Application of LATE-PCR to Detection of Genetic Trisomies:

Construction of an Assay for Down’s Syndrome

Oze Koreh
Senior Thesis
Wangh Laboratory
Department of Biology, MS008
Brandeis University
Waltham, MA 02454

Research Mentors:
Lawrence J. Wangh, Ph.D., J. Aquiles Sanchez, Ph.D., Kenneth Pierce, Ph.D., John Rice, M.S., Arthur H. Reis Jr, Ph.D. and Cristina Hartshorn, Ph.D.

Research Period:
January 2006 – May 2009
April 2009

1. Abstract:

LATE-PCR, an advanced form of asymmetric PCR amplification developed in the Wangh laboratory, offers a novel approach to construction of a clinically-compatible, convenient, reliable and sensitive assay for detection of Down's syndrome, a genetic condition caused by trisomy of chromosome 21. The assay described in this thesis interrogates genetic markers in chromosome 21 to distinguish between diploid genomes having two copies of chromosome 21 and triploid genomes having three copies of the same chromosome. These genetic markers, also known as heterozygous markers or alleles, are comprised of single nucleotide polymorphisms (SNPs) that differ in the paternal and maternal copies of chromosome 21. To increase the likelihood of finding a heterozygous (i.e. informative) SNP marker I developed LATE-PCR assays for a set of high frequency SNP sites within a region of chromosome 21 that is tightly linked to Down's syndrome. Starting with samples containing as little as a single cell, the assay can amplify as little as a single SNP and then can analyze the SNP using a single fluorescent probe at several temperatures, in order to establish the fraction of chromosome 21 copies that have one SNP sequence among the total number of chromosome 21 copies in the sample. For instance, the normal diploid genome of a heterozygous individual has two copies of chromosome 21, either one of which (50%) has its own SNP variant. In individuals with trisomy chromosome 21, one of the variants at a heterozygous SNP will be present in either 66% or 33% of all copies of that SNP on all copies of chromosome 21. Utilization of SNPs with a high frequency of heterozygosity in the European and global populations to ensure a 98.5% chance of obtaining at least two informative SNPs for every subject.

2. Conclusion:

This thesis demonstrates that LATE-PCR assays based on the logic described in the abstract, readily detect chromosome 21 trisomy with 99.0% accuracy in commercially available cells lines derived from Down's syndrome patients.
INTRODUCTION:

Long Term Research Goal: Genetic duplications and deletions of either small segments of the human genome or entire chromosomes are responsible for a wide array of clinical conditions which range in severity from negligible to lethal. However, routine diagnosis of these conditions is hindered by the complexity and costs of detection. The goal of my research is to develop a novel molecular diagnostic method that can be widely applied to detection of genetic duplications of virtually any chromosomal segment. This method is demonstrated here by detection of Down’s syndrome (DS), which involves a trisomy of human chromosomes 21.

In accord with our goal, this thesis reviews current clinical detection methods and evaluates their strengths and weaknesses. This thesis also introduces a new method for detection of chromosomal duplications that overcomes all of the limitations of previous methods. It uses LATE-PCR for chromosome counting, as first described by Aquiles Sanchez in the Wangh laboratory.

Down’s syndrome: Overview

Down’s Syndrome (DS) is the most common genetic alteration due to an extra chromosome copy, occurring in 1 out of 750 births (0.13%). This corresponds to 22-43% of all chromosomal non-disjunction cases, which occur in ~0.3-0.5% of live births (1). The frequency of chromosomal non-disjunction leading to trisomies is actually higher, since trisomies are frequently found in spontaneous abortions. Among the aborted conceptuses, trisomy 21 is found in 1 out of every 43; trisomy 16 found in 1 out of 13. A review of several fetal loss studies indicates a 23%-43% loss of DS diagnosed pregnancies post CVS and amniocentesis analysis, respectively (2).

The clinical presentation of DS is highly complex and variable; most traits only affect a portion of the population, such as congenital heart disease occurring 40-50%. Several features present for the entire population, such as cognitive impairment, facial dysmorphology, and hypotonia as newborns; furthermore, the histopathology of Alzheimer disease can be detected by the fourth decade in most DS patients.

Diagnosis of DS presents the challenge of detecting the difference between normal individuals with 2 chromosome 21 copies versus Down’s syndrome individuals with 3 chromosome 21 copies resulting from meiotic non-disjunction. The DS population consists of about 95% complete or partial trisomies. Partial trisomies of over 5Mb are less common than whole genome trisomies and result from abnormal meiosis and
segregation. About 2% of DS have acrocentric translocations of chromosome 21 with one of the other acrocentric chromosomes, most often with chromosomes 14 or 21, but may also occur with 13, 15, and 22 (3). Recognizable mosaicism of normal and trisomic cells exists in 2-4% of cases, resulting from a mitotic chromosome segregation error. Microtrisomies, caused by unequal crossover in meiosis, may occur due to the presence of intra-chromosomal duplications or low copy repeats; however their incidence is unknown due to the lack of routine high quality cytogenetic analysis.

Since DS is characterized by a third copy of chromosome 21, it would follow that the dosage of chromosome 21 genes would be expressed 50% more relative to euploid individuals. Gene over-expression in trisomy 21 has been studied and determined to occur in only 39% and 62% of lymphoblastoid and fibroblast cells, respectively. On average, genes were found to be up-regulated by 1.44 and 1.67 compared to normal diploid lymphoblasts and fibroblasts, respectively. Importantly, it was observed that many genes present in triplicate exhibited no up-regulation (4). The same has been confirmed in murine models (3). In an attempt to identify a region specific to DS, classification of genes based on degree of expressivity has been conducted. The diploid expressivity level has been compared to that of trisomics in order to understand if certain parts of human chromosome 21 are preferentially over-expressed. Three categories were created: those identical, under-expressed, and slightly over-expressed, and highly elevated expressivity genes. Despite these efforts, there do not appear to form any continuous genomic region and are spread out throughout chromosome 21 (3).

Studies of human chromosome 21 translocations show that individuals expressing the DS phenotype share a common triplicated minimum region. This region, known as the Down's syndrome Critical Region (DSCR), is located within 21q22 and is about 5.4Mb long. The actual boundaries of the DSCR have been controversial, however, due to the discovery of individuals with the DS phenotype with smaller partial duplications. For example, a 2.3Mb partial trisomy for 21pter-q22.13 presented with the DS phenotype (5). The concept of the DSCR itself (i.e. that the DS phenotype is caused by the genes in the DSCR) has also been questioned.

Studies showing that a triplicated DSCR alone does not necessitate the DS phenotype have lead researchers to develop other models of understanding the disease (5). An example of an alternate model implicates up-regulation of coding non-genic regions (CNG) on human chromosome 21 as cis acting elsewhere in the genome.
Despite the controversy associated with the DSCR, many diagnostic tests focus on this region in an attempt to detect Down’s syndrome cases due to translocations.

**Screening for Down’s Syndrome**

The current standard of care in the United States involves the use of up to four non-invasive methods, followed by the offer of invasive testing if the initial testing indicates a possible trisomy. Current methods for early disease detection include invasive sample collection techniques, including amniocentesis and chorionic villus sampling (CVS), which allow collection of enriched fetal cells, but increase the risks of miscarriage and infection. Alternatively, non-invasive techniques, such as ultrasonography and maternal serum screens pose minimal risk to the mother or fetus, but do not provide definitively determination of trisomy due to their high rate of false positive.

Pregnancies are first monitored for Down’s syndrome by ultrasound and serum tests in those cases at high risk (i.e. women older that 35). Other high risk factors for Down’s syndrome include women with a medical condition (diabetes, asthma, high blood pressure, or epilepsy), paternal or maternal family history of genetic disorders, or previous premature pregnancies or birth defects. If fetal abnormalities are detected or the pregnancy is determined high risk, then parents may elect more invasive procedures acknowledging the accompanying risks (see below).

The ultrasound detection, typically recommended at 7 weeks, may be used to confirm the existence of a pregnancy, establish a date of conception, and check for multiples. Ultrasound is repeated at 11-13 weeks to assess the nuchal translucency (NT), observing the amount of fluid around the neck; high fluid volume indicates a high risk for DS. NT over 3mm at 7 weeks correlates with a 12-fold increase in fetal aneuploidy; NT can detect up to 72% of DS cases with a 5% false-positive rate. The additional scan can be used to observe the fetal nasal bone for indication of DS, as well as detect other neural tube disorders.

During the first trimester maternal serum can be checked for levels of free human chorionic gonadotropin (ß-hCG) and pregnancy-associated plasma protein A (PAPP-A). Combination of both measurements during the first trimester allows for a 60% detection rate for trisomy 21 with a false positive rate of 5% (6). During the second trimester, alpha fetoprotein (AFP), inhibin-A, ß-hCG, and unconjugated estriol (uE3) levels are observed for abnormalities; combined they are known as the quad screen. They allow for 75-80% detection rate for women over 35 with a similar false positive rate. Low maternal serum
AFP and uE3 with elevated levels of β-hCG and Inhibin A, for example, is associated with DS. The combination of both NT and first trimester screening allows for detection of 80% of cases with a 5% false-positive rate; combination of the first trimester serum test, the NT test, and the second trimester test, raises the detection rate to 85% with a 5% false-positive rate, however results cannot be delivered to parents until late in the pregnancy. Those tests that utilize two hormones in the first trimester and two in the second, combined with NT measurement, are known as the Combined Test and are the most comprehensive approach to non-invasive screening. Only if abnormalities are found are invasive methods recommended to the pregnant woman.

The current screening practices have limitations associated with the timing of screening, certainty of being affected, and delay associated with receiving results. During most of the first trimester, detection cannot be performed due to increased risk of the fetus, due to insufficient amniotic fluid, which does not begin to accumulate until the 9th week of gestation. Some detection may occur late in the first trimester via chorionic villus sampling (CVS), however, most detection, which is performed by amniocentesis, cannot be done until the 15th week. As such, if a genetic abnormality is identified late in a pregnancy, the mother has only a short period of time to decide whether to continue her pregnancy. The preferred approach of screening, the Combined Test, uses NT (from ultrasound) and the Quad Test along with maternal age, to identify women at risk of over 1:250 of being affected (7). Those women at a greater risk are interpreted as screen-positive for Down's syndrome and are offered a diagnostic test, usually amniocentesis. Approximately 1 in 20 of all screened women fall into the screen-positive group, as the false positive rate is set at 5% and the occurrence rate for the group is 1/250 (p=0.004). The Combined Test has the Odds of Affected given a Positive Result (OAPR) of 1 in 23, meaning that 22 out of 23 of all women who screen-positive will be relieved to learn through detection that they are carrying a normal pregnancy. Current detection methods rely on obtaining the amniocentesis or CVS, culturing the sample for 2-3 of weeks, and then proceeding to analysis. The current detection methods have a high sensitivity and reliability, however, they require significant waiting time that causes the expecting parents anxiety. This means that, 22 out of 23 of all parents worry for 2-3 weeks when they screen-positive until they are relieved by a false positive diagnosis.
New “Non-Invasive” Methods of Sample Collection for Genetic Diagnosis:

Whether elected by the patient or recommended by the genetic counselor, a sample of fetal DNA needs to be obtained for genetic diagnosis. Fetal genomic sample can be collected by several traditional methods, all of which are invasive, including chorionic villus sampling (CVS), amniocentesis, and percutaneous umbilical blood sampling (PUBS). Recently, noninvasive approaches to detection have been described using DNA or mRNA’s circulating in maternal blood plasma. Prenatal detection, through analysis of the mother’s blood, has been demonstrated by Dennis Lo, et al. (8) who have used circulating mRNA’s in maternal serum to quantify gene expression from fetal chromosome 21. In a related effort, Chim, et al, (9) illustrated that non-invasive detection is possible using known DNA methylation markers associated with particular disorders. They identified a panel of CpG islands that is differentially methylated between the maternal and fetal genomes and established CpG sites that are either completely methylated in the maternal sample and less than 20% methylated in the fetal sample, or completely methylated in the fetus and less than 20% methylated in the mother. A caveat of this study is that differentially methylated markers do not exhibit complete penetrance in the population (i.e. these markers are not consistent among different individuals).

Another limitation of this approach lies in the method of PCR amplification used to distinguish methylated and unmethylated cytosine residues. PCR involving methylation sensitive markers, or using methylation-sensitive PCR (MSP) requires modification of all DNA by sodium bisulfite, to convert all unmethylated, but not methylated, cytosines to uracil. This makes the subsequent amplification with primers specific for methylated versus unmethylated DNA. The use of sodium bisulfite is damaging to DNA and counter indicated for PCR. As such, the application for LATE-PCR would have to be modified as to not interfere with amplification. The alternative, previous approach to studying methylated utilizes differential restriction enzymes to cleave and distinguish methylated from unmethylated DNA (10).

Traditional Invasive Methods of Sample Collection for Genetic Diagnosis

Amniocentesis involves insertion of a needle from the outside through the wall of the uterus into the amniotic sac with the guidance of ultrasound for the collection of an amniotic fluid sample which contains fetal cells. A variety of techniques of amniocentesis exist, resulting in a wide range of miscarriage rates, ranging from 0.1% to 1%. The risk
for miscarriage from amniocentesis was investigated in the US using 33,500 pregnant women with singletons, known as First and Second Trimester Evaluation of Risk (FASTER) Trial, by the Society of Maternal-Fetal Medicine. This large scale trial identified the risk of first trimester miscarriage as 0.15% and created the guideline that amniocentesis should not be performed before 14 weeks (11). Amniotic fluid is the product of fetal urination. Following kidney maturation in the 9th week of gestation, a sufficient amount of fluid has to build up to buffer the fetus from the placenta before using amniocentesis. As such, it is recommended that amniocentesis be performed between week 15 and week 20 of the gestational period, when sufficient amniotic fluid has been collected for sampling. If amniocentesis is attempted too early, anhydramnios, or lack of amniotic fluid, may ensue. In short, amniocenteses should only be performed for specific indications during the first trimester; otherwise it should be reserved for the second semester when the risk of miscarriage is reduced.

Chorionic villus sampling (CVS), the current standard for the end of the first trimester diagnosis, harvests placental tissue after 10-13 weeks of conception, which is earlier than possible with amniocentesis. First trimester CVS has a risk of 0.2-0.42% miscarriage, comparable to second trimester amniocenteses (6, 12). CVS has a 2% sampling failure requiring secondary sampling, in comparison with 0.4% for amniocentesis.

Percutaneous umbilical blood sampling (PUBS) provides the means for chromosomal analysis in the absence of indicative results from amniocentesis, CVS, and ultrasound. Similar to amniocenteses, a needle directly samples fetal blood from the umbilical cord using the guidance of advanced imaging ultrasound. The procedure poses a significant risk, 1-2% of procedures result in miscarriage; to decrease the risk, the procedure is only performed after the 17th week of gestation.

During genetic sample collection from the fetus, a possibility exists for maternal DNA contamination. As a result, an uncertainty exists in any sample regarding the ratio of maternal to fetal contributions. Amniotic fluid is normally clear; but in the case of maternal blood contamination, the fluid turns red and is discarded. CVS or PUBS could potentially have maternal contamination which would be difficult to identify with the naked eye. Most studies assume that the genetic origin is predominantly, if not entirely, fetal; however, the only way to make certain of this would be to use fetal markers as part of the diagnostic testing.
Current Methods of Genetic Testing: Critique of Their Strengths and Weaknesses

Once collected, fetal DNA can be analyzed for chromosome 21 trisomy by an assortment of methods.

Cytogenetic Methods:

Karyotyping is the current standard practice to identify human chromosome 21 and all other aneuploidies and balanced translocations. A chromosome spread (karyotype) permits the detection of structural chromosomal abnormalities; however the technique is limited to only gross deletions and duplications over 5Mb, requires the growth of cells over several days, and is labor intensive. In order to increase the resolution for chromosomal abnormalities, Fluorescent in situ hybridization (FISH) is used.

FISH is a cytogenetic technique that detects the presence or absence of specific DNA targets based on signals from fluorescently labeled DNA probes hybridized to a substrate, typically cells. There are several types of FISH techniques (i.e. regular, interphase, and fiber) which vary in the nature of the substrate used for chromosomal probe hybridization. Regular FISH is carried out on metaphase chromosomes that are tightly coiled; in interphase FISH, hybridization is performed on intact (interphase) cell nuclei where chromosomes adopt a random confirmation; in fiber fish, the cell nucleus is broken and the interphase chromosomes are tightly stretched out on a slide to achieve maximal resolution of closely spaced targets (i.e. <5 kbp). Sample preparation for Fiber FISH is an art that only a few laboratories can perform, thus accounting for the rare use of this technique. Detection of duplication and deletions using FISH is based on the number of fluorescent signals which corresponds to the copy number of the target sequence in each nucleus.

FISH has distinct advantages. FISH provides the ability to detect large sequence deletions/duplications, probes can be made in-house in most labs, and several FDA approved commercial probe sets are available in the market. Although having much potential, FISH is limited by the requirement of specifically labeled probes, significant cost, and high labor requirement. The principal limitations of FISH, are however, low throughput and the limited ability to detect only deletions that are the exact probe length.

Comparative genomic hybridization (CGH) is a cytogenetic method for assessing copy number changes. Patient DNA and control DNA are both labeled with different fluorescent dyes and hybridized to normal human metaphase preparations or on a DNA molecules arrayed on a slide. The resulting regional differences in fluorescence between
the unknown sample and the control are identified as loss or gain of genomic DNA. CGH allows high resolution of 20-80bp detection of full and partial trisomies, but can only detect unbalanced translocations. It cannot detect balanced reciprocal translocations or inversions since they do not change copy number. Lastly, a significant amount of DNA is required for the assay (1-5mg).

**PCR Methods:**

Quantitative fluorescence PCR (QF-PCR) permits the relative quantification of polymorphic microsatellite markers for the determination of copy number. Microsatellite alleles differ in size. Following amplification of sequences flanking microsatellites alleles using fluorescently labeled primers, amplicons are separated by size on a gel. Once individual alleles are identified, allele peak measurements are obtained by a semi-automated analyzer to compare their relative amounts. Peak heights and areas are the current standard of allele copy number comparison. Assays can be designed in-house; simultaneous amplification of multiple microsatellite markers (multiplexing) allows for fast, cheap, and minimally laborious requirement. Difficulties arise in interpretation of microsatellite alleles of PCR product ratios due to preferential amplification of the smaller allele. Testing relies on the availability of parental DNA and informative markers.

Homologous gene quantitative PCR (HGQ-PCR) (13) determines the copy number of chromosome 21 by simultaneous amplification of two highly homologous genes one located in chromosome 21 (for example, the human liver-type phosphofructokinase) and the other located on another chromosome as a copy number reference (i.e. the human muscle-type phosphofructokinase on chromosome 1). In this method, both genes are amplified using a single pair of primers which preserves the relative number of these targets among amplification products. Identification of gene copy number by relating ratios of the two genes can detect mosaicism and deletions, enjoys equal amplification of the two assayed genes, and has demonstrated 95% reliability in a clinical trial. However, HGQ-PCR is labor intensive, requiring serial dilutions of the amplicons, and the use of a fluorescent DNA sequencer followed by electrophoretic separation (although each gene target could also be distinguished by gene-specific probes). Lastly, HGQ-PCR requires high quality DNA; otherwise amplification of homologous genes PFJL and PFKM may result in erroneous ratios.

Paralogous sequence quantification (PSQ) is similar to HGQ-PCR in that PSQ uses the same primers to amplify two genes on different chromosomes to quantitatively
measure the copy number of human chromosome 21. Contrary to HGQ-PCR, PSQ uses as targets paralogous genes that have a high degree of sequence relatedness, i.e. a gene and a pseudogene that are located at different loci and accumulate single nucleotide polymorphisms (SNP’s) at different rates. Since identical primers are used, the efficiency of PCR is identical for the two genes and any difference in amplicon yield identifies allele imbalance. Relative dosage difference can be determined from pyrosequencing the paralogous sequences.

Multiplex probe ligation assay (MLPA) can detect dosage changes at the single nucleotide level. This technique is based on comparative quantitation of specifically bound probes amplified using universal primers allows high order multiplexing, potentially achieving wide genomic coverage. Genomic DNA is hybridized to two halves of probe, both flanked with universal primer sequences. One half has a fixed length of 20-30nuc, whereas the other half has a variable length fragment (25-43nuc) allowing for differentiation by length in electrophoresis; both halves have a target specific sequence. During PCR, both halves bind, are joined by a DNA ligase, and are amplified by the universal primers. Following PCR, amplicons are separated via electrophoresis and relative peak heights indicate duplications or deletions (14). MLPA allows multiplexing of up to 40 targets using a single fluorescently labeled primers, while allowing for the identification of mid-sized deletions or duplications missed by cytogenetic and DNA techniques. MLPA probes creation via M13 cloning is a tedious task; the assay requires at least 20ng DNA, however, reproducible reliable results have been reported to require upwards of 100-200ng DNA (14). MLPA may produce erroneous results if a polymorphism exists in the sequence where probes bind; a SNP would prevent the probes from binding and ligating, thus appearing like an exon deletion.

Multiple amplifiable probe hybridization (MAPH) requires 1ug of DNA that is fixed and hybridized to a membrane. A set of probes with flanking sequences corresponding those to the primers are added; every probe has a different length corresponding to its target, but with identical flanking sequences. Excess probe is washed away, leaving the bound probe in equal amount as its target. The probe are then removed from the membrane and then amplified by the primer pair. The products are separated by electrophoresis and quantified based on their relative band intensities or peak heights. Just like MLPA, MAPH provides the potential for high order multiplexes. Probe design presents the challenge of making every probe significantly different in length to allow separation by electrophoresis; furthermore, probes are created by cloning the target
sequence into a plasmid vector in order to create identical flanking primer sequences, which in itself is a tedious task. MAPH poses a contamination risk due to the repeated use of identical primers; the washing step creating unbound probe contamination, but also requires 2-3 days to complete the experiment due to the need of overnight hybridization. Just like MLPA, MAPH presents a risk of false indications of segmental deletions, such as in the case of no deletion when 100-200bp probes binds to part of the sequence but anneals to the deleted region (14).

Quantitative Real-Time PCR (qRT-PCR) allows for a continuous detection of product throughout the amplification. Amplification takes place in a thermocyclers with an optical system for exciting fluorochromes and monitoring their emitted wavelength. PCR products can be monitored by intercalating dyes or by various probes. Intercalating dyes, while economical, pose a risk of non-specific binding to double stranded products. Fluorescent probes bind their complementary DNA target and provide the specificity that intercalating dyes lack. Use of multiple fluorochromes with discrete wavelengths allows for PCR multiplexing. Amplified DNA can be quantified as an absolute number of copies compared in relative amounts to other amplified products, all in real time. This technique has been used for allele discrimination (genotyping) and gross quantitative assays. However, there have been very few reports on the use of real-time PCR for detection of large deletions and duplications. RT-PCR eliminates the need for gel electrophoresis and post-PCR processing, thus reducing the chance of laboratory contamination with amplification products. Other benefits include the requirement of minimal amounts of DNA, high reproducibility, and a protocol that is technically straightforward and easily automated. Unfortunately, RT-PCR does not provide size resolution, multiplexing is largely limited by the number of fluor that can be simultaneously screened in the same tube, and by the high cost of the probes, roughly $250 per design. Lastly, the challenge in this technique is the need to detect less than two-fold (2x) difference in copy number for detection of chromosome duplications (the actual difference between in chromosome copy number between a Down syndrome individual and a normal individual is 1.5), thus any scatter decreases resolution. As a result, this method requires much optimization.

Melt curve analysis employs the characteristic dissociation of dsDNA as a function of temperature to confer the identity of a SNP. PCR amplification is performed using either an intercalating dye that binds within dsDNA or utilizing molecular probes with fluor and quenchers as a source of fluorescence. The dissociation of the two DNA strands in a reaction is dependent on their complementarity, strand length, and GC content (GC's
have 3 hydrogen bonds, so higher energy binding). Heating up, or melting, the complementary dsDNA while measuring change in fluorescence as a function of temperature, followed by normalization, establishes a quantification of the allele as percent allowing for characterization of the SNP. The technique uses SNPs which are readily available, allows single reaction analysis with no intermediate handling, permits rapid analysis in about 1 hour, has the potential for multiplexing (although limited to about 4 dyes per well), and may easily be adapted to a high-throughput format. Melt curve analysis as applied to DS detection illustrates the limitations of this method for assessment of chromosomal copy number differences. Pont-Kingdon et al applied melt curve analysis to DS detection and concluded that this method could be used as an adjunct for traditional cytogenetic analysis (15). Melt curve analysis lacks a high degree of reliability, as alleles are only differentiable to 2 standard deviations. Furthermore, intra-assay variability requires the use of positive controls in every run to which other samples are standardized; this requires creating new confidence intervals during each assay. Additionally, the assay lacks correction for background noise, extent of probe binding, or the effect of temperature on fluorescence. The major issue encountered by Pont-Kingdon et al is that the assay is subject to extreme experimental variability.

**Figure 1: Graph of Melt curve analysis from Pont-Kingdon**
Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry is the newest approach for prenatal detection of chromosomal aneuploidies. The technique has the advantages of the compatibility for high throughput analysis, as well as the potential for high degree of multiplexing. The current multiplexing capacity is 24 assays in one reaction using iPLEX system developed by Sequenom. The disadvantage of the procedure is that it requires hundreds of replicates samples, and use of gel electrophoresis to separate the different samples. Dr. Dennis Lo et al looked at 9 proteins strictly associated with fetal growth and development through 2 dimensional gel electrophoresis (2-DE) and MALDI-TOF to identify abnormal biomarkers for DS. Dr. Lo has identified plasma placental RNA markers for noninvasive prenatal detection (16). He then created the approach of MALDI-TOF, 2-DE assay, specifically looking at a SNP on PLAC4 mRNA, transcribed from fetal chromosome 21, to identify DS individuals (17). The results of his work have allowed him to identify cases of DS in the laboratory, following extensive enrichment of fetal mRNA's from the maternal serum (17). The results of his work have allowed him to identify cases of DS in the laboratory, following extensive enrichment of fetal mRNA's from the maternal serum (17). His accomplishment sparked a rush to develop commercial prenatal detection assays, a challenge taken up by a Bioengineering group at Stanford University who came up with Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood (18) and an effort by Sequenom, who have commission the work of Dennis Lo, himself. Based on Lo’s work, Sequenom released a MALDI-TOF mass spectrometry assay for the reliable detection of trisomy 21 (19). Their future perspective for noninvasive prenatal diagnostics indicates that they have exclusively licensed intellectual property rights to develop non-invasive prenatal genetic tests using the MassARRAY System and other platforms. Their goal is to create the SEQureDX technology, based on the work of Professor Dennis Lo from the Chinese University of Hong Kong, analyzing circulating cell-free fetal (ccff) nucleic acids from maternal blood.

Dr. Lo was able to use digital PCR to create a strategy able to detect allelic imbalance of SNPs transcribed from human chromosome 21 (20). PLAC4 mRNA, a placental mRNA transcribed from fetal human chromosome 21, was chosen as a marker.
Maternal plasma of women carrying trisomy 21 fetuses had blood drawn peripherally, enriched for ccff mRNAs to 25%, and then he could reliably detect allelic imbalances between the marker and a reference. To carry out the analysis, multiple 96 and 384 well plates were used per individual; some individuals had as many as 7 384 well plates used, meaning 2688 wells for a single individual. That means that 2688 digital PCRs were done and 2688 lanes were used in the 2-DE gel. Such extreme amounts of work are necessitated by this technique, rendering it unconventional for the clinical setting. Below are the results of the MALDI-TOF 2-DE method.

**Figure 2: Results of MALDI-TOF 2-DE method**

![Graph showing G/A ratio comparison between placenta and maternal plasma](image)

**Description:** RNA-SNP allelic ratios of PLAC4 in placentas and maternal plasma of euploid and trisomy-21 pregnancies. The ratios of the trisomy-21 samples segregated into two groups. Trisomy samples with an extra G allele showed a higher G/A ratio than the euploid group, whereas samples with an extra A allele showed a lower G/A ratio than the euploid group. The reference intervals, defined as the mean ± 1.96 s.d., are delineated by dashed lines (Figure from 19).
Figure 3: Trisomy Detection as Performed by Dennis Lo, et al.: Proportion of informative wells positive for Chr21 vs. the number of informative wells

![Graph showing proportion vs. number of informative wells]

Description: Artificial DNA mixtures of 25% placental/maternaluffy coat. The numbers at the top of the bands indicate the number of 384-well plates required before the data set was classifiable for the cases delineated by the dotted lines surrounding each number (Figure from 19).

Although the approach is highly automatable and multiplexable, it has several hindrances that make it clinically incompatible. Primarily, between 4 and 7 384-well plates have to be analyzed through digital PCR, followed by 2-DE after analysis—meaning that between 1536 and 2688 samples must be analyzed in order to obtain a sample diagnostic. Digital PCR, expensive and time consuming on its own, would be impractical to carry out in every testing facility across the country, for several hundreds or thousands of samples to be tested daily.

The Logic of LATE-PCR

In order to overcome the challenges observed in all of the described techniques above for the detection of genomic duplications or deletions, we turned to a new method of asymmetric amplification known as Linear-After-The Exponential (LATE)-PCR.

Asymmetric amplification in LATE-PCR occurs by using uneven concentrations of amplification primers. While both primers are present in the reaction, amplification occurs in an exponential manner; however, after several amplification cycles the limiting primer depletes, and the reaction switches to a linear amplification phase where only one strand is made. This is in contrast to conventional (symmetric) PCR where both
strands are amplified equally and thus present at identical concentrations to the end of the reaction. Since the strands produced by symmetric PCR are complementary, they eventually anneal to each other interfering with probe binding. The accumulating double stranded DNA also inhibits Taq DNA polymerase. The single stranded DNA produced by asymmetric PCR bind the detection probe without competition from the complementary strand binding, which results in improved detection sensitivity down to detection of the single genome as starting material.

LATE-PCR differs from conventional asymmetric PCR in several aspects. Conventional asymmetric PCR uses a reduced concentration for one of the primers used for symmetric PCR in order to preferentially amplify one of the DNA target strands. LATE-PCR improves on conventional asymmetric PCR by adjusting the length or GC content of the limiting primer Tm to insure complete binding at the annealing temperature despite its lower concentration. LATE-PCR employs allele-specific mismatch tolerant probes that bind to their complementary allele target with a high affinity and to non-complementary targets with a significantly reduced affinity at high temperature. At sufficiently low temperatures the probes binds to both matched and mismatched targets with similar affinities. Thus, mismatch-tolerant probes provide a measure of both their allele-specific targets as well as a measure of the total amount of targets in the reaction. Affinity correlates to stability, which can be manipulated by temperature and measure fluorescence to ascertain complimentarily. In general, measurement of affinity correlates to the temperature at which oligonucleotides (primer and probes) anneal. At low temperature, probe binding would occur even in the presence of a mismatch or a deletion in the probe target sequences, but at a higher temperature, the probe binds only to the perfectly matched target.

As a result, probe binding at low temperatures detects the totality of amplification products while binding at high temperatures measures the specific allele being evaluated. The percent of the interrogated SNP allele in the sample would be derived from the ratio of the probe fluorescent signals at high temperature divided by that the probe fluorescent signals obtained the low temperature.

**Thermodynamic Theory of Mis-Match Tolerant Probes:**

The Gibbs Free energy equation, \( \Delta G = H - TS \), where \( G \) = Gibbs Free Energy, equals to \( H \), enthalpy (heat, measured in joules) minus \( T \), Temperature (measured in Kelvin) multiplied by \( S \), entropy (distortedness, measured as joules per Kelvin). Probe
hybridization occurs when $\Delta G$ is negative, as when the reaction temperature (heat) is low [manipulation of $H$], the probe to target $T_m$ is high [manipulation of $T$], or when entropy is large [manipulation of $S$].

**Figure 4: Visual OMP Simulation of Mis-Match Tolerant Probe Binding to Two Alleles**

Description: Concentration of bound probe to target fluctuates as a function of temperature, as it alters thermodynamic stability. In this case, a single nucleotide polymorphism (SNP) creates the differentiation between targets.

As illustrated by Figure 4 (above), at high temperature, the probe only binds to the matched allele, but not to the mis-matched allele. The mis-matched allele does not bind at high temperature. At low temperature, the probe binds to all targets, matched- or mis-matched.

**LATE-PCR Genotyping:**

The LATE-PCR method for measuring allele percentages has already been applied to SNP genotyping. In the original publication, Sanchez *et al.* used two-temperature (2T LATE-PCR) end-point analyses to genotype alleles near human p53 tumor suppressor gene, as well as the human HEXA gene responsible for Tay-Sachs, with >99.7% accuracy (21). The method proved capable at distinguishing between heterozygous genotypes of a SNP site from the two possible homozygous genotypes samples, as seen on the right in figure 3. These genotypes differed in the number of copies of individual alleles. For a SNP site (from Figure 5, below) consisting of C and G alleles, homozygous CC has 2 copies of the C allele, while heterozygous CG has one copy of each allele, whereas homozygous GG has no copies of the C allele. Since the probe
only detects the matched allele, in this example the C allele, the assay distinguishes between 0, vs. 1 vs. 2 copies. Application of this strategy to DS requires that we distinguish smaller differences in the number of copies (2 vs. 3 copies). Since a theoretical trisomy would fall between the homozygous and the heterozygous, where little space remains, probe design must change in order to make it work. As a result, DS genotyping presents additional challenges in resolution.

**Figure 5: 2T LATE-PCR End-Point Analysis**

Description: Control replicate of DNA samples corresponding to the various genotypes were used in the experiment (21). The probe, binding to the C allele, binds the same amount at high temperature as low temperature and so is 100%, the GC sample it binds to 50% of it, and to the GG sample it does not bind, so 0%.

In order to increase the sensitivity of 2T LATE-PCR, which can reliably distinguish between three vastly distinct genotypes, Three-Temperature (3T) LATE-PCR was developed with several optimizations. 3T LATE-PCR, by normalization at an additional temperature, increases the sensitivity, versatility, and reliability of detection among variant alleles to 99.7% accuracy (22). Most impressively, the sensitivity of 3T LATE-PCR rivals that of Pyrosequencing, but with a couple advantages. 3T LATE-PCR can be carried out in a single tube, on the single cell level, requires only a thermaycler and fluorescence meter, and does not require high expertise. As such, the approach could be used to increase the spacing between heterozygous (50% bound) sample and the homozygous boxes (0% and 100%) in order to allow the detection of duplications or deletions that may create ratios other than those three.
MATERIALS AND METHODS:

Primer and Probe Tm:

The probes and primers for the DS detection assay were designed using Visual OMP software (DNA Software Inc., Ann Arbor, MI). Design specifications for the primers and probe follow the 3T LATE-PCR protocol (22) and are described in further detail below. The design criteria for the detection of DS are more stringent than for the detection of Loss of Heterozygosity (LOH), as more allele combinations are possible. When detecting DS, five possible genotypes are possible, whereas only three are possible in LOH. As such, the design of the probes for DS detection followed protocol and through optimization, differential binding of the probe to the matched and mismatched targets was achieved.

The primers were designed according to LATE-PCR specifications and the probes were designed according to 3T LATE-PCR (22). The specific protocol for LATE-PCR primer and mismatch-tolerant probe design and concentrations using Visual OMP is available upon request. LATE-PCR primers and probes were purchased from Operon Technologies (Huntsville, AL) and the dual labeled linear probes were purchased from Biosearch Technologies (Novato, CA). The oligonucleotides sequences used in the assay can be found in Materials and Methods, table 1.

Probe and Primer Design

The mismatch tolerant probes used in this technique are constructed using a FAM fluorochrome and a Black Hole Quencher (BHQ-1) at their ends. When the probe is not bound it acquires a random coil configuration. Under this condition, the quencher tends to reside in close proximity to the fluor and absorbs the signal emitted. When the probe binds to the target, the molecule is straighten and the quencher and the fluor are apart and fluorescence can be measured. The two terminal ends of the probe have complementary nucleotides to keep the fluorochrome quenched in the unbound probe. 

Probe design uses VisualOMP, a primer/probe design program, which allows simulation of probe binding against various targets. Probe design criteria requires that the matched probe bind over 95% of the matched allele while binding less than 5% of the mismatched target at the same temperature.

Primer creation was performed in VisualOMP using suggested primers for the target sequence matching the 3T LATE-PCR criteria for design (22). The found primers were then converted in their concentrations to further fill the criteria and then optimized. Several of the primers have been manually designed based on experience placing
primers around the probe. Primer design protocol, describes both the automated and
manual design, is available upon request.

DNA samples

Purified genomic DNA's were obtained from the Coriell Cell Repository (Camden, NJ). The corresponding DNA's, with SNP genotyping are available upon request.

Amplification

Amplification was performed in an ABI Prism 7700 Sequence Detection System
(Applied Biosystems, Foster City, CA). The thermal cycling profile for assay was 95°C
for 3 min, followed by 50 cycles of 95°C for 10 seconds, 64°C for 10 seconds, and 72°C
for 20 seconds. Following LATE-PCR amplification, the tubes were cooled down to 30°C,
incubated for 20 minutes to in order to allow for maximal probe binding and fluorescent
readings from the hybridization probe were collected every degree Celsius for 90
seconds for melt curve analysis.

Normalization for Data Analysis

Data analysis for the assay occurs in several steps which are generally sample
collection, normalization, and scoring. The ABI Sequence Detector collects fluorescence
signals at every degree during the final melt and saves the data. The data is then
exported from the ABI Sequence Detector Software into Excel, where Normalized
Fluorescence Ratios (NFR) is calculated for analysis. In the 3T LATE-PCR method,
normalized fluorescence is calculated by the following algorithm: NFR= (Fm-Fr)/(Fb-Ft),
where Fb and Ft are the fluorescence readings towards the Bottom and the Top of the
temperature window of allele discrimination, respectively, and Fm is the fluorescent
reading at any given temperature during Melt curve analysis. The temperature chosen
for the melt-curve analysis varies between the various amplicons, and is based on the
point of maximal differentiation of the probe among the possible alleles. The point of
maximal differentiation, Fm, occurs when the matched allele binds >95% of the target,
while the mismatched allele binds <5%, ideally 2%. This creates the maximal
differentiation, because the ratio of the [Specific Allele] / [All Targets] is compared to that
of [Non-Target Allele] / [All Targets]. The rational of probe design has been extensively
described in the Three Temperature LATE-PCR paper (22).
In order to construct differentiable confidence intervals to define the control genotypes, a training set of 12-24 replicates was used. The number of replicate wells per each control sample varied depending on the standard deviation from the mean for each control genotype. For example, standard deviation from the mean varied between samples. The homozygous samples, as they were homogenous, had the least variability among replicate wells and could in most cases create a 2.57σ interval with as few as 8 well replicates. The heterozygous samples, consisting of two different types of alleles, have an equal Passion distribution of the two alleles. Just like flipping a coin with two faces, if 5 trials are performed, the average likelihood of having each face is 2.5 times; if the result is 4 heads and 1 tail, then an insufficient sample size problem exists. In allele detection, as in flipping a coin, large sample numbers are required to obtain an equal statistical probability for the independent outcomes. Since the heterozygous sample, using <1000 cells had a higher degree of variability between wells, they had a larger standard deviation from the mean. As such, to define the heterozygous control, some cases require 14-24 well replicates. Boxes were created by determining the average and standard deviation fluorescence ratios for each control genotype, then creating a confidence interval of 2.57 standard deviations above and below, using the equation:

\[
\text{Confidence Interval} = (\mu_{\text{genotype}} - 2.57\sigma_{\text{genotype}}; \mu_{\text{genotype}} + 2.57\sigma_{\text{genotype}})
\]

The results of the assay would be scored utilizing three control DNA's per each SNP of known genotypes for the two homozygous and single heterozygous combinations to create confidence intervals for detection of normal individuals. Once the three confidence intervals of the controls are set, then any samples falling between the confidence intervals of the homozygosis and the controls would be a possible trisomy ratio. Since 10 SNPs are interrogated, the confirmation of two informative SNP's is necessary. If in two different SNP's an individual has either 33.3% or 66.7% heterozygosity, they would be identified as trisomy; instead if at least two SNP were found to have 50% heterozygosity, then the individual would be normal. If an individual has a mixture of normal and abnormal allele ratios, then the individual would either be a partial trisomy (identified correctly), a mosaic trisomy (identified correctly), or an error may have occurred in analysis; in this situation, verification would be recommended by an alternative method. If an individual fails to have at least two informative SNP's, as occurs in 1.48% of cases, then the individual was not screened by the assay and no results can be made. In order to eliminate this problem, more SNPs could be developed and added to the assay. At this point, it would simply be a turn of the crank.
Table 1: Complete list of oligonucleotides used in LATE-PCR DS Assay

<table>
<thead>
<tr>
<th>SNP</th>
<th>Oligos</th>
<th>Sequence 5' to 3&quot;</th>
</tr>
</thead>
</table>

Note: LP = Limiting primer sequence, EP = Excess primer sequence
RESULTS:

Logic of DS Detection Using LATE-PCR:

Figure 6: A Single Mismatch-Tolerant Probe Distinguishes Genotypes

Description: The probes at high temperature only binds to the match target, denoted by the red dot, but not to the mismatched target, denoted by the white dot. At low temperature, the probe binds to the totality of targets. The ratio created between high and low temperature is what creates the fluorescence ratios.

Identification of Testing Markers

Many benefits arise from the use SNPs as markers. An assay for DS detection would incorporate the use of heterozygous SNPs as genetic markers to distinguish allele copy number. Recently, the human genome project has successfully sequenced and mapped the human genome, allowing for the identification of common single nucleotide polymorphisms with a high heterozygosity index. As of April, 2009, there are nearly 20.6 million SNPs sites identified in the human genome, of which 292,495 are located on human chromosome 21. These polymorphisms are inherited genomic elements that in some instances experience selective pressure. The frequency of certain allele combinations correlates with ancestral origins and can be identified as a percentage of each genotype within the population, identifying the percent of each homozygous subgroup as well as the percent heterozygosity, known as the heterozygosity index (HI).
According to the Hardy-Weinberg principal, genotype frequencies within a population will remain in a constant equilibrium from generation to generation, unless subject to selective pressures. These selective pressures include mutations, natural selection, limited population, and non-random mating. This describes why populations vary in their heterozygosity index among SNPs – humans have evolution in discrete regions creating diversity; however, some SNPs exist with a high global heterozygosity index. As such, in selecting SNPs for the assay HI had to be ascertained for a particular population. Since all of the cell-lines obtained from Coriell Cell Repository were of European origin, all SNP's were selected based on a high European HI. In addition, a high Global HI was sought out in order to make the assay most universal, by obtaining representative data from averaging HI from the ethnicity categories. The assay would be most universal if it used SNPs informative for the general world population; a significant HI in the European population is required since the control samples from the repository are ethnicity specific. In order to produce a low false-positive identification rate, the simultaneous identification of two SNPs will be used to reliably identify and confirm diagnosis of aneuploidy.

**Utilization of the Down's Syndrome Critical Region (DSCR)**

Since the DSCR is the minimal region that leads to the DS phenotype when duplicated, interrogation focuses on this region to identify heterozygous samples with an allele imbalance. The 10 designed SNP's reside on human chromosome 21 between 34Mb and 47Mb, as depicted in figure 7. The DS phenotype necessitates a triplication of at least a portion of the region, therefore to catch as many cases as possible, this is where the markers were made.

**Figure 7: Locations of 10 SNP's relative to the DSCR**
Description: The red box around human chromosome 21 shows the region observed in greater detail in the lower white region. The red lines delineate the hashed location of the SNPs relative to human chromosome 21 in nucleotides, demonstrating complete coverage of the Critical Region.

Table 2: The 10 SNP's with Associated Hl and Relative Location on DSCR

<table>
<thead>
<tr>
<th>#</th>
<th>SNP</th>
<th>Hl European</th>
<th>Hl Global</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>Genes</td>
</tr>
</tbody>
</table>

Average: 0.493 0.495 0.497

Description: The 10 assay SNPs are listed here with relative Hl for the European and Global populations, as well as their respective genes. As observed, the variability of Hl in the Global population for some of the SNPs is greater than others, signifying selective pressures among genes in humans who have evolved in different parts of the world. Those SNPs with higher variability would likely not be used in an assay targeted for a global population. * refers to "HapMap-CEU" population.

In short, genetic marker selection criteria selects SNPs located on human chromosome 21 in the DS critical regions with a high, well established heterozygosity index in both European and Global populations.

How A Single Probe Can Discriminate Matched from Mismatched Targets:
In VisualOMP, the simulated binding of the probe to the matched and mis-matched sequences should consist of two parallel curves that come together around 40°C and are sufficiently spaced, to allow differential binding between the matched and mismatched targets. As the reaction temperature decrease from a high temperature, the probe starts to selectively bind the matched sequence, as it has a higher thermodynamic stability than the probe to the mismatch. Ideally, the rightmost curve, the binding of the probe to the matched sequence, reaches over 95% binding (max concentration) before a significant amount the mismatch target binds to the probe, which is the leftmost curve. Measuring the amount of fluorescence, as seen in figure 8 at 50°C would indicate that 95.0% of the match is bound while 2.3% of the mismatch is bound. At this point the fluorescence is measured as 1, quantifying the total amount of matched target. Then the temperature is lowered to 30°C, and fluorescence is measured again. Creating a ratio between the high and low temperature, we create a ratio between the bindings of the matched allele to the mismatched allele. In theory, if two alleles of A existed and one of G, so it was an AAG trisomic individual, we would get a ratio of 2:3, or a 2/3 ratio; such a result would be positive for trisomy.

**Figure 8: VisualOMP Melt Profile for Mismatch Tolerant Probes**

Description: LATE-PCR Probes used for DS detection. The rightmost curve illustrates probe binding to the matched allele target. The leftmost curve illustrates probe binding to the mismatched allele target. Concentration of fluorescence divided by total fluorescence, so 1.5e^-0.07 actually equals 100% bound, whereas 0.00e^-0.00 equals 0% bound.

**Statistics for how many SNPs required**

Statistically, (Table 3) the average HI for the selected SNPs is between 40-50% and the lower index of 48% is used for safety. The probability of obtaining zero or only a
single informative SNP with a HI of 40% out of 10 sites tested is only 1.48%; there is a 98.52% of obtaining at least 2 or more informative markers out of the 10 (23).

Table 3: showing probability of achieving each combination of positives for HI=48%

<table>
<thead>
<tr>
<th>Probability of Finding a discrete k number of Heterozygous SNPs out of n sites examined (10)</th>
<th>n = # of SNP sites examined</th>
<th>k = number of heterozygous among 10 SNP sites at HI=48%</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.00%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P of 0 or 1 Heterozygous out of 10 =</td>
<td></td>
<td>0.14%</td>
</tr>
<tr>
<td>P of 2 or more Heterozygous out of 10 =</td>
<td></td>
<td>98.52%</td>
</tr>
</tbody>
</table>

Description: Indicates the probability of the exact, discrete number of found heterozygous samples found out of 10 SNPs using a low measure of heterozygosity of 48.0%. For example, there is a 0.14% chance to have exactly 0 heterozygous samples in a 10 SNP assay; whereas there is a 24.41% chance of finding exactly 5 heterozygous SNPs in a 10 SNP assay. To determine the probability of obtaining 2 or more heterozygous SNPs in a 10 SNP assay, the individual discrete probabilities are added up and found to be 98.52%.

**Progress in Probe Design**

Mismatch-Tolerant probes were designed in VisualOMP, a primer/probe design program, which allows simulation of probe binding against various targets. As described in the Materials, probe design criteria requires that the matched probe bind over 95% of the matched allele while binding less than 5% of the mismatched target at the same temperature. This creates the maximal window for analysis. In preparation of the 3T LATE-PCR paper (22) two probes were used as examples with known simulated allelic ratios of 53% G:47%C, 55.6%G:45.5%C, and 60%G:40%C, displayed in figure 9. The ability of a probe to differentiate alleles depends on the read temperature, which should occur at the location of maximal distinction between the two alleles. If the distinction between the alleles is minimal, as in Figure 9A, then allele differentiation will be minimal
as well, as seen below in 9C. In Figure 9B, where the probe can distinguish the two alleles at 95.5% and 2.2%, the separation is much greater, as observed below in 9C.

**Figure 9:** Improvements in linear probe design increases assay resolution

Description: (A and B) Probes illustrate different discrimination of the matched and mismatched alleles. (C) The assay resolution increases as the Tm of the matched and mismatched alleles increase, i.e. the more they are discriminated by the probe, the larger the difference. Figure obtained from *Detection of SNP Allelic Imbalances Using an Optimized Three-Temperature Method for LATE-PCR Endpoint Analysis* (22).

**Measurement of fluorescent ratios, homozygous vs. heterozygous ratios:**

The mismatch tolerant probes used in LATE-PCR detect copy number variations using relative quantitation of the percentage of an allele among amplification products. At low temperature fluorescentconco is measured to determine the absolute amount of all alleles present and at high temperature where only the allele complementary to the
probe is bound. A euploid sample at a heterozygous SNP would have at a high temperature 50% of the normalized fluorescence as it did at a low temperature, indicating that the allele bound at a high temperature was present at a 1:1 ratio as the other allele. If a chromosomal duplication existed, a 33.3% or 66.7% ratio would result, from having the matched allele bound 1:3 or 2:3. In this technique, amplification of an informative heterozygous locus yields ssDNA to which the allele-specific, mismatch tolerant probes bind; the ratios of probe signals collected at three temperatures reveals the percentage of the heterozygous marker needed to assess duplications and deletions.

**Normalization**

Subtracting the background fluorescence, normalizing to high and low temperatures, and determining the standard deviation for the average of controls normalizes the raw detected fluorescence and results in improved reliability of fluorescent ratios among replicate samples. Normalization of the fluorescent signal is paramount to sample identification as several variables in the reaction contribute to scatter of fluorescent ratios indicative of allele percentages. For example, the fluorescence intensity varies as a function of temperature, the amount of starting DNA varies among replicates, and the amplification yield varies within a group of otherwise replicate samples.
**Steps of Normalization:**

**Figure 10A:** 1st Derivative Graph of Probe Binding to SNP in PCR to Determine Read Temperature.

Description: Here the background is subtracted and the first deviation of the data is graphed in order to determine the normalization temperatures at low and high ends, as well as the read temperature. The read temperature is where the maximal separation occurs between the two alleles, which graphically occur in the intersection of the matched and mismatched alleles.

**Figure 10B:** Normalization to High Temperature
Figure 10C: Following normalization at high temperature, the background fluorescence from the NTC's is subtracted.

Figure 10D: Normalization to Low Temperature and Demonstration of Size of Separation using the Black line of equal length in 4 locations.
Figure 10E: Final analysis of 24 well replicates of three control genotypes
Following normalization as observed in Figures 9A-9E, clearly reproducible genotypes are distinguished to 3 standard deviations, or 99.7% certainty, that no overlap occurs.

**Assay Results**

After determination that the LATE-PCR method for detection of deletions and duplications could distinguish control, diploid DNA to 3 standard deviations reproducibly, then the next step integrated 11 genomic samples from individuals with DS, in order to see if the sensitivity of the assay could readily distinguish them from controls. In addition to testing the resolution of the probe, the 11 DS samples had to be genotyped so that large scale analysis could be performed with only informative markers. Below are the results of a primary run.
Figure 11: Endpoint Melt Curve Analysis Showing Genotyping of 11 Trisomies into 5 genotypes.

Description: 11 cell-lines from DS individuals (1-11) were tested with probe rs11910515 along with 3 control genotypes (two homozygous and one heterozygous) which served to designate the first homozygous, the heterozygous, and second homozygous controls. The 11 trisomy samples were genotyped in replicates of 2. As can be seen, DS samples 2, 5, 8, 1, 3, 9, 10, and 11 are informative as they are heterozygous, forming an unusual ratio of 33.3% or 66.7%, thus falling between the homozygous samples and the control diploid. Note: the boxes depicted indicate no statistical significance and are for visual organization only.

The LATE-PCR assay is designed as an end-point assay, meaning that measurement of fluorescence at 3 temperatures are read, but not displayed or analyzed until the end. This approach contrasts with Real-Time PCR which collects fluorescence data at every single temperature. While Real-Time PCR is more informative, it is less clinically applicable as it requires a Real-Time PCR machine, which is far more costly than traditional PCR. Endpoint PCR could be performed using a single thermalcycler and a fluorescence meter, both of which are clinically compatible and are significantly more affordable. Genotyping was performed on a Real-Time PCR device and for
endpoint analysis only three temperature readings were used. Figure 12 (below) is a Real-Time run for the genotyping of 6 DS samples (Samples 1-6) at SNP rs2968 in real time, which can be contrasted to endpoint analysis in Figure 11 (above).

**Figure 12: Real-Time PCR analysis of 6 DS Samples, identifying informative DS samples for the SNP.**

Description: SNP rs2968 using non-coded trisomy-21 samples with a single control homozygous and a heterozygous. NOTE: The assay lacks the positive homozygous control.

The assay has been able to demonstrate 2.57 standard deviation discrimination among replicate samples, establishing at least 99.0% sensitivity, as observed below.
Figure 13: SNP site rs2836931 distinguishes 12 well replicates at 50°C to 2.57 standard deviations.

Internal Controls were used to normalize fluorescence ratios, which also proved effective at creating confidence intervals for the various control genotypes. Thus, trisomies could be identified as not falling within the heterozygous box at an informative SNP site, as illustrated (below) in Figure 14.
**Figure 14:** SNP rs1125040 differentiates between Internal Control and PCR Fluorescence at 54C to 3 Standard deviations, illustrated by colored boxes.

Description: A single SNP site, rs1125040 was used to genotype 3 control diploid samples, as well as two trisomies. 3-standard deviations above and below the mean were calculated and a colored box was fitted for each of the 5 resulting genotypes. Here analysis is displayed in a bar graph, relative to 0, the difference between the terminal PCR fluorescence and that of the internal control.

**Ability to Identify DS:**

To date, DS can be identified in individuals to at least 99.0% certainty, using markers that have complete coverage of the DSCR. Since 10 SNP's are used, the failure rate of not finding two informative SNP's would be 1.5%. To decrease the failure rate, additional SNP can easily be added to the assay, by following the procedure outlined.

**Table 4:** Probe Database Indicating Informative DS Samples and DSCR location

<table>
<thead>
<tr>
<th>Informative</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td># SNP: Nucleotide</td>
<td>Samples</td>
</tr>
</tbody>
</table>

---
Application of LATE-PCR to Detection of Genetic Trisomies

** Description:** **The numbers denote the coded DS cell-lines used for assay design purposes. The Coriell Cell Repository catalog numbers corresponding to the cell lines can be found in table 5.**

<table>
<thead>
<tr>
<th>Trisomy Coding</th>
<th>ID#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5: DNA ID# from Coriell Cell Repository**

**DISCUSSION:**

Linear-after-the-Exponential Polymerase Chain Reaction (LATE-PCR) can be used to replace existing detection methods and thereby genomic duplications has the ability to successfully address the limitations of existing detection methods by reliably detecting genomic duplications and deletions in a rapid, low-cost manner. The constructed assay reduces detection times from 2-3 weeks to same day results. Current screening approach efforts using the Combined Test only capture 85% of all DS cases of all singleton pregnancies, whereas if the assay were used it could detect at up to 96.5% of all cases (excluding mosaic trisomies) with a high sensitivity (99.0%) of discrimination between alleles. The actual certainty of the assay would increase from 99.0% if more than 2 informative SNP's are found per individual. If 4 informative SNP's are found, then the certainty of the assay would be higher than if only 2 informative were found, while yielding the same definitive result.

The proof of principal experimentation with DS as a model system illustrates that detection of duplications is achievable using the described LATE-PCR method. There are many disorders arising from allele dosage imbalance caused by chromosomal DNA deletions and/or duplications. As such, the approach can be used to detect any disease or disorder arising duplications or deletions of DNA. Provided funding and time, SNP
assays could be created for the detection of any disease arising from DNA deletions or duplications. The table below shows examples of genetic disorders caused by DNA deletions or duplications.

Table 6: Clinically relevant disorders arising from allele dosage imbalances

<table>
<thead>
<tr>
<th>Gene Target</th>
<th>Function</th>
<th>Genetic Alteration</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In practice, the assay can be expanded to cover many genetic mutations caused by genomic deletions or duplications and be applied to many labs across the country. Readily accessible testing facilities would increase the availability of testing and would offer parents cheaper detection options than currently available. If applied in practice, expecting parents can expect several positive outcomes, such as higher certainty in screening, potentially a lower FPR, and a significant decrease in Odds of Being Affected given a Positive Result (OAPR). This would significantly decrease the anxiety associated with being one of the 22 out of 23 parents who are worried about a possible positive screen, if this became the standard for screening. In detection, application of LATE-PCR would dramatically reduce the waiting period from 2-3 weeks to a single day result. This would eliminate the period in which parents worry about the possibility of receiving the worst news – an element of fear would be gone.
The LATE-PCR method would be difficult to implement for screening purposes as access to genetic fetal samples still requires invasive means. However, it would be the perfect solution for pre-implantation applications where a dividing morula at the 8+ cell stage \textit{in vitro} could be sampled through digital PCR. This would be the first clinical location to test the LATE-PCR method for detection of deletions or duplications.

\textbf{Advantage of LATE-PCR}

LATE-PCR benefits from being a single tube, end point assay that could be easily automatable due to the simplicity of the approach. In principle, the technique should be clinically compatible as it can supply high-throughput, rapid, reliable results. Additionally, on large scale, the reagents for the LATE-PCR assay are cheap and can be lyophilized for easy, quick use. Utilizing Smith Detection's portable PCR device, this would even permit detection in remote regions of the world where access to high-technology testing facilities may not be readily accessible.

LATE-PCR is also unique because the algorithm used to calculate fluorescent ratios of probe bound at 3 temperatures includes normalization of background probe fluorescence and difference in amplification yield among replicate samples. This normalization step provides additional tools to improve reliability of fluorescent signal ratios among replicate samples.

\textbf{Disadvantage of LATE-PCR – Including Solutions}

Disadvantages of the technique include the reliance on heterozygous SNPs and the need for external reference control samples of known genotype for identification of fluorescent ratios corresponding to DS. The largest hindrance for the approach to prenatal detection rests in access to fetal genomic sample, which would ideally be amniocentesis.

Several solutions exist to overcome the lack of access to fetal genomic samples. The first solution rests in the method of obtaining the amniocentesis. Current amniocentesis involves withdrawing a significant volume of amniotic fluid. As shown in this thesis, a definitive diagnosis of trisomy can be carried out using LATE-PCR by analyzing as a little as \textasciitilde100 single cells. The need for such small cell samples could mean that the volume of amniotic fluid drawn could be greatly reduced from the current amount of about 40mL. Additionally, the size of the needle used for amniotic sampling could also be reduced, thus further decreasing miscarriage risk. A priori it seems likely that such a
change in protocol would reduce the risks associated with amniocentesis. The second solution would utilize detection based on circulating targets in maternal plasma. This could be performed on mRNA’s or on methylated fetal DNA fragments, as Dr. Lo and Dr. Chim have attempted, respectively (8, 9). The third solution to overcome difficulty of sampling would apply the LATE-PCR method to pre-implantation applications. This would eliminate the risk of miscarriage, as the process is performed in vitro, and not on the pregnant mother.

**Future Prospective**

The future of LATE-PCR detection should focus on exploration of ways to obtain access to fetal DNA non-invasively. As described in the solutions above, exploration should be made into detection of circulating fetal DNA’s and RNA’s in maternal plasma. In addition, exploration should be made into differentially methylated CpG islands specific to DS fetuses; this could be thought out via use of microarrays.

At this point, the proof of principal illustrates that detection of small variations in allele presence, i.e. 2 vs. 3 is possible using this technique and methods. As such, creating a similar assay for any disorder arising from genomic deletions or duplications would be a function of a turn-of-the-crank. The method exists, now it can be simply applied to other disorders and applications.

**Acknowledgements**

I thank the members of the Wangh laboratory for four years of support, encouragement, and mentoring towards this thesis. Thank you most particularly to Dr. Aquiles Sanchez for his devoted to guidance and his constructive criticism during all phases of this project.

This work was supported by a grant from Smiths Detection (Edgewood, MD) to L.J.W.
REFERENCES:


7. Wolfson Institute of Preventive Medicine, Barts and The London School of Medicine and Dentistry: Antenatal Screening for Down’s Syndrome and Open Neural Tube Defects. *Information for Health Professionals* March, 2008.


