not of other WGA methods. With a larger template, MDA generally provides a higher representation of the genome, but due to the non-specific nature of the low-copy template amplifications, further research into the use of DOP as an alternative WGA technique may be warranted.

The possibility that the excess hybridization was an artifact of the stronger Alexa Fluor dyes was successfully minimized since the negative DOP samples did not hybrid even when labeled with one Alexa Fluor while the DNA-containing sample demonstrated strong hybridization signals. There is little scientific basis to suggest that the dyes could be a cause of non-specific hybridization, but the possibility of that occurring could concomitantly be ruled out nonetheless.

In attempts to overcome the creation of non-specific DNA, several cycles of PEP were attempted and an aliquot was removed, then subjected to MDA. This was performed to enable the MDA reaction to begin with higher quantities of template DNA than what would be otherwise initially limited from a single cell. With the higher quantity of DNA in the starting MDA reaction, it was surmised that less non-specific DNA would be amplified since there is more target available for the Φ29 polymerase to anneal to. Unfortunately, the initial attempts at amplifying DNA with PEP then MDA failed to demonstrate subsequent PCR amplification at the attempted volumes and concentrations. The reason for failure could be as simple as sub-optimal reaction conditions since there are many variables in both reactions that must be compensated for including differences in reaction volumes, concentration of reagents, and cycling conditions. Both PEP and MDA use an alkaline lysis method for template preparation. The PEP product was not subjected to further lysis since those components will remain in the aliquot used for MDA. Excess reagent conditions such as potassium and magnesium can lower amplification efficiencies (Rolfs *et al.*, 1992). It is difficult to ascertain reagent concentrations due to the proprietary nature of the MDA kit; reagent concentrations cannot be adjusted accordingly to identify optimal conditions. PCR clean-up kits such as the QiaQuick kits that are used after a

complete MDA might have a role in optimization. PCR purification kits remove components less than 100 bp while recovering 90-95% of the DNA. This could remove any remaining random hexamers from the PEP reaction, but would not be effective for removing other reagents in the reaction. Further investigations into optimization of these variables are warranted as well.

Microarray containing spotted oligonucleotides or bacterial artificial chromosomes (BAC) are also available. Although oligo arrays have the advantage of also providing genotyping data as well as DNA copy number (which could be advantageous in PGD cycles), they appear to have a higher degree of variability than do the BAC arrays; accurate genotyping has been reported while changes in chromosome number were not reliably detected which may rule out their use in our aneuploidy research (Bignell *et al.*, 2004). BAC array CGH is an alternative to the cDNA arrays as BAC clones are becoming more widely available (Cheung *et al.*, 2001). Genomic DNA can be cloned in BAC vectors and spotted onto arrays; the BAC clones serve as a more stable environment for large fragments of DNA (Shizuya *et al.*, 1992). The preparation of the BACs can be problematic since BAC cultures yield very low quantities of DNA. Additionally, it is difficult to maintain ample concentration of DNA to spot the array (Snidjers *et al.*, 2001). Once these obstacles are overcome, the BAC arrays yield more intense signals and are superior over the cDNAs at detecting single chromosome gains and losses (Segraves *et al.*, 2003; Guillaud-Bataille *et al.*, 2004). As a supplement to the experiments here, two attempts were made at BAC array hybridization; no signal was detected (data not shown). Although the manufacturer recommends sophisticated hybridization stations that are not currently available at our facility, further study is warranted to optimize the conditions for the use of BAC arrays.

7.4 Conclusions for Specific Aim # 4

These experiments in Specific Aim # 4 lay the foundation for future experiments as suggested above to continue optimization of the use of microarrays for single cell analysis. From the results obtained, several conclusions can be drawn:

1) MDA can generate microgram quantities of DNA from a single cell
2) Alexa Fluor dyes can be used to produce strong microarray hybridizations
3) DOP may be a substitute for MDA as the WGA prior to microarray analysis

It appears that post-MDA samples are not optimized at this time for microarray analysis due to the generation of non-specific products. However, the larger quantities of DNA may have other applications that would allow for multiple loci to be analyzed from a single sample. This could include experiments for DNA fingerprinting or sequence analysis, which require large amounts of starting DNA (Fiorentino *et al.*, 2004).

SECTION 8
SUMMARY

PGD is an option that allows at-risk couples to have their embryos evaluated for genetic diseases prior to the establishment of pregnancy. The adaptation of PCR methodologies to amplify DNA from a single-cell has resulted in limitations of sensitivity and specificity for a technique that was designed for larger amounts of starting template. The studies presented in this dissertation are part of an ongoing process to evaluate the sensitivity and specificity, and optimize single cell diagnosis. Reproducible and reliable single cell diagnoses can be achieved, and the limitations can be overcome. The development of these methodologies can be costly and time-consuming, but are of the utmost importance for a clinic offering the technology of PGD. The knowledge gained from these studies has contributed both to the direction of research and clinical applications in the field.

From these experiments, the following conclusions were made:
Specific Aim #1: The process of whole genome amplification and subsequent PCR testing can be performed as a means of rescuing samples that previously failed to amplify in initial tests of PCR. The time involved to perform these tests limits the use of the PEP protocol initially, but the method still can serve as an alternative if amplification fails and the genetic status of the embryo is not known. This could abrogate the need to re-biopsy the embryo since an additional non-invasive technique is used to obtain the genetic answer. In addition, these studies laid the foundation for the clinical application of a reamplification procedure assessing a different genetic locus that resulted in the embryo transfer of male embryos whose previous genetic status was unknown in an at-risk X-linked carrier. The studies of Specific Aim #1 introduced a novel method to achieve a genetic diagnosis from previous amplification failure in a single cell (Gibbons *et al.*, 1995b). The techniques have been applied to embryos about which little was previously known in order to provide further genetic information.

Specific Aim #2: Different lysis techniques can affect both the sensitivity and specificity of single cell diagnosis. The KOH lysis method produced a significantly higher rate of amplification and higher accuracy when compared with the other methods tested. Using this method, there was no statistical difference in amplification rate and diagnostic accuracy when testing one versus two cells. These studies also demonstrated the importance of optimizing the PCR conditions by evaluating heterozygous cells that contain both alleles so that the incidence of ADO can be assessed. The study for Specific Aim # 2 was one of the first and certainly the most comprehensive study investigating different lysis buffers and their effect on sensitivity and specificity of single cell analysis. Most PGD clinics today use the alkaline lysis buffer protocol and have referenced these studies (Gitlin *et al.*, 1996).

Specific Aim #3: This set of studies demonstrated the importance of biopsying a blastomere with a visible nucleus. The integrity of the blastomere membrane or the integrity of the cytoplasm itself does not affect the amplification results if the nucleus and nuclear membrane remains intact. The studies for Specific Aim # 3 have provided insight to the optimal blastomere morphology for the improved chances of successful amplification (Gitlin *et al.*, 1997). Blastomere morphology continues to be the preferred method for blastomere selection prior to biopsy.

Specific Aim # 4: From the results obtained, several conclusions can be drawn:

1) MDA can generate microgram quantities of DNA from a single cell
2) Alexa Fluor dyes can be used to produce strong microarray hybridizations
3) DOP may be a substitute for MDA as the WGA prior to microarray analysis

The experiments for Specific Aim # 4, though preliminary, lay the foundation for future experiments as the field of detecting chromosome number expands to include the use of array CGH.

The technology used in PGD is rapidly evolving. New gene sequences and mutation analyses will increase the repertoire of conditions that can be diagnosed. With continuing studies to optimize sensitivity and specificity, one could imagine the future ability to obtain a complete chromosome or genetic profile from a single cell using microarray technology. Other advances could be

in the direction of stem cell research and/ or gene therapy. Currently embryos that are diagnosed as affected are not transferred to the uterus. Most couples discard those embryos although some elect to have them cryopreserved in the event that future technology may enable their transfer to initiate a pregnancy. Technologies for gene therapy and/or embryonic stem cells may provide a promising role in the study of human diseases and the possibility to actual correct the cause of these devastating medical conditions.

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APPENDIX A

PRIMER DESIGN REPORT

Molecule Name: TAY SACHS 11 494 BPS Report Date: 02/05/05

PCR Primer Design Report

---- L E F T P R I M E R ----------

Sequence: 5'-GCCAGACACAATCATACAGG-3'

Length: 20 Bp-pos: 21
%GC: 50; Tm C: 66; Composition: A:8 C:6 G:4 T:2

Criteria: % GC: Min 45, Max 55; Tm C: Min 55, Max 80
Energy cutoff: 0.0 kcal; Reject runs >= 3 bases
Meets criteria selected: YES

---------- R I G H T P R I M E R --------

Sequence: 5'-CAACAACAGTCTGGTGATGG-3'

Length: 20 Bp-pos: 388
%GC: 50; Tm C: 67; Composition: A:6 C:4 G:6 T:4

Criteria: % GC: Min 45, Max 55; Tm C: Min 55, Max 80
Energy cutoff: 0.0 kcal; Reject runs >= 3 bases
Meets criteria selected: YES

Primer Balance:	Left	Right	Comment
Length	20	20	
% GC	50	50	
Tm C	66	67	1C difference

Primer Cautions:	Left	Right	
3 or more G or C at 3' end	NO	NO	
Runs of bases	NO	NO	(see criteria for
Secondary structure	NO	NO	(primers, above

Primer Interactions:

(Number of adjacent bases in largest duplex)

	L:L	R:R	L:R
Any Primer-dimers	2	3	6
3'end involved	2	1	3

Figure 18. Primer design report. Primer design reports are software generated after sequence and preference input, in this case for Tay-Sachs disease.

APPENDIX A (continued)

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Largest Duplexes reported in Primer Interactions
--------------------------------------------------------------------------------
Primer-Dimers with 3'end involvement:


Left:Left:
      L  5'-GCCAGACACAATCATACAGG-3'
                                ::
       L  3'-GGACATACTAACACAGACCG-5'

Right:Right:
      R  5'-CAACAACAGTCTGGTGATGG-3'
                               :
               R  3'-GGTAGTGGTCTGACAACAAC-5'

Left:Right:
      L  5'-GCCAGACACAATCATACAGG-3'
            :::
       R  3'-GGTAGTGGTCTGACAACAAC-5'
--------------------------------------------------------------------------------
Other Primer-Dimers:


Left:Left:
                   L  5'-GCCAGACACAATCATACAGG-3'
                             ::
L  3'-GGACATACTAACACAGACCG-5'

Right:Right:
      R  5'-CAACAACAGTCTGGTGATGG-3'
                     :::
     R  3'-GGTAGTGGTCTGACAACAAC-5'

Left:Right:
      L  5'-GCCAGACACAATCATACAGG-3'
            ::::::
 R  3'-GGTAGTGGTCTGACAACAAC-5'
```

APPENDIX B
CONTAMINATION CONTROL

As a complement to the experiments reported here in this dissertation, extraneous DNA contamination was simulated by adding genomic DNA into PCR reaction tubes containing 60μL sterile water overlaid with oil (Gitlin and Gibbons, 1995). Tubes were placed in 4 typical PCR lab settings under conditions of timed exposure to UV germicidal lighting at 254nm: 1) bench top
2) microcentrifuge tube rack (tubes were placed in rack as normal)
3) inside a glass beaker, closed with aluminum foil (used for sterilization)
4) inside a plastic beaker (used for storage). Tubes were removed from UV exposure after 10, 20, 30, 60, 90, 210 minutes and 14, 18 hours (simulating overnight irradiation). Ten or twenty tubes were assigned to each datum point although some data points were not collected if the results were anticipated to be repetitive. After exposure, all samples along with non-exposed control tubes were analyzed for a known DNA sequence by nested PCR for greater sensitivity. PCR was carried out as previously described in the Materials and Methods section. Fisher's exact statistical test was used to compare results of amplification for the different genes used. The computer program GRAPHPAD INSTAT (Graphpad Software, San Diego, CA) was used to assist in analysis.

Results for the UV light exposure studies are shown in Table XVII and Figure 19. All non-exposed control samples (n=20) demonstrated amplification at all time durations as expected. A decrease in amplification rates was seen in the bench top specimens in as little as 10 minutes after UV exposure. Even though 75% of the samples amplified, the decrease over the 100% amplification rate of the controls was still significant ($P = 0.0471$). A 30-minute exposure completely eliminated PCR amplification in the bench top samples.

Thirty-minute exposure was not sufficient to irradiate the DNA from PCR tubes that were in a housed in a PCR rack or glass beaker. However, PCR tubes that were in a plastic beaker for 30 minutes began to display the significant

APPENDIX B (continued)

effects of UV irradiation and only 40% of those displayed PCR amplification (P = 0.004). It required more than one hour for samples held in the PCR rack to exhibit the effects of the UV exposure. At one hour, there was 100% amplification while after a three hour, thirty minute exposure, there was a slight, but not significant decrease in amplification rates to 85%. Significance was achieved after a longer exposure that ranged between 3.5 hours to 14 hours. At 14 hours (simulating overnight exposure), the samples in the PCR rack amplified at a significantly lower rate than the non-exposed controls (20% versus 100% respectively, P<0.001).

The DNA samples in PCR tubes housed in a glass beaker did not show a decrease in amplification rates, even after an 18-hour exposure.

These studies were undertaken to minimize the risk of contaminating DNA. From this set of studies, we conclude that a 30-minute exposure to ultraviolet irradiation is sufficient to decontaminate most bench top and other surfaces in the PCR lab. However, caution must be exercised when relying on UV irradiation, as some laboratory settings will not be affected. The inability of the UV radiation to penetrate glass should be considered when storing sterile tubes and reagents in these types of containers.

APPENDIX B (continued)

Table XVII. Amplification results after timed UV exposure

Time of exposure	Amplification of Sample on Benchtop	Amplification of Sample in Plastic Beaker	Amplification of Sample in PCR rack	Amplification of Sample in Glass Beaker
Time Zero (controls)	20/20[a,b,c,d,e] (100%)	→	→	→
10 minutes	15/20[a] (75%)	N/A	N/A	N/A
20 minutes	9/20 (45%)	N/A	N/A	N/A
30 minutes	0/10 (0%)	4/10[b] (40%)	N/A	N/A
1 hour	N/A	4/20 (20%)	10/10[c] (100%)	10/10[e] (100%)
1.5 hours	N/A	7/20 (35%)	10/10 (100%)	10/10 (100%)
3.5 hours	N/A	3/20 (15%)	17/20 (85%)	10/10 (100%)
14.5 hours	N/A	0/10 (0%)	2/10[d] (20%)	10/10 (100%)
18 hours	N/A	0/10 (0%)	3/10 (30%)	10/10[e] (100%)

[a] $P = 0.0471$.
[b] $P = 0.004$.
[c,e] no significant difference.
[d] $P < 0.001$.

APPENDIX B (continued)

Table XVIII. Basic guidelines for quality control

- Perform Pre- and post- PCR in separate rooms.
- Maintain sterile/DNA-free techniques.
- All reagents should be aliquoted for single-use where possible.
- Centrifuge aliquots prior to use and use microcentrifuge tube opener.
- Wear caps, masks, gloves. Change Often
- Restrict PCR laboratory access to minimize traffic.
- Autoclave and filter water, buffer, oil, etc.
- Designate aliquoted reagents for clinical use.
- Randomly test in PCR.
- Use forceps to handle tubes, use lid openers, etc.
- Use positive displacement pipettes or use aerosol resistant tips with standard pipettors
- Clean and decontaminate lab surfaces, instruments, racks, etc. with 10% bleach or UV lights.
- Use underpads or liners on benchtop surfaces for easier containment and clean-up.
- Save remaining aliquots of all materials for reference.

APPENDIX B (continued)

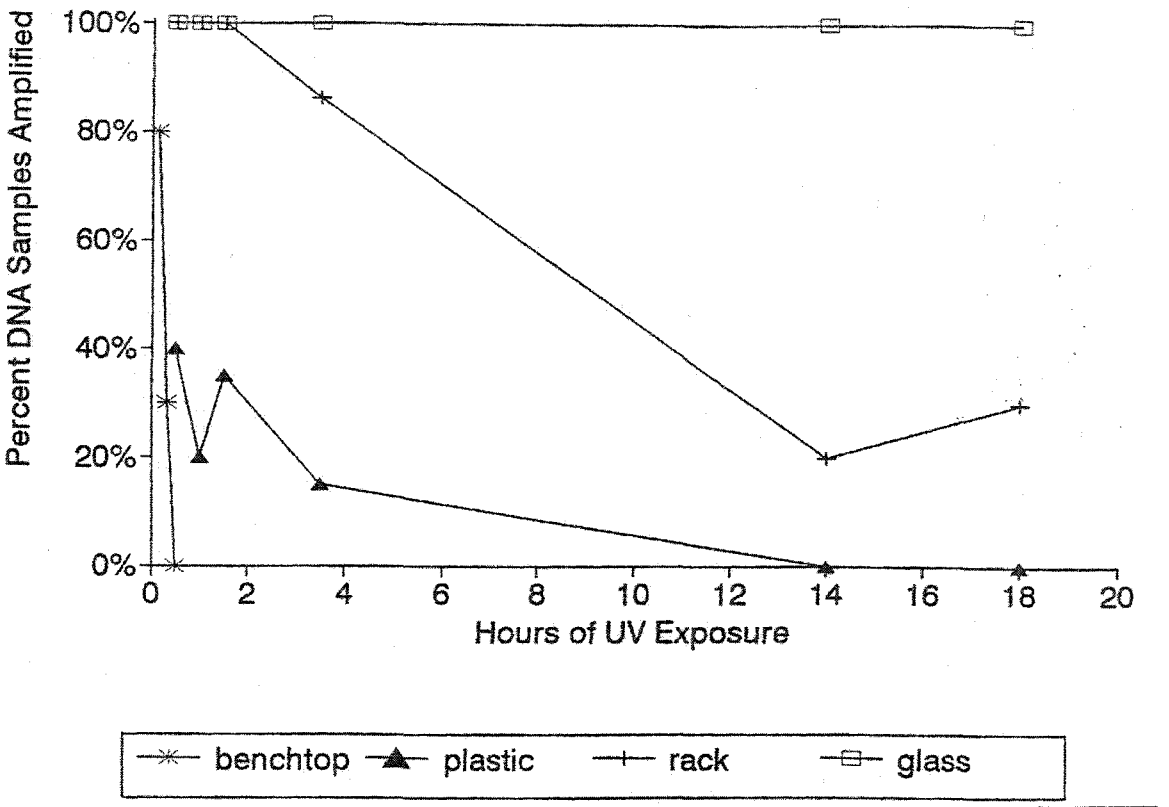

Figure 19. The effect of Ultraviolet irradiation. Reaction tubes containing DNA were placed in four common PCR lab settings and time-exposed to UV lights mounted 18 inches above.

APPENDIX C
STATISTICS

The materials and methods section for each set of experiments lists the statistical method that was applied for data analysis. The *P* value is the probability that a study will show a statistical significance when no real difference exists (Dawson and Trapp 2004). This is known as a type I error – that a true null hypothesis (the hypothesis being tested) is rejected or that a difference is detected when a difference does not truly exist. A *P* value less than 0.05 means that there is less than a 5% chance of a type I error – that the results show a false statistical difference. Thus, the lower the *P* value, the greater the statistical significance.

Another aspect of experimental design is power analysis, or the determination of the ability of the statistical test to an actual difference of effect (Dawson and Trapp 2004). This is in relation to a Type II error – a false null hypothesis is not rejected or a difference is concluded when a difference does not exist. The power is calculated as (1 minus a type II error, or 1-β). This relates to the number of samples needed to detect a statistical significance when one truly does exist.

The main statistical test used in this dissertation is Fisher's exact test. This is used when the sample size is too small for the Chi-Square analysis. An explanation of both follows. It was Ronald Aylmer Fisher in 1922 that defined three fundamental issues of statistics:

1) specification of the kind of population that the data came from
2) estimation (choice of the method to calculate)
3) distribution (Fisher 1925).

Fisher arranged experiments into sub-experiments that differ from each other by several factors, treatments, or other variables. The differences could then be detected by statistical outcomes; this advance allowed for greater flexibility in experimental design and allowed for more than one experimental variation rather

APPENDIX C (continued)

than the inefficient existing approach at the time of only varying one technique at a time.

Chi-Square is used to test the hypothesis that all classifications or factors have the same level of frequency or distribution (Armitage 1994). Chi-Square tests are appropriate for *actual numbers* of occurrences, not on percentages, proportions, means of observations, or other derived statistics (Swinscow 1997). This is one reason why Chi-Square (and Fisher's exact test) are the most suitable for statistical computation here. Chi Square is an approximate and versatile test in that it does not matter how a question is stated – it measures the observed versus the expected frequency. Although most statistical computations are now calculated by computer software packages, the calculation for Chi-square (χ^2) is listed in Equation 6.

$$\chi^2_{(df)} = \frac{\Sigma\,(\text{Observed frequency}-\text{Expected frequency})^2}{\text{Expected frequency}} \qquad (6)$$

df = degrees of freedom, calculated as *df* = (r-1)(c-1), the number of rows minus one times the number of columns minus one (Dawson and Trapp 2004).

Yates correction may be applied to a 2x2 contingency table (Dawson and Trapp 2004). This correction involves subtracting half the difference between the observed and expected frequencies before squaring. This makes the χ^2 value smaller. Smaller χ^2 are attractive because it means that the null hypothesis is rejected less often; thus, it is a more conservative test. Going back to the definitions, this reduces the chance of a Type I error, although it does increase the chance of a type II error.

The following demonstrates a 2x2 contingency table used to calculate if there is a difference in a treatment group, as in the dissertation when a different lysis method is used. The difference between Chi-Square and Fisher's is calculated in Table XIX.

APPENDIX C (continued)

Table XIX. Example of contingency table for statistical calculation

	Amplification	No Amplification	Total
Control	a	b	a+b
Treatment	c	d	c+d
Total:	a+c	b+d	a+b+c+d=n

Where Chi-square =

$$\chi^2_{(df)} = \frac{\Sigma\,(\text{Observed frequency}-\text{Expected frequency})^2}{\text{Expected frequency}}$$

Following the table:

$$\chi^2_{(1)} = \frac{n(ad-bc)^2}{(a+c)(b+d)(a+b)(c+d)}$$

And Fisher's exact test is:

$$P = \frac{(a+b)!(c+d)!(a+c)!(b+d)!}{a!b!c!d!n!}$$

where ! is the symbol for factorial (n-1)(n-2), etc., (Dawson and Trapp 2004).

APPENDIX C (continued)

As previously stated, a 2x2 frequency table with small numbers of expected frequencies (in case the total number of observations is less than 20) should have the statistics calculated differently. Fisher's exact test should be used rather than the Chi-Square. Many times, the computer software will apply the most appropriate test when the sample size is in question.

There are other statistical tests that compare two data sets as does the Chi-Square and Fisher's exact (Dawson and Trapp 2004). One is the Kolmogorov-Smirnov test (KS-test); however, it is not as sensitive. Fisher's exact test is more appropriate since it retains more power with small sample sizes.

APPENDIX D

LIST OF COMMON ABBREVIATIONS USED

A	Adenine
ADO	Allele drop-out
BAC	Bacteria artificial chromosomes
BI	Boiling
bp	Base pair(s)
BSA	Bovine serum albumin
C	Cytosine
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductor regulator gene
CGH	Comparative Genomic Hybridization
DNTP	Deoxynucleotide triphosphate
DOP	Degenerate oligonucleotide primers
DTT	Dithiothreitol
FISH	Fluorescent in situ hybridization
G	Guanine
H_2O	Water
ICM	Inner cell mass
IVF	In vitro fertilization
kb	Kilobases
KCl	Potassium Chloride
LN_2	Liquid Nitrogen
MDA	Multiple displacement amplification
mFISH	Multifluor fluorescent in situ hybridization
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
PB	Polar body
PBI	First polar body
PBII	Second polar body
PCR	Polymerase chain reaction
PEP	Primer extension preamplification
PGD	Preimplantation genetic diagnosis
SDS	Sodium dodecyl sulfate
SKY	Spectral karyotype analysis
SSC	Saline-sodium citrate
T	Thymine
Taq	*Thermus aquaticus*
TE	Trophectoderm
T_m	Melting temperature
TSD	Tay-Sachs disease
UV	Ultraviolet
WGA	Whole genome amplification

APPENDIX E

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VITA

Susan Adele Gitlin was born in Bluffton, Indiana and completed her secondary education there. She received her Bachelor of Science degree from Purdue University in West Lafayette, Indiana, in May 1980, majoring in Animal Science. Her post-graduate training began at Texas A&M University in College, Station Texas where she received her Masters of Science in Physiology of Reproduction in May 1983, under the mentorship of Dr. Paul Harms. Her thesis was entitled: Bovine pituitary release of luteinizing hormone during gestation and early postpartum. During her two years at Texas A&M, Susan taught the laboratory section for Reproduction in Farm Animals.

Her professional career began at Baylor College of Medicine in Houston Texas where she was the embryology supervisor for the In Vitro Fertilization Laboratory in the Ob/Gyn department. After leaving Baylor, Susan became the laboratory director for Infertility Associates of Indiana in Indianapolis, Indiana.

Susan joined the faculty of Eastern Virginia Medical School as an instructor in the Ob/Gyn department to perform the genetic analysis for the Preimplantation Genetic Diagnosis (PGD) program. In addition to that position, Susan assists with the patient education for couples involved in the PGD process. The Jones Institute PGD program is recognized as one of the leaders in the field. Susan has presented papers on PGD at national and international meetings and is a member of the European Society of Human Reproduction's consortium on preimplantation genetic diagnosis

Susan is an instructor for the Master of Science program in Clinical Embryology and Andrology as well as the Mammalian Reproduction course. Susan began the Biomedical Sciences Ph.D. program under the mentorship of the late Dr. Gary Hodgen.