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Establishing a Method to Detect Fragile X CGG Repeats within the FMR1 Gene of Embryo Trophectoderm Biopsies

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ABSTRACT

Fragile X Syndrome (FXS) is an X-linked disorder characterized by a CGG trinucleotide repeat located within the 5' untranslated region of the Fragile X Mental Retardation 1 gene (FMR1). The FMR1 gene is further categorized into classifications of protein function such as normal, intermediate, premutation or full mutation depending on the number of CGG repeats present[23]. A normal FMR1 gene exhibits anywhere between 5 and 44 repeats. An allele in intermediate range displays 45-54 repeats. A premutation allele occurs when 55-200 CGG repeats are present and levels of the FMR1 gene product, Fragile X Mental Retardation Protein (FMRP) start to deplete. When alleles fall into this range, they are subject to expansion when transmitted into subsequent generations. Additionally, female premutation carriers are at risk for inheriting Fragile X-associated Primary Ovarian Insufficiency (FXPOI), which causes the ovaries to not work correctly. A full mutation displays complete methylation of the FMR1 gene, no FMRP production, and greater than 200 repeats. An individual diagnosed with a full mutation for FXS exhibits developmental and behavioral issues that include, but are not limited to, intellectual impairment, failure to meet milestones, and lack of impulse control[20].

Due to the severity of a full mutation of FXS, and risk of FXPOI and expansion, female patients seek reproductive assistance from infertility specialists. A current method of testing for FXS involves the use of quantitative Polymerase Chain Reaction (qPCR)-based single nucleotide polymorphism (SNP) genotyping for linkage analysis. The inheritance pattern of the affected allele is tracked throughout this process; however, the number of repeats present is not observed. This project aims to establish a method for detecting Fragile X CGG repeats within embryo trophectoderm (TE) biopsies. The

determination of CGG repeats is a crucial addition to current methods of testing to avoid the risk of expansion and to assist patients in obtaining a healthy pregnancy[12].

This study was broken down into the completion of four phases. It began with the validation of Asuragen's Amplidex PCR/CE FMR1 reagent kit to detect CGG repeats within the FMR1 gene of genomic DNA (gDNA) samples. The use of gDNA with this kit served as a gold standard for comparisons throughout this project. The next phase included, cell lines with known CGG repeat sizes to mimic TE biopsies. They were then tested on four different amplification methods. Those methods included GenomePlex WGA4, SurePlex DNA, REPLI-g Single Cell Kit, and targeted pre-amplification. Next, arrested whole embryos were used to test the ability of the chosen amplification method on embryonic samples. Lastly, TE biopsies from discarded aneuploid whole embryos to observe the developed methodology's accuracy on clinical samples.

The gDNA results were as expected and provided a source for the validation of this methodology. Although three out of the four amplification methods being tested did not provide usable results, the REPLI-g Single Cell Kit showed very promising results. It showed 100% consistency with the expected results from the cell lines used. This amplification method was then used on arrested whole embryos and showed CGG repeat sizes as expected from parental genotypes. The TE biopsies showed encouraging results, suggesting that this procedure has potential for clinically implementation.

CGG repeat sizes and expansion can be observed using this new methodology. It has the potential application to assess the amount of expansion for patients with limited numbers of usable embryos. Transferring embryos with FMR1 premutation alleles will encompass more diligent genetic counseling and detailed consents.

MONTCLAIR STATE UNIVERSITY

ESTABLISHING A METHOD TO DETECT FRAGILE X CGG REPEATS WITHIN

THE FMR1 GENE OF EMBRYO TROPHECTODERM BIOPSIES

by

Jenna Bedard

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

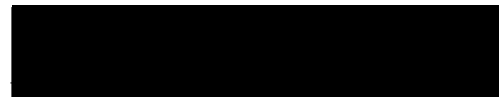Masters of Science

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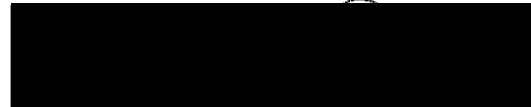
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INTRODUCTION

Infertility affects approximately 15% of the reproductive aged population. It is defined as the inability to conceive a clinical pregnancy after one year of unprotected intercourse and can affect both males and females[1]. Causes of infertility in males can be due to hormonal imbalances, genetic disorders, or disruption of the testicular and ejaculatory functions. Additionally, females experience issues with fertility due to disruption of ovarian function from genetic disorders, obstruction of the fallopian tubes, and abnormal uterine contour[2]. Assisted reproductive technologies (ART) are used to treat infertility and contribute to increased delivery rates. Over the past few decades, advances of ART continuingly increased, with the most effective treatment option being in-vitro fertilization (IVF). IVF is a process in which ova are retrieved from the ovaries of the female patient; while simultaneously, a sperm sample from the male partner is collected. The ovum is then fertilized in-vitro and transferred into the uterus of the female patient[3]. Although seemingly effective, there are still limitations that are involved with the use of IVF. The failure of embryos to implant is a main concern for reproductive specialists.

There are many possible explanations why embryos fail to implant; such as, uterine environment, embryo quality, the age of the egg retrieved, or chromosomal abnormalities[4]. All embryos are expected to inherit forty-six chromosomes; twenty-three from each the contributing mother and father. When an embryo exhibits the correct number of chromosomes it is referred to as euploid; while an embryo that displays an incorrect number of chromosomes is considered aneuploid. Aneuploidy is likely the most prevalent cause for the formation of genetic abnormalities and the leading genetic cause

of miscarriages[5,6]. There is an aberrant chromosomal segregation that occurs; which results in an abnormal number of chromosomes inherited by the embryo[7]. A previous study collected data to support the notion that aneuploidy, additionally, increases with maternal age[8]. This is problematic for reproductive specialists as they counsel women in pursuit of IVF. They observed a steady increase of aneuploidy observed within the embryos of female patients above the age of 30. Furthermore, the same cohort of women are at risk for having little to no euploid embryos available for transfer[8].

To overcome the limitations of IVF, researchers developed technologies used currently in clinical practice to assist in the evaluation of human embryos during the IVF process. Preimplantation genetic diagnosis (PGD) of aneuploidy significantly reduces the risk of delivering a chromosomally abnormal offspring[9]. PGD is a process in which a biopsy of 5-10 cells is taken from the trophectoderm (TE) of the blastocyst stage embryo and chromosome copy number is determined prior to transfer back into the female patient's uterus. It has been established that blastomere biopsies on day 3 embryos during the cleavage stage had a significant reduction in sustained implantation. However, most clinics conduct biopsies from the TE of a day 5 or 6 blastocyst, which showed to be comparative to controls with no statistical difference between the sustained implantation rates[9].

The recent developments of comprehensive chromosome screening (CCS), a form of PGD, have been validated and clinically implemented to assess chromosome copy number of all 24 chromosomes (chromosomes 1-22, X and Y)[10]. Quantitative Polymerase Chain Reaction (qPCR) based methods of CCS are the most efficient in respect to experimental time and analysis[11]. In 2004, Handyside *et al* isolated small quantities of

genomic DNA (gDNA) from cleavage stage embryos that underwent whole genome

amplification (WGA) to produce microgram quantities of amplified DNA. This DNA was

used for PGD using a qPCR based method for chromosome screening[11]. Although

effective, there were some limitations using WGA. Allele drop out (ADO) occurs and

amplification failure occurs frequently with the use of WGA[12]. ADO occurs when one or

both alleles of a heterozygote fail to amplify. Additionally, the primers anneal randomly

and cause only about 60% of the inputted DNA to be amplified. Due to these limitations,

Zimmerman *et al* focused on constructing a technology that did not need to assistance of

whole genome amplification[12]. Successfully, Zimmerman and her group developed and

validated a platform for simultaneous chromosome screening and single gene disorder

(SGD) testing[12]. qPCR can provide additional genotypic information that is useful for

detecting, and diagnosing many SGD's[13]. Currently, many groups shifted focus towards

the use of next generation sequencing as standard CCS method. The use of targeted next

generation sequencing (tNGS) has been recently validated by Zimmerman et al as a more

advanced and efficient method of CCS; however, it is not yet been established to work

for SGD testing[14].

In 2015, Werner *et al* conducted a study to clarify the accuracy of CCS using

PCR-based methodologies and how they affected implantation and delivery rates. The

researchers observed equivalent accuracy to commonly used CCS techniques, increased

throughput, and simultaneous detection of SGD's[12,15]. The list of different SGD's that are

commonly tested for is extensive; however, one specific disorder commonly tested for

among infertility clinics is Fragile X Syndrome (FXS).

FXS is the most inherited form of mental retardation and affects about 1 in every 4000 males and 1 in every 8000 females[16]. FXS can be characterized by a tri-nucleotide CGG repeat within the 5' untranslated region (UTR) of the Fragile X Mental Retardation 1 (FMR1) gene. The FMR1 gene exists in four different allelic forms. The first is a normal FMR1 allele, that exhibits less than 45 CGG repeats and paired with normal production of the FMR1 gene product; Fragile X Mental Retardation Protein (FMRP)[17]. The next allelic form is an intermediate, or gray zone, FMR1 allele. These alleles display between 45 and 55 CGG repeats with normal FMRP production. The next form is a premutation FMR1 allele, which exhibits between 55 and 200 CGG repeats[17]. When an allele reaches this range the levels of FMRP start to deplete. Individuals with alleles that fall into these first three ranges display between 0 and 3 AGG interruptions. These interruptions create stability for the gene by revealing an AGG approximately every 9 or 10 CGG repeats[17]. The fourth and final allele form is a full mutation FMR1 allele. A full mutation allele exhibits greater than 200 CGG repeats and complete methylation (the addition of a methyl group to the CpG islands) of the 5' UTR of the FMR1 gene. In turn, this causes transcriptional silencing and no FMRP production[16].

FXS demonstrates an x-linked inheritance pattern[16]. The first observation of a variant X chromosome, that was associated with intellectual disabilities, was used as a marker X chromosome to gain knowledge about X linkage inheritance[18]. These researchers determined that the expression of a chromosomal variant may vary from generation to generation. To go into further detail, Martin et al's group developed the first pedigree describing inheritance patterns of mental defects caused by sex linkage[19]. For example, when females are carriers of FXS, their offspring will have a 50% chance of

inheriting the affected X chromosome. Comparatively, male carriers will always pass on their affected X chromosome to their daughter[9].

When an individual expresses a full mutation FMR1 allele, they exhibit a variety of phenotypic indicators. There is the presence of a fragile site at Xq27.3[20,21] and displays of moderate to severe mental retardation[16,20]. Penetrant males of FXS almost always display severe intellectual disabilities; or mental retardation. They are also subject to abnormal facial features such as large ears, prominent jaw, and macroorchidism. Other complications, such as anxiety, hyperactivity, and autism-like behaviors, also accompany the previously discussed list of phenotypes. Females are likely to have more mild forms of mental retardation and variable accompanying physical, or psychiatric, features[16,2]. This is due to the mutation being x-linked and females undergoing the process of x-inactivation. It has been established that FMR1 expression does not occur from the inactive X chromosome; therefore, is subject to X chromosome inactivation[23].

The lack of FMRP consequently causes the expression of the previously described phenotypic indicators. FMRP is a ribonucleic acid (RNA) binding protein[24]. It is part of a ribonucleoprotein complex responsible for translating polyribosomes, which can travel between the cytoplasm and the nucleus of a cell. It is known and well established that FMRP is involved in nuclear export, cytoplasmic transference, and/or translational control of specific messenger RNA (mRNA) molecules. It is suggested by Bardoni *et al* to play a role in the regulation of protein synthesis at the dendrite postsynaptic sites of neurons within the brain[24]. In particular, FMRP is accountable for the maturing of dendrite spines, which receive sensory input and transmit electrical signals to the neurons cell body. These structures of the neuron play important roles in motivation, learning[25],

and memory[6]. FMRP is suggested to play a role in the maintenance of memory over a

lifespan because long-term memory is facilitated by the growth of dendritic spines, or

elongation of existing spines. Therefore, when there is an absence of FMRP, the dendrite

spines experience complications with their maturation process leading memory and

learning abilities to be affected[27]. FMRP is also found to play a role in the development

of the reproductive organs and can cause severe issues with fertility, especially in

women**Error! Bookmark not defined.**[7,24].

The slow depletion of FMRP when a FMR1 allele falls within the premutation

range is paired with an increase of FMR1 mRNA transcripts. It is still unclear how this

mechanism occurs; however, there are some theories[28]. There is a hypothesis suggested to

explain the paradox of increased FMR1 transcripts unexpectedly associated with

decreased levels of FMRP. This theory suggests that the increased number of CGG

repeats leads to the translational inhibition of the FMR1 transcripts by causing

conformational changes of the transcripts themselves. This conformational change in the

FMR1 transcript impacts the commencement of translation and hinders the 40S

ribosomal subunits evidently causing the reduced production of FMRP[28].

Fertility becomes problematic specifically for FMR1 permutation carries.

Premutation FMR1 alleles attributes to a spectrum of different phenotypic indicators

depending on the CGG repeat length[2]. Clinically significant phenotypes and risks are

suggested to have a positive correlation with the increase of the CGG repeat size; which

plateaus around 100 repeats for alleles within this range. Premutation carriers are at risk

for inheriting other Fragile X associated disorders, including fragile X tremor/ataxia

syndrome (FXTAS)[29]. FXTAS most commonly affects males; however, females can

inherit this disorder as well. FXTAS is a neurodegenerative disorder characterized by late-onset of progressive cerebellar ataxia and tremors. It is also associated with short-term memory loss, executive function deficits, cognitive decline, muscle weakness and autonomic dysfunction to name a few symptoms[9]. Another Fragile X associated disorder is Fragile X Primary Ovarian Insufficiency (FXPOI), which only affects female premutation carriers. FXPOI is a disorder in which the ovaries do not function properly and is defined as the cessation of menses before the age of 40 years old[22]. Females affected by this disorder need assistance in reproducing; such as seeking the use of IVF.

Additional to the risk of FXPOI, premutation carriers have molecular modifications to be cautious of as well. As an allele reaches the premutation range of 55 to 200 CGG repeats, the gene becomes remarkably unstable and is at risk to undergo expansion during transmissions into subsequent generations[8]. This instability is influenced by multiple factors. The first factor includes the ability of these repeats to expand to a full mutation, exclusively when transmission involves a maternal premutation allele. Although expansion from a premutation allele to a full mutation allele does not occur in every case, an expansion to a full mutation have been documented as low as 56 CGG repeats[30]. In contrast, when the paternal allele is transmitted to their daughters the allele may either expand, contract, or remain unchanged[31]. The second influential factor is that the level of instability is correlated with CGG repeat size. The larger the number of repeats being carried, allows for a greater risk of expansion. The last associated factor is the disappearance of the AGG interruptions that are present in the normal range population[32]. Typically, premutation alleles have either none or one AGG interruption towards the 5' end of the repeats and long uninterrupted CGG repeats towards the 3' end.

These are the main factors that influence the instability of premutation alleles and what makes the transmission of these alleles worrisome for female and male carriers.

The assistance needed by female premutation carriers at risk for FXPOI and the associated risk of expansion of premutation alleles collectively classify the importance for IVF in reproduction. Female patients attend infertility clinics to gain insight on their probability of conceiving a healthy offspring. Throughout the IVF process, these patients must elect to have their embryos undergo PGD testing to ensure their optimal health. PGD for FXS is done concurrently with qPCR-based aneuploidy screening to evaluate the chromosome copy number of the biopsied embryo. To assess the inheritance of the affected allele, qPCR-based SNP genotyping for linkage analysis is utilized. This method of testing was developed and validated by Zimmerman et al in 2016[12]. This technique enables researchers to determine which embryos inherited the fragile X (FX) affected chromosome by tracking linked markers. These linked markers are chosen based on the genotypes of the informative SNPs determined by microarray of the patient and partner's gDNA. An informative SNP is when the mother is heterozygous and father is homozygous. A chromosome is labeled as affected if there is the presence of an intermediate, premutation, or full mutation FMR1 allele (or greater than 45 repeats). Therefore, if an embryo inherited the affected X chromosome, that embryo is marked as 'not recommended for transfer' without determination of the CGG repeat size within the biopsy and is then discarded. Without the repeat sizes, there is a significant amount of embryos discarded, when in fact they may be able to produce a viable pregnancy.

Asuragen's Amplidex PCR/CE FMR1 Reagants Kit was created by Asuragen's research and development department to provide reduced complexity and an optimized

workflow to produce a more sensitive method for determining CGG repeat sizes. There

are two PCR methods: a gene specific PCR and CGG repeat primed (RP) PCR. The gene

specific PCR involves the use of the FMR1 forward primer and the FMR1 reverse primer

and simply displays the gene specific peaks when analyzed. The use of a three-primer

CGG RP PCR on gDNA was established by Liangjing Chen from the Asuragen team[33].

The development of this kit helped created the idea to pursue this project and to alter the

current protocol for testing TE biopsies for FXS. The CGG RP PCR was chosen for this

study and is distinguished for two reasons[33]. The first, it includes 3 primers: FMR1

forward primer and the FMR1 reverse primer to flank the FMR1 gene, and a CGG

primer. The CGG primer anneals to each CGG repeat. When the data is analyzed, one can

determine zygosity because each CGG repeat will be detected. The presence of CGG RP

peaks in the display plot provided by the analysis program communicates that there is the

presence of a longer gene specific peak. If a homozygous female was tested with 30 CGG

repeats on each chromosome, then there will be one gene specific peak present in the

normal range and no CGG primed peaks after the detection of the first gene specific peak.

The second reason is this PCR method is able to show the presence of the AGG

interruptions. Gaps will represent the AGG interruptions when each CGG RP peak is

displayed[33]. An obstacle with this kit is to adapt this workflow to be usable with TE

biopsies; which contain limited amount of DNA to start with. One main goal of this

project is to significantly lower the number of embryos that get discarded, when they may

in fact be able to produce a successful pregnancy. Diverging from their qPCR-based SNP

genotyping for linkage analysis and developing a more accurate method for testing these

biopsies for FXS could be useful for physicians when counseling their female patients as well.

Along with the risk for FXPOI, females of advanced maternal age would also greatly benefit from a testing that offers CGG repeat sizes. These older females may only produce one or two good-quality embryos. For example, if a woman of 42 years of age is a premutation carrier of FXS, produces one euploid blastocyst that inherited the FX affected haplotype and another aneuploid blastocyst that inherited a normal X chromosome, a physician would not recommend the transfer of either embryo. Therefore, both embryos get discarded and the woman is unable to try for a sustainable pregnancy. However, with a newly developed test using Asuragen's Amplidex PCR/CE FMR1 Reagant kit can provide the CGG repeat size of that euploid embryo the female patient had available. If her premuation did not expand, the embryo would be feasible for transfer and be at risk for very limited FX symptoms if the premutation has few CGG repeats.

Overall, the addition of the Asuragen Amplidex PCR/CE FMR1 Reagent kit into the clinic's protocol for testing embryo TE biopsies can help physicians, as well as patients. The physicians will be able to counsel patients more accurately about the potential risks of FXS and provide more detailed information about their embryos being tested. While the patients can benefit by potentially saving some embryos from being discarded by using this more accurate methodology. If the test results comes back that the CGG repeat size has not expanded, the physicians can recommend it usable for a transfer. Therefore, giving the patient the opportunity for a sustained pregnancy and a healthy offspring as a result. This project aims to adapt this new protocol to the already existing

procedures by testing different amplification methods' accuracy along with the Asuragen Amplidex PCR/CE FMR1 Reagent kit to determine which provides the most accurate and reliable results for TE biopsies.

MATERIALS AND METHODS

Phase 1:

<u>Genomic DNA isolation</u>

Three different sample types were used when extracting gDNA. Bloodwork, buccal swabs, or chorionic villi samples were all taken from female patients seeking IVF treatment for FXS.

<u>*Bloodwork*</u>

Blood was drawn from IVF patients seeking SGD treatment for FXS. gDNA was isolated using the epMotion 5075, following the protocol as recommended by Eppendorf (Eppendorf, Mount Laurel, NJ, USA). The gDNA was quantified using a Nanodrop 8000 spectrophotometer (ThermoFisher Scientific, Santa Clara, CA). These samples were then normalized to 25ng/µl placed at -20°C until ready for PCR amplification using the Amplidex kit.

<u>*Buccal swabs*</u>

CytoSoft Cytology Brushes (ThermoFisher Scientific, Santa Clara, CA) were sent to relatives of the couple seeking IVF treatment. Due to the chosen analysis method, qPCR-based SNP genotyping linkage analysis, relatives must be involved to properly

track the affected alleles. The recipient of the swab brushed inside the cheeks of their mouth for 10 seconds each side and mailed the swabs back to the clinic. Once received, the gDNA isolation began.

Using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) for extraction, specific alterations were made to ensure proper isolation of the gDNA. 300µl of Cell Lysis Solution is added to a clean 1.5mL Eppendorf tube. The swab, brush first, is placed in the same tube and held with sterilized forceps; while the handle of each swab is cut with sterilized scissors to fit into the tube. 1.5µl of Puregene Proteinase K is added to each sample and mixed by inverting approximately 25 times. The samples were then incubated overnight at 55°C.

The following day, the samples are spun down to remove any condensation from the caps of the 1.5mL tubes. Using sterile forceps, the collection brush was scraped along the sides of the tube to recover as much liquid as possible. Once enough solution is recovered, the brush head is discarded. 300µl of 99-100% ethanol is then added to each sample and vortexed to mix thoroughly. The entire sample was then transferred to an appropriately labeled DNeasy Mini Spin column placed in a 2mL collection tube. The samples were centrifuged at 8,000 rotations per minute (RPM) for 1 minute. The supernatant is discarded and the spin column is placed into a new 2mL collection tube. 500µl of Buffer AW1 is then added to the membrane of each column and centrifuged for 8000RPM for 1 minute again. The flow-through is discarded and the spin column is placed into a new 2mL collection tube. 500µl of Buffer AW2 is, again, added to the membrane of each column and centrifuged for 14,000RPM for 1 minute again. The filtrate is discarded and the spin column is added to a new 2mL collection tube. Without

adding any reagents, the samples are placed directly in the centrifuge at full speed

(14,000RPM) for 2 minutes to remove any excess solutions. The remaining filtrates are

discarded and the spin column is placed in a new appropriately labeled 1.5mL tube. 50µl

of AE Buffer is added directly to the membrane of the column and incubated at room

temperature for 1 minute. The samples were then centrifuged at full speed for 1 minute,

with the caps placed counterclockwise to avoid them breaking off. The column is then

removed from the 1.5mL tube and discarded, for the gDNA is now eluted into the AE

buffer. The gDNA in each sample is then quantified using the Nanodrop 8000

spectrophotometer. These samples were then normalized to 25ng/µl placed at -20°C until

ready for PCR amplification using the Amplidex kit.

Chorionic Villi Samples

Chorionic Villi Samples (CVS) are pieces of tissue taken from the villi of the

chorion of a developing fetus, which will eventually form into the placenta. This is a type

of prenatal test used to detect congenital abnormalities; such as the presence of FXS.

gDNA of these samples were isolated using the DNeasy Blood and Tissue Kit

(Qiagen, Valencia, CA). The protocol specifications for gDNA extraction from tissue was

followed. 180µl of Buffer ATL was added to each sample, along with 20µl of proteinase

K. The samples were vortexed for mixing and incubated at 56°C until completely lysed.

This incubation lasted anywhere from 1-2 hours. 200µl of Buffer AL was added to each

sample and mixed thoroughly by vortexing. The samples were incubated at 56°C for 10

minutes. 200µl of 96-100% ethanol was added and mixed by vortexing. The entire

mixture was then transferred into a DNeasy Mini spin column placed in a 2 mL collection

tube. The samples were spun at 8000 rpm for 1 minute. The flow-through was discarded

and the spin column was added to a new 2mL collection tube. 500µl of Buffer AW1 was

added to each sample and centrifuged for 1 minute at 8000 rpm. The flow-through, again,

is discarded and the spin column is added to a new 2mL collection tube. 500µl of Buffer

AW2 is then added to each sample and centrifuged for 3 minutes at 14,000 rpm. The

flow-through and collection tube were discarded. The spin column was then placed in a

new 1.5mL tube, properly labeled. The DNA was eluted by adding 200µl of Buffer AE to

the membrane of the spin column. The samples were incubated at room temperature for 1

minute and then centrifuged for 1 minute at 8000 rpm. The samples' DNA concentration

were quantified using the Nanodrop 8000 spectrophotometer because of its ability to

detect larger amounts of DNA. These samples were then normalized to be 25ng/µl placed

at -20°C until ready for PCR amplification using the Amplidex kit.

Amplidex PCR Amplification

 All samples were normalized to 25ng/µl, as recommended by Asuragen's

PCR/CE FMR1 Reagents Protocol Guide (Asuragen, Austin, TX). A positive and

negative control was included. The positive control was provided by Asuragen and is a

Process Control, which contains peaks in all expected allelic ranges. The negative control

consists of 2µl of the diluent, provided by the Amplidex FMR1 Reagent Kit instead of a

DNA sample. The samples were prepared for amplification using the AmplideX FMR1

Reagents Kit. The protocol for CGG RP PCR was followed because of its ability to

produce additional informative data. The PCR master mix consists of 11.45µl of GC-

Rich Amp Buffer, 0.5µl FMR1 F,R FAM Primers, 0.5µl FMR1 CGG Primer, 0.5µl

diluent, 0.05µl GC-Rich Polymerase Mix, 2µl of each pre-amplified DNA sample

(including the positive and negative controls) per one reaction. The samples are then placed in a 2720 thermocycler (Applied Biosystems, Foster City, CA) for the appropriate cycling protocol as recommended by Asuragen. It began with an initial denaturation step at 95°C for 5 minutes. Followed by 10 cycles of a continued denaturation at 97°C for 35 seconds, then primer annealing at 62°C for 35 seconds, and ending with extension at 68°C for 4 minutes. There is then another set of 20 cycles, with the same conditions as the previous 10 cycles; however, 20 seconds is added after every cycle to the last extension step at 68°C. There is further extension that takes places during a 10 minute hold at 72°C and then ends with the samples at a 4°C hold until ready for the next step.

Capillary Electrophoresis for Fragment Analysis

The amplified samples are now prepared to be run on the 3730xl DNA Analyzer (ThermoFisher Scientific, Santa Clara, CA) for capillary electrophoresis (CE). A master mix is prepared, consisting of 11µl of Hi-Di Formamide and 2µl ROX 1000 Size Ladder for each expected reaction. 13µl of the prepared master mix is combined with 2µl of PCR product within a MicroAmp Optical 96 Well Reaction Plate. These samples are then denatured for 2 minutes at 95°C and stored at 4°C until ready for injection on to the instrument.

The 3730xl DNA Analyzer is prepared for data acquisition according to manufacturer's directions. The run buffer used for this instrument consisted 180mL of distilled water from a Millipore Ultrapure Water System (Hach, Loveland, CO) and 20mL of 3730 Buffer (10X) with EDTA. The samples are then run on the instrument using Fragment Sizing Analysis protocols specific to Asuragen's calibration requirements. The POP-7 polymer is used, along with a recommendation for 2.5kV at 20

seconds for each injection on a standard 3730xl with 96 capillaries at 50cm of length. The run time on the instrument was approximately 1 hour and 45 minutes.

Data Analysis

Data collected from the CE is inputted into GeneMapper5 software. Each sample shows a display plot exhibiting peaks for each allele that is detected by the instrument. The display plot is analyzed for each of the samples and size of the peak is determined. Because the CGG RP PCR protocol was followed, each time a CGG is discovered the software will automatically call it. To begin analysis, all automatic allele calls are deleted and the size of gene-specific peaks are then converted into CGG repeat length. One can determine repeat length by using the following formula:

$$CGG_i = \frac{Peak_i - c_0}{m_0}$$

Where $Peak_i$ is the size in base pairs of the gene specific peak, c_0 is a size correction factor, and m_0 is the mobility correction factor for each CGG repeat. C_0 and m_0 are specific for each standard instrument, ours being 3730xL with 50cm capillaries for its configuration. Therefore, c_0 is equal to 231.9 and m_0 is equal to 2.937. From here, after analyzing the peaks for each sample we can determine the number of repeats and compare each result against their expected repeat size.

Phase 2:

Cell lines

Human lymphocytes were obtained from Coriell Repository with different CGG repeat sizes and were cultured in RPMI 1640 with 2X nonessential amino acids, 15% fetal bovine serum, 2mM L-glutamine at 37°C and 5% CO_2. The six different cell lines

and their respective repeat sizes can be found in table 1.

Table 1: *Cell lines used to mimic TE biopsies.*

Cell Line	Gender	FMR1 Gene	CGG Repeat Size
GM06889	Female	Normal	30;23
GM06890	Male	Normal	30
GM06891	Male	Premutation	118
GM06894	Female	Premutation	30;78
GM06896	Mosaic Female	Mosaic Premutation	23;95-120-140
GM06897	Male	Full Mutation	477

A total of 12 replicates were collected for each cell line and placed into a 0.2mL

PCR tube, making the total sample number 72. Each sample had 6 cells collected in 1μl

of RPMI 1640 using a 100μm stripper tip (Midatlantic Diagnostics, Mount Laurel, NJ,

USA) under a dissecting microscope and placed into a PCR tube. 6 cells were chose to

mimic a TE biopsy to establish the use of the desired method on a limited number of

cells. These samples were used in testing method one, GenomePlex WGA4

Amplification, and method four, targeted pre-amplification. Another set of 72 samples,

containing 6 cells were collected in phosphate buffered saline (PBS), using the same

100μm stripper tip and under the same dissecting microscope as previously described.

These samples were used in method 2, SurePlex DNA amplification, and method 3,

REPLI-g Single Cell Kit for amplification. Negative controls consisted of 1μl of clean RPMI 1640 culturing media and 1μl of PBS. 6 replicates for each cell line were used to test each of the four following amplification methods.

Using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) gDNA was isolated from each of the 6 cell lines. The protocol was followed for the gDNA extraction of cells. 2mL of cells were spun down for 5 minutes at 190 rpm; the supernatant was then removed. The pellet was re-suspended in 200μl of PBS and transferred into a clean 1.5mL tube. 200μl of Buffer AL was added to each sample and mixed thoroughly by vortexting. The samples were incubated at 56°C for 10 minutes. 200μl of 96-100% ethanol was added and mixed by vortexing. The entire mixture was then transferred into a DNeasy Mini spin column placed in a 2 mL collection tube. The samples were spun at 8000 rpm for 1 minute. The flow-through was discarded and the spin column was added to a new 2mL collection tube. 500μl of Buffer AW1 was added to each sample and centrifuged for 1 minute at 8000rpm. The flow-through, again, is discarded and the spin column is added to a new 2mL collection tube. 500μl of Buffer AW2 is then added to each sample and centrifuged for 3 minutes at 14,000 rpm. The flow-through and collection tube were discarded. The spin column was then placed in a new 1.5mL tube, properly labeled. The DNA was eluted by adding 200μl of Buffer AE to the membrane of the spin column. The samples incubated at room temperature for 1 minute and centrifuged for 1 minute at 8000 rpm. The samples' DNA concentration were quantified using the Nanodrop 8000 spectrophotometer.

Method #1: Single cell lysis and GenomePlex WGA4 Amplification

As mentioned above, these samples were collected in 1μl of RPMI 1640 culturing

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media. The total sample number is 36; 6 replicates from each cell line. The samples are lysed in alkaline lysis buffer. 7ul molecular biological grade water was added to the PCR tubes, followed by adding 1μl alkaline lysis buffer [200 mM KOH and 50 mM DTT]. The samples are then incubated at 65°C for 10 min. 1ul neutralization buffer [0.9 M Tris–HCl, pH 8.3, 0.3 M KCl and 0.2 M HCl] is added to the sample to neutralize the lysis buffer. The lysates were snap frozen with liquid nitrogen and stored at -20°C until ready for amplification using the Amplidex kit.

DNA of the lysed cells is amplified by WGA using the GenomePlex WGA4 kit as recommended by the supplier (Sigma Aldrich Inc). 2μl of 1X library preparation buffer was added to the side of the tube, above the 10μl sample. The sample was spun down to consolidate the solutions, vortexed to ensure adequate mixing of the solutions, and then spun again. 1μl of library stabilization solution is then added to the tube. The samples is the spun, vortexed and spun again. The samples were incubated at 95°C for 2 minutes in a 2720 thermocycler (Applied Biosystems, Foster City, CA). The samples were briefly spun down to consolidate the solutions and moved to ice for cooling. 1μl of library preparation enzyme is added to the samples and flicked to ensure mixing. The samples were spun down before incubating in the 2720 thermocycler for the following conditions: 16°C for 20 minutes, followed by 24°C for 20 minutes, then 37°C for another 20 minutes, finishing with 75°C for 5 minutes and ending at 4°C until ready for the next step. Next, the amplification master mix was made consisting of 7.5μl of the 10X amplification master mix, 48.5μl of nuclease-free water, and 5μl of WGA DNA polymerase for each reaction. The master mix was vortexed and spun to consolidate the solution. Each sample received 61μl of this master mix and was vortexed and spun to guarantee appropriate

mixing for each sample. The samples incubated at 95°C for 3 minutes for the initial denaturation, 25 cycles of another denaturation at 94°C for 30 seconds, annealing and extension at 65°C for 5 min and ending with a hold at 4°C. The WGA DNA is purified using GenElute PCR cleanup columns (Sigma Aldrich Inc, St. Louis, MO). Before conducting the purification the tubes needed preparation. 3 tube racks were obtained and one 1.5mL tube for each sample, closed and empty, were placed in the first rack. Opened 1.5mL tubes were placed in the second rack and 375µl of binding solution was aliquoted into each. 2mL tubes from the clean-up kit were placed in the third rack and a spin column was placed into each. 500µl of column prep solution was added to each column and spun at 12 RCF for 1 minute using the 5424 centrifuge (Eppendorf, Mount Laurel, NJ, USA). All tubes had appropriate labels for their corresponding samples.

The amplified samples were spun before beginning the clean-up protocol. Each sample was added to the 1.5mL binding solution tubes and vortexed and spun for mixing. The sample, now combined with the binding solution, was added to the spin column and spun at 12 RCF for 1 minute. The supernatant was discarded. 500µl of diluted wash solution was added to the spin columns and spun at 16.1 RCF for 1 minute. The supernatant was discarded. The samples were spun again at max speed for 2 minutes to further dry the column. The spin column was transferred from the 2mL tube to the clean empty 1.5mL tubes. 50µl of elution solution was added to the spin column and incubated at room temperature for 1 minute. The samples were spun at maximum speed for 1 minute and the spin columns were discarded. The samples were quantified using a Nanodrop 8000 spectrophotometer. The only alteration being that the blank used for this method was the elution solution provided in the clean-up protocol. These samples were

then normalized to be 40ng/µl placed at -20°C until ready for Amplidex PCR amplification.

Method #2: SurePlex DNA Amplification System

The SurePlex DNA Amplification System requires cell collection to begin with 1.5µl of PBS into PCR tubes, with the addition of the 6 cells from each cell line in 1µl of PBS. The lysis protocol is then followed as recommended by Illumina. 2.5µl of collection buffer was added to each sample and centrifuged at 200g for 3 minutes, using the same centrifuge as mentioned in the first method. 2.5µl of cell extraction buffer was added to the samples. They were spun, vortexed and spun again to ensure sufficient mixing. The samples were placed on ice while the extraction cocktail master mix was prepared. For one reaction, the master mix contained 4.8µl of extraction enzyme dilution buffer and 0.2µl of cell extraction enzyme. 5µl of the master mix was added to each sample and spun down to consolidate the solutions. They were vortexed to mix the solutions thoroughly and spun again. The samples incubated at 75°C for 10minutes, then 95°C for 4 minutes and then placed at 25°C until ready for pre-amplification.

DNA of the lysed cells is amplified by WGA using the SurePlex DNA Amplification Kit as recommended by Illumina (San Diego, CA). The pre-amplification master mix for one reaction contained 4.8µl of SurePlex pre-amp buffer and 0.2µl of SurePlex pre-amp enzyme. 5 µl of the master mix was added to each sample and vortexed and spun to ensure consolidation. The samples incubated at 95°C for 2 minutes to begin enzyme activation then endured 12 cycles at 95°C for 15 seconds, 15°C for 50 seconds, 25°C for 40 seconds, 35°C for 30 seconds, 65°C for 40 seconds, followed by 75°C for 40 seconds. Finishing with a 4°C hold until ready for amplification.

For one reaction, the amplification master mix contained 25µl of SurePlex amplification buffer, 0.8µl of SurePlex amplification enzyme, and 34.2µl of nuclease-free water. The master mix was vortexed and spun down for consolidation. 60µl of the master mix was added to each sample and vortexed. The samples were spun before incubation at 95°C for 2 minutes to start enzyme activation, then 14 cycles of 95°C for 15 seconds, 65°C for 1 minute, and 75°c for 1 minute. Ending with a 4°C hold until ready for quantification.

The samples are then quantified using the Qubit 2.0 Fluorometer (ThermoFischer, Santa Clara, CA) as recommended by Illumina. To begin, the Qubit dsDNA HS working solution was prepared for 48 samples consisting of 11.945mL of dsDNA HS buffer and 55µl of dsDNA HS reagent. We only had 36 samples, so the left over master mix was stored at 4°C. 190µl of the working solution was added to the Qubit assay tubes, one for each sample. A 1:10 dilution was conducted for each sample. 10µl of each dsDNA HS standard and 10µl of each sample were added to the Qubit assay tubes, containing the 190µl of the working solution. The samples were spun, vortexed, and spun again. They incubated for 2 minutes at room temperature, while calibration of the instrument occurred using the dsDNA HS standards. Each sample was then quantified and the concentration was recorded and converted to ng/µl. A GeneVac from Eppendorf (Eppendorf, Mount Laurel, NJ, USA) was used to evaporate excess water from samples with concentrations lower than 40ng/µl. After being dried, the samples were normalized to 40ng/µl and stored at -20°c until ready for the Amplidex PCR amplification.

Method #3: REPLI-g Single Cell Kit

The samples used for this method were collected using PBS. The PCR tubes were

prepped with 3µl of PBS before collection occurred. After collection, 3µl of Buff D2 is added to the 4µl of cell material (supplied with PBS) and incubated at 65°C for 10min. 3µl of the stop solution is added to halt the reaction and flicked to ensure thorough mixing. DNA of the lysed cells is amplified by WGA, utilizing a multiple displacement amplification (MDA) methodology, using the REPLI-g Single Cell Kit as recommended by Qiagen. For one reaction, the master mix consisted of 9µl of nuclease-free water, 29µl of REPLI-g reaction buffer, and 2µl of REPLI-g DNA polymerase. The master mix was vortexed and spun to ensure adequate mixing. 40µl of master mix was added to each 10µl sample of denatured DNA. The samples incubated at 30°C for 8 hours. Followed by a 3 minute incubation at 65°C to inactive the REPLI-g DNA polymerase.

Before quantification, the samples needed to undergo a purification procedure. The procedure chosen was a beads purification protocol using Ampure XP Beads. Before beginning, fresh 70% ethanol was prepared and the Ampure Beads were removed from the refrigerator to reach room temperature. The full 50µl volume of each sample was transferred to a 96 well plate. The amount of beads needed for each sample is 1.8 times the volume of the sample; i.e. 50µl x 1.8 = 90µl of Ampure beads. The plate, containing the samples and beads, was sealed, vortexed and spun to ensure thorough mixing. The samples incubated at room temperature for 5 minutes and were then put onto a magnetic rack. Once the magnetic beads formed a pellet and the liquid cleared, the supernatant was removed. After use with the REPLI-g amplification kit, the samples were very sticky and required additional scraping of the excess beads off of the sides of the wells. 200µl of the freshly prepared 70% ethanol is added to each well containing a sample. The plate was moved around the magnetic rack a couple times to ensure adequate washing. The

supernatant was removed and another 200µl of 70% ethanol was added. This last step was repeated 4 times, resulting in 4 washes. Once the last supernatant was removed, the plate was sealed and the residual ethanol was spun down. All ethanol was removed from each of the samples. While removing the remaining ethanol that settled after being spun, the pellets were dried for 3 minutes at room temperature. 50µl of nuclease-free water was added to each well, while off the magnet, and mixed properly to re-dissolve the pellets. The plate was sealed and incubated at room temperature for 2 minutes. It then was vortexed and spun down. Lastly, the plate was placed back onto the magnetic where the pellet reformed. The supernatant was removed and placed into a new, properly labeled, PCR tube.

These samples were then quantified using the Agilent 2200 TapeStation System (Agilent Technologies, Santa Carla, CA). The specific protocol used was the one for gDNA to allow visualize of larger bands. 10µl of Genomic DNA Sample Buffer was mixed with 1µl of the Genomic DNA Ladder within the first well of the plate being used. Following down the same column, 10µl of Genomic DNA Sample Buffer was mixed with 1µl of each sample. The plate was vortexed and spun down to consolidate all reagents. It was placed into the 2200 TapeStation system. Because of the TapeStation's ability to only detect concentrations between 0 and 100ngµl, it was unsuitable for use with our samples. The next option was the Nanodrop 8000 spectrophotometer because of its ability to detect larger concentrations. This method had better specificity for our samples because of their high DNA concentrations (~600ngµl). Each sample was normalized to 40ng/µl using the concentrations from the Nanodrop and stored at -20°c until ready for the Amplidex PCR amplification.

<u>Method #4: Single Cell Lysis and Targeted by Pre-Amplification of Specific FMR1 Gene</u>

All replicates of 6 cell aliquots were lysed according to the same lysis protocol as described in method 1.

Initially, primers had to be created to specifically target the FMR1 gene and was done using Integrated DNA Technologies (IDT, Corlaville, IA). Additionally, Jean Wilson's publication in the Journal of Molecular Diagnostics[34] was used to create a forward and reverse FMR1 primer to target the entire FMR1 gene. The forward primer read as follows: 5' – 6FAM-GGAACAGCGTTGATCACGTGACGTGGTTTC-3' and the reverse primer read as: 5'-GGGGCCTGCCCTAGAGCCAAGTACCTTGT-3'. For one reaction the master mix for the pre-amplification consisted of 25µl of Taqman PreAmp master mix (ThermoFisher Scientific, Santa Clara, CA), 2.5µl of the 10µM FMR1 forward primer, 2.5µl of the 10µM FMR1 reverse primer and 10µl of nuclease-free water were all combined to create the master mix used in this amplification method. The master mix was vortexed and spun to guarantee suitable mixing of the reagents. Then, 40µl of the master mix was added to each sample. The samples were then spun, vortexed and spun down again. The thermocycling conditions for the 2720 thermocycler (Applied Biosystems, Foster City, CA) began with 95°C for a 10 minute enzyme activation hold, then 18 cycles of 95°C for 15 seconds, 60°C for 4 minutes, and ending at 4°C until ready for the next steps. This part of the protocol was altered from 18 cycles, to 24 cycles, then to 30 cycles, then to 36 cycles and finally ended with 42 cycles of pre-amplification.

No quantification is necessary for these samples since the targeted amplicon is too small to produce a needed concentration of 40ng/µl. The samples were stored at -20°C

until ready for Amplidex PCR Amplification.

To ensure the IDT FMR1 primers were outside the FMR1 region, next-generation sequencing (NGS) was conducted to confirm its position. A control aneuploid male cell line 1359 with a normal FMR1 gene was used sequencing, along with a negative control of water. The sample underwent the pre-amplification steps as previously described and were stored at -20°C until the Amplidex Gene-Specific protocol was conducted.

FX Gene-Specific PCR Amplification for Sequencing in Method 4

The two samples, control sample 1359 and a negative control were prepared for Gene-Specific PCR using the same Amplidex FMR1 Reagent Kit as recommended by Asuragen. The PCR master mix consisted of 11.45µl of the GC-Rich amplification buffer, 0.5µl of the FMR1 F,R FAM primer, 1µl of the diluent, and 0.05µl of the GC-Rich Polymerase Mix per one reaction. The master mix was thoroughly mixed by vortexing and 13µl was dispensed into each new PCR tube. 2µl of each sample was added to its appropriately labeled tube. The samples were then vortexed and spun down to consolidate the reagents. They were then put into a 2720 thermocycler and were subjected to the following conditions: 98°C for a 5 minute hold, then 25 cycles at 97°C for 35 seconds, 62°C for 35 seconds, 72°C for 4 minutes, and finished off with another extension at 72°C for 10 minutes. A simple way to describe the difference between the Gene-Specific PCR protocol and the CGG RP PCR protocol is the use of the third primer, FMR1 CGG primer. It makes up part of the master mix in the CGG RP PCR but is not included in the Gene-Specific protocol. There is also only one set of PCR cycles in the Gene-specific protocol compared to the CGG RP PCR. Following this PCR, the Agilent

Tape Station was used to determine the size of the amplicon.

Next-Generation Sequencing using PGM

Due to the size of our amplicon being less than 400bp, the protocol was started at the end-repair section. 79µl of nuclease-free water was added to each sample because there was 100ng of input DNA. Additionally, there was 20µl of 5X end repair buffer and 1µl of end repair enzyme added to each sample. The samples were incubated for 20 minutes at room temperature. To purify the samples, 180µl of the AmPure XP Reagent beads was added to each sample. The samples were vortexed to mix thoroughly and placed on a magnet to for 3 minutes, or until the solution cleared. The supernatant was removed and 300µl of freshly made 70% ethanol was added to each. The samples were moved around the magnet to thoroughly wash the beads. The supernatant again, was removed and the washing step was repeated one additional time. The samples were spun down to remove all residual ethanol and left at room temperature to dry for about 5 minutes. The pellet of beads was removed from the magnet and re-dissolved in 25µl of low TE buffer and mixed by vortexing. The samples were spun down and placed back on the magnet until the solution cleared. The supernatant was removed and placed in a new appropriately labeled tube.

Ligate and nick-repair was the next step followed. Because barcodes were added to the samples, the reaction was setup for barcoded libraries. Added to the 25µl of eluted DNA was 10µl of 10X ligase buffer, 2µl of Ion P1 adapter, 2µl of Ion Xpress Bardcode X[3], 2µl of dNTP Mix, 49µl of nuclease-free water, 2µl of DNA ligase, and 8µl of nick-repair polymerase. Each sample was vortexed and then spun down before placed in the

2720 thermocycler. The conditions followed were 25°C for 15 minutes, 72°C for 5 minutes, and held at 4°C until ready for the next purification. The purification began with adding 150μl of the AmPure XP Reagent beads and then the steps were followed identical to the purification that occurred after the end-repair protocol.

The amplification began with 25μl of DNA, 100μl of the Platinum PCR SuperMix High Fidelity and 5μl of library amplification primer mix was added to each sample. The tubes were placed in a 2720 thermocycler under the following conditions: 95°C for 5 minutes to denature the DNA, followed by 8 cycles of 95°C for 15 seconds for additional denaturing, 58°C for 15 seconds for annealing to occur, 72°C for 1 minute of extension, ending with a 4°C hold until ready for the final purification. The amount of beads added to each sample was 195μl. The rest of the protocol was followed identically to the purification that occurred after end-repair. The elution step involved the addition of 20μl of low TE buffer, instead of the previous 25μl.

The Personal Genome Machine (PGM) (ThermoFisher, Santa Clara, CA) was setup as recommended by Thermofisher Scientific.

Amplidex PCR Amplification for All Four Methods

All pre-amplified samples are then prepared for amplification (protocol provided by Asuragen) using the AmplideX FMR1 Reagents Kit, as described in phase 1. A positive and negative control were included, as described previously in phase 1. The samples are then placed in a 2720 thermocycler for the appropriate cycling protocol as recommended by Asuragen, also described previously in phase 1.

Capillary Electrophoresis for Fragment Analysis

The amplified samples are now prepared to be run on the 3730xl DNA Analyzer for CE, aforementioned in phase 1. The 3730xl DNA Analyzer is prepared for data acquisition according to manufacturer's directions (Sanger Sequencer Genetic Analyzer, Applied Biosystems). The samples were run on the instrument using Fragment Sizing Analysis protocols specific to Asuragen's calibration requirements, also mentioned previously in phase 1.

Data Analysis

The data to be analyzed is exported from 3730xl Genetic Analyzer and inputted into GeneMapper5 software, as described previously in phase 1. All automatic allele calls are deleted and must be manually chosen based on the criteria provided by the Asuragen manual. After analyzing the peaks for each sample, we can determine the number of repeats and compare each of our methods.

Continuation of Confirmation using REPLI-g Single Cell Kit for Amplification

To progress onto phase 3, the REPLI-g Single Cell Kit for amplification was chosen. An additional 48 samples, consisting of either cells from cell lines or gDNA, were collected and de-identified to provide extra confirmation that the amplification and following methodology works accurately. These samples underwent the lysis method as recommended by Qiagen using the REPLI-g Single Cell kit, along with the amplification procedure. The samples were purified using the beads purification protocol as previously described. The samples were normalized to 40ng/µl and followed by PCR using the Amplidex FMR1 reagent kit. Lastly, CE on the 3730xl DNA analyzer was conducted and the data was analyzed using the GeneMapper5 software.

Phase 3:

To begin, 3 more replicates from each cell line were used to test whether or not the single cell lysis protocol will work successfully with the REPLI-g Amplification system. The cells were lysed using the single cell lysis method described in phase 2, method 1. Then they underwent the REPLI-g amplification, followed by the Amplidex PCR and the CE. Once validated, the amplification kit could be used on arrested embryos that were already stored as lysates.

Use of Discarded Arrested Embryos

12 arrested embryos from patients seeking IVF treatment for FXS were obtained from long-term cyro storage in a liquid nitrogen dewer. The quality of each embryo at the point it arrested was either 5 cells, 8 cells, or a morula. No PGD testing had been conducted to observe chromosome copy number because each embryo did not reach the required blastocyst stage. Therefore, the arrested embryos were lysed as described in phase 2, method 1 before being provided for this study.

REPLI-g Amplification

The REPLI-g amplification kit was used, aforementioned in phase 2, method 3. The samples underwent a beads purification, previously described in phase 2 under method 3. The samples were then quantified using the Nanodrop 8000 spectrophotometer. The samples were treated similarly to the cell lines and normalized at 40ng/µl.

<u>Amplidex PCR Amplification, CE, and Data Analysis</u>

Amplidex PCR amplification was conducted as previously mentioned in phase 1. Followed by the CE using the 3730xl DNA analyzer. The data analysis was conducted in the same fashion as in the previous phases using GeneMapper5 software.

<u>Genotyping</u>

After conducted the previous experiments, genotyping was done to determine the gender of each embryo. The previously normalized samples were diluted from 40ngµl to 5ng/µl in 50µl of total volume. Simultaneously, a master mix is made consisting of 2.5µl of Taqman Gene Expression Master Mix, 0.125µl of the AmelXY assay, and 0.375µl of water per 1 reaction. A separate master mix was made for another version of a gender assay called ANACKX assay. The same volume was added to another master mix created with the same parameters. 3µl of the master mix is added to each appropriate well on a 384 well plate. Then, 2µl of each DNA sample is then added to the appropriate wells. Each DNA sample was run with the two different assays. The plate is vortexed and spun to ensure adequate mixing. An SDS file is then made using SDS 2.4 software to create the plate layout for the 7900 Fast Real Time PCR System (ThermoFisher, Scientific, Santa Clara, CA). The plate is initially ran on a GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA) under the following conditions: 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minutes, ending with a final hold at 4°C. The plate was then run on the 7900 Fast Real Time PCR System (ThermoFisher Scientific, Santa Clara, CA) to conduct a post read for analysis.

Phase 4:

Collection of Discarded Whole Aneuploid Embryos

5 discarded whole aneuploid embryos were used throughout this phase and are

listed in table 2. They were biopsied using Olympus TH4-100 Biopsy and microinjection

microscope working system.

Table 2: *Discarded Whole Aneuploid Embryos Used*

Embryo #	Ploidy Status	PCR 24 Results	FX Affected?	Gender
1	Aneuploid	47,XX,-1,+3,+10	yes	F
2	Aneuploid	47,XX,+10	no	F
5	Aneuploid	47,XY,+2	yes	M
4	Aneuploid	47,XXX	Inconclusive (XXX)	F
3	Aneuploid	45,XX,-9	no	F

Single Cell Lysis

Lysis was conducted on all biopsies from each embryo, previously described at

the beginning of amplification method 1. After following the necessary steps, the lysates

were stored at -20°C until ready for amplification.

Amplification using REPLI-g Single Cell Kit

DNA of the lysed cells were amplified by whole genome amplification (WGA)

using the REPLI-g Single Cell Kit as recommended by Qiagen, aforementioned in

amplification method 3.

Before quantification, the samples underwent a purification procedure, as

mentioned above in amplification method 3. The procedure chosen was a beads

purification protocol using Ampure XP Beads.

The samples were quantified using the Nanodrop 8000 spectrophotometer. Each

sample was normalized to 40ng/µl using the concentrations from the Nanodrop and

stored at -20°c until ready for the Amplidex PCR amplification.

Amplidex PCR Amplification

All pre-amplified samples are then prepared for amplification (protocol provided

by Asuragen) using the AmplideX FMR1 Reagents Kit, as described in phase1 and again

referenced in phase 2 under Amplidex PCR amplification for all four methods.

Capillary Electrophoresis POP-7

The amplified samples are now prepared to be run on the 3730xl DNA Analyzer

for CE, previously mentioned in phase 1. The 3730xl DNA Analyzer is prepared for data

acquisition according to the manufacturer's directions (Sanger Sequencer Genetic

Analyzer, Applied Biosystems). The samples are then run on the instrument using

Fragment Sizing Analysis protocols specific to Asuragen's calibration requirements, also

mentioned previously in phase 1.

Data Analysis

The data to be analyzed is exported from 3730xl Genetic Analyzer and inputted

into GeneMapper5 software, as described previously in phase 1. All automatic allele calls

are deleted and must be manually chosen based on the criteria provided by the Asuragen

manual. The data from this final phase will serve as additional validation of the

developed method and confirmation that the method works on clinical samples.

<u>Next-Generation Sequencing using the Ion Torrent Proton</u>

Because the aneuploid whole embryos were testing using CCS a few years ago, the CCS platforms have changed. Previously, PCR-24, a qPCR based CCS method for aneuploidy screening was used for all biopsies. Currently NexCCS, a NGS based CCS method is now used as a more sensitive method for CCS. NexCCS was conducted on biopsies from the aneuploid whole embryos to compare the CCS methods. A 10µl lysate from each aneuploid whole embryo was used. They first underwent pre-amplification. A master mix was created using 25µl of Taqman PreAmp Master Mix, 2.5µl of a universal reverse primer, and 10µl of water for every 1 reaction. The master mix was mixed thoroughly before added to each lysate. Then, 2.5µl of a unique fusion primer, or barcode, was added to each sample as an identifier. The samples were mixed thoroughly and spun down to consolidate the solutions. The samples were then placed in a 2720 thermocycler under the following conditions: 95°C for a 10 minute hold, then 24 cycles of 95°C for 15 seconds, 60°C for 4 minutes, and ending with a 4°C hold until ready for the next step.

The samples were then quantified using the Agilent TapeStation as described previously to ensure the amplicons are present. All the samples are then normalized to 15ng and pooled together. They then underwent a beads purification protocol, as previously described. The prepared library is then quantified on the Agilent TapeStation again. The library is then normalized to 50pmol and placed at 4°C until ready to be loaded on the Ion Chef Instrument (ThermoFisher Scientific, Santa Clara, CA) according the manufactures recommended instructions. The ion chef creates the chip that will then be loaded onto the Ion Torrent Proton for Next-Generation Sequencing (ThermoFisher

Scientific, Santa Clara, CA). Once the chip is loaded, it is placed on the Proton following the manufactures recommendations. From there, the data is analyzed using an algorithm created by a bioinformatics team and the chromosome copy number plots are created needing further analysis.

RESULTS

Phase 1:

PCR and CE was conducted to determine the CGG repeat size for all the known samples that were used in this phase. gDNA was previously isolated from blood or buccal swabs from patients seeking IVF treatment for FXS, or chorionic villi samples from the fetus of a similar patients. After amplifying the gDNA with the Amplidex PCR and following with CE, each sample's CGG repeat sizes were compared to that of two reference labs. As seen in table 3, the CGG repeat sizes for each sample listed under the column 'In House' was within 0-3 CGG repeats[33], as expected, compared to the results provided by the two reference labs. With all 17 samples showing an exact match to at least one of the reference labs' results, this method has shown to be very reliable for testing for CGG repeat sizes on gDNA.

Table 3: *Results of gDNA run using Asuragen's Amplidex Kit*

	CGG Repeat Numbers					
Sample ID	Reference Lab #1		Reference Lab #2		**In House**	
1	31	125	30	126	30	126
2	31		30		30	
3	31	75	30	74	30	74
4	29	92	28/29	93/94	29	93
5	29	56	29	56	29	56
6	24		22/23		23	
7	30	>200	29/30	>200	30	319
8	>200		>200		287	
9	30	74	29	74	29	74
10	10	89	10	87	11	87
11	29	75	30	75	30	75
12	31	85	31	86	31	86
13	24	79	23	79/80	23	79
14	>200		>200		316	
15	30	30	29	30	29	30
16	55		54		54	
17	90		93		93	

Figure 1 is a display plot from the analysis program GeneMapper5 software and is an example of an electropherogram after use of the CGG RP PCR method. Each time a CGG repeat is detected, it is marked on this display plot with a blue line. To clarify, the x-axis is the number of base pairs and the y-axis is the signal intensity. The space between the first 10 CGG peaks detected and the next 10 is representative of the AGG interruption, denoted by the black arrow. These interruptions, aforementioned in the introduction, help maintain stability of the gene. The two, more intense, peaks are labeled as the gene-specific peaks. The sizes of these peaks can be converted to CGG repeat length using the formula discussed in the methods of phase 1. Because the FMR1 gene is on the X chromosome, samples from a female will display two intensified peaks, such as

in figure 1, and males will exhibit only 1 intensified peak. Specifically for figure 1, the sample is a female exhibiting a peak within the normal range and a peak within the premutation range for CGG repeat size. Therefore, it can be determined that this female patient has one normal X chromosome with 29 repeats and her other X chromosome exhibits a FMR1 gene within the premutation range with 93 repeats. She is also fortunate to have two AGG interruptions that were not lost in transmission, since she does exhibit a premutation FMR1 allele.
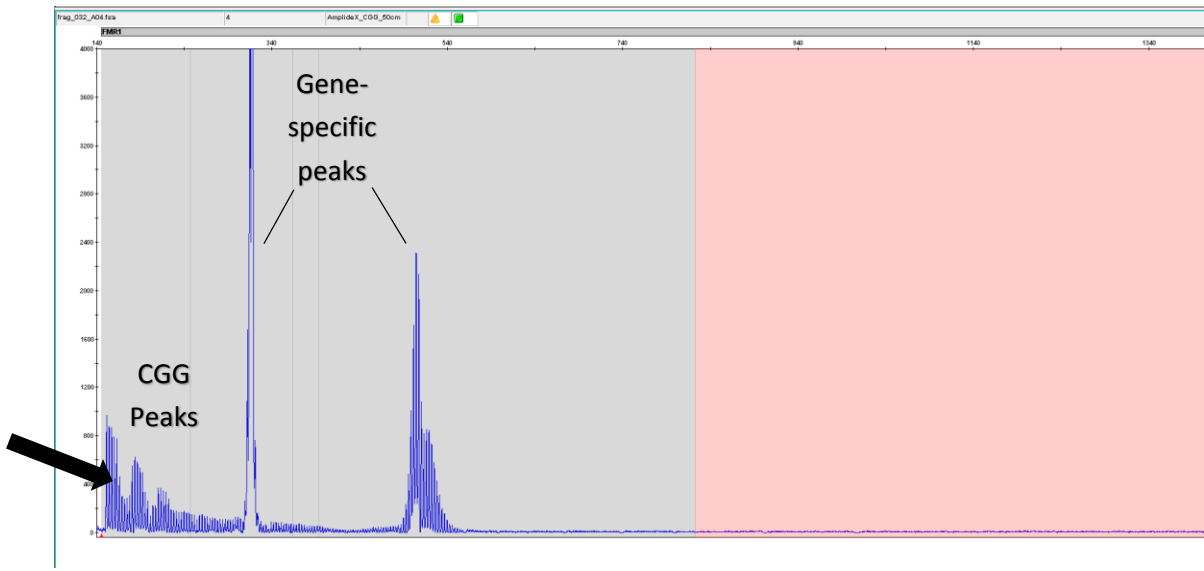

Figure 1: Display plot for gDNA sample number 4.

Phase 2:

6 replicates from each cell line, containing 6 cells to mimic a trophectoderm biopsy, were used to test the four previously mentioned amplification methods. The same Amplidex PCR, CE and data analysis were conducted for all four methods. The only difference is method 4 had additional tests done for confirmation purposes.

Amplification Method #1 Single cell lysis and GenomePlex WGA4 Amplification & Method 2: SurePlex DNA Amplification System

After conducting PCR and CE on the whole genome amplified DNA described in methods 1 and 2, there were no CGG or gene-specific peaks detected in the display plots. As seen in figure 2, there were no gene-specific peaks detected at all for the GenomePlex WGA4 Amplification method. There were 6 replicates run for each of the 6 cell lines, totaling 36 samples for this method and all samples exhibited this same result. Figure 3 displays results for the SurePlex DNA Amplification system. There were no peaks detected using this WGA method as well.
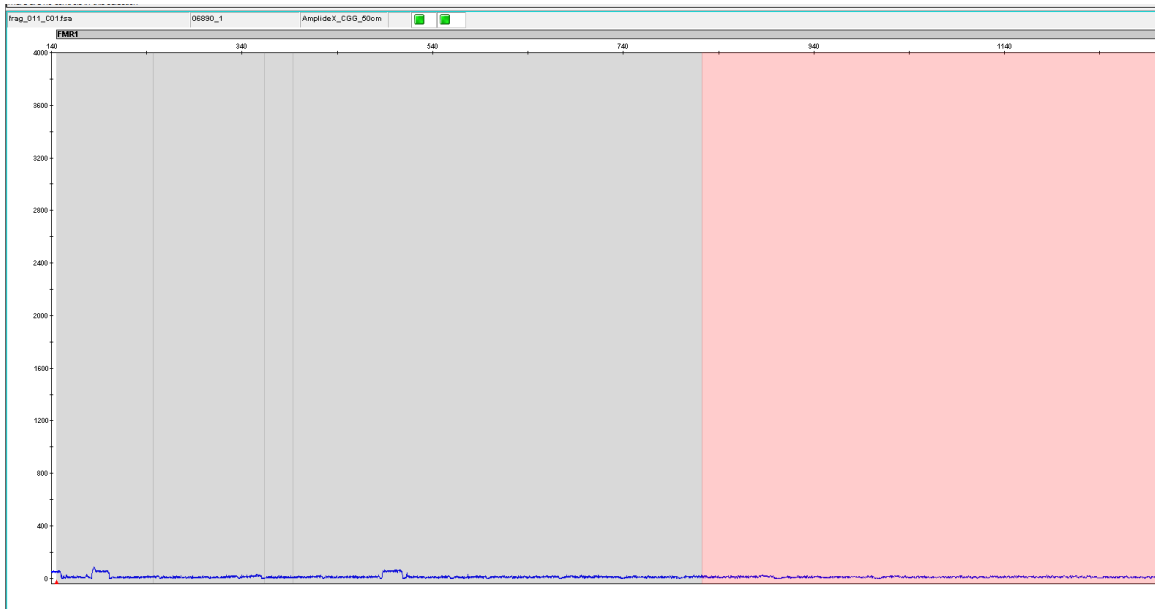

Figure 2: Display plot for sample 06890_1 used with GenomePlex WGA

Figure 3: Display plot for sample 06894_V used with SurePlex amplification.

Method #3: REPLI-g Single Cell Kit

   Although the REPLI-g Single Cell Kit for amplification is also a WGA method, it
is specifically a multiple displacement amplification (MDA). It was expected for this kit
to work more efficiently for longer products compared to the two previous WGA
methods that were not MDA versions. As seen in figure 4, there are CGG peaks present
at the beginning of the display plot, along with 2 AGG interruptions present. The gene-
specific peak shown on the display plot has a high signal intensity, which gave great
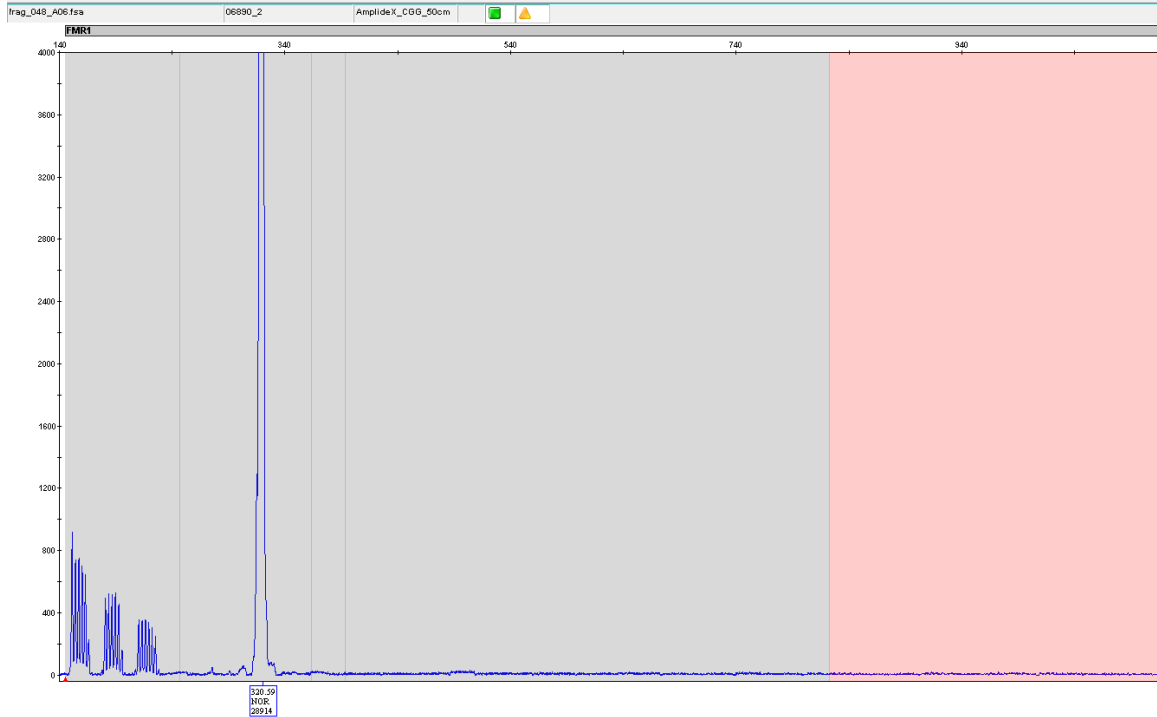confidence that this method worked accurately.

Figure 4: *Display plot for 06890_2 used with the REPLI-g amplification.*

Method #4: Single Cell Lysis and Targeted by Pre-Amplification of Specific FMR1 Gene

During the last and final amplification method, a lot of trial and error occurred. To

ensure the FMR1 primer that was created would anneal outside our desired region, NGS

using the PGM was conducted. The sequencing results can be observed in figures 5A and

5B. Figure 5A shows the presence of the forward FMR1 primer that was created; while

figure 5B shows the presence of the reverse FMR1 primer. As discussed in the materials

and method section, the number of cycles conducted for the pre-amplification step was

altered many times. The number of cycles began at 18 and was increased by 6 cycles.

After every 6 cycles, the Amplidex PCR and CE was conducted to observe if any peaks

would be detected in the display plot. No peaks were observed until the samples

underwent a total of 42 cycles and those results can be seen in figure 6. The signal

intensity of the peak is very low, but still remains above the cut-off threshold for the

3730xl DNA Analyzer. In order to not waste lysates from the FX cell lines, a lysate from

a control aneuploid cell line 1359 that was available was used until a peak was detected.
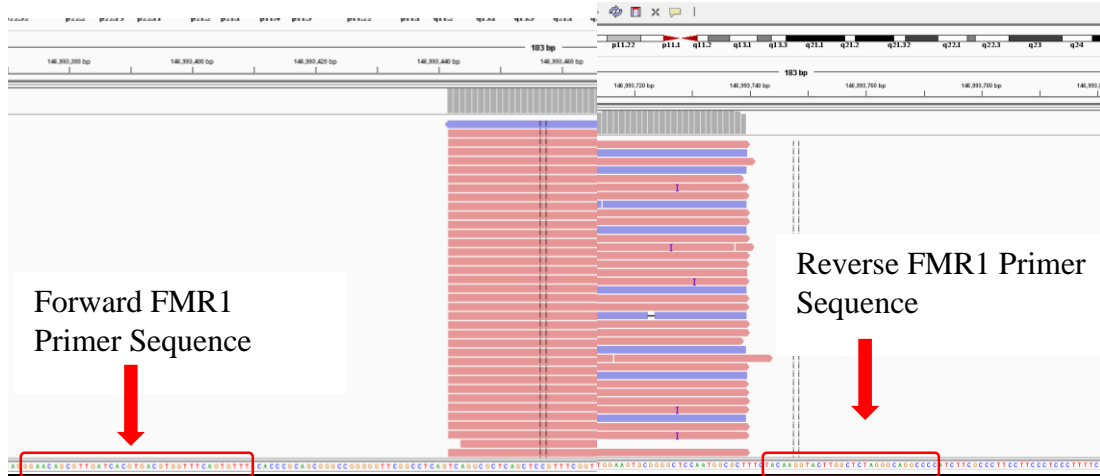

Figure 5A*: NGS results confirming the presence of the forward FMR1 primer created.*

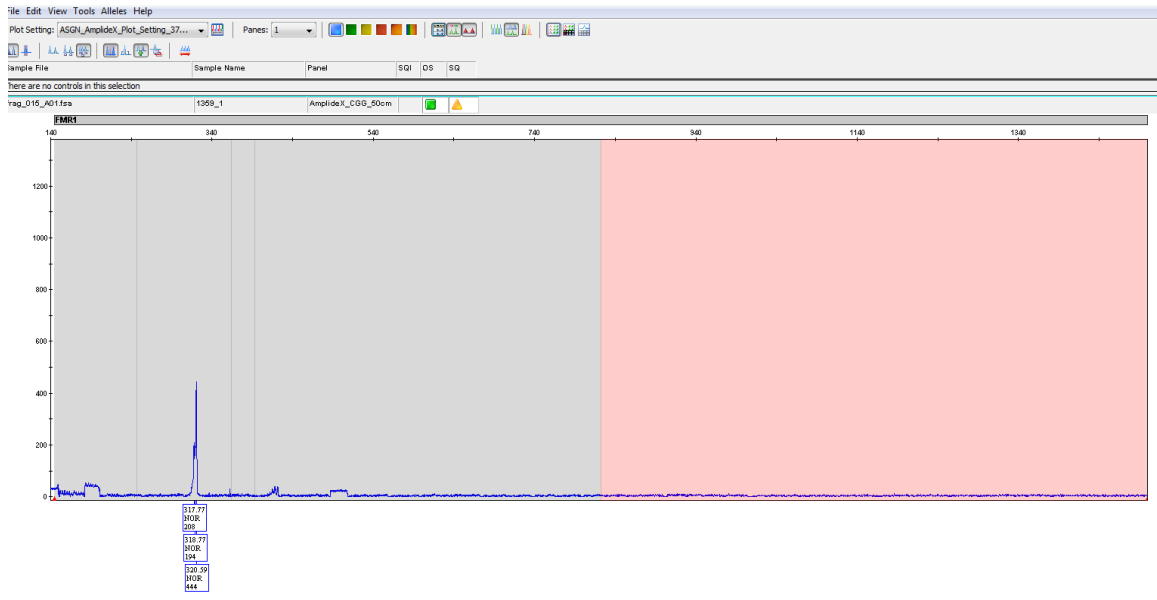***Figure 5B:*** *NGS results confirming the presence of the reverse FMR1 primer created.*

Figure 6*: Display plot of only peak displayed with targeted amplification.*

When 42 cycles of pre-amplification were conducted on samples from the cell

lines the results look abnormal. There are too many peaks that reach above the gene-

specific signal intensity threshold. All 6 replicates from each of the 6 cell lines exhibited

similar results. From these results, the decision was made to move forward onto phase 3

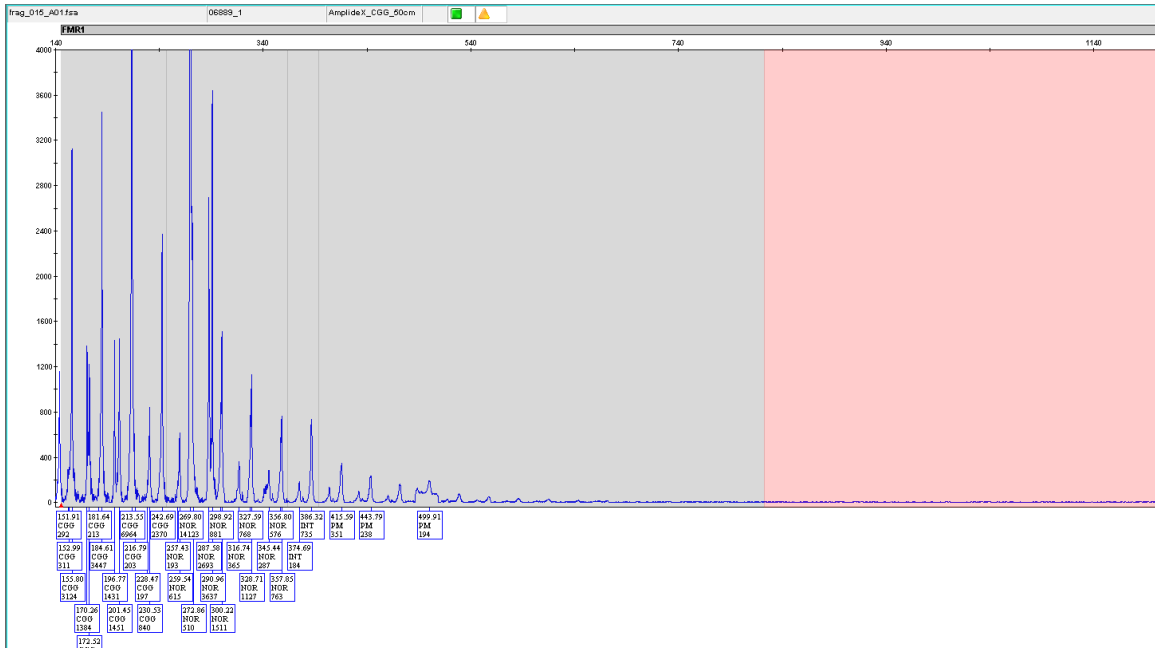using the REPLI-g Single Cell Kit for amplification.

Figure 7: *Display plot of samples with IDT FMR1 primer and 42 cycles of preamp.*

Continuation of Confirmation using REPLI-g Single Cell Kit for Amplification

To evaluate the method further, the cell line samples were blinded and then run

through the same steps as previously described. The samples were de-identified and

given new ID's before the amplification using the REPLI-g kit began. The same process

was followed and results are shown in figure 8. Two peaks within the normal range, with

satisfactory signal intensity, were observed in the electropherogram. Therefore, blinding

the samples allows for further evaluation of the methodology when the expected results

are unknown.

Figure 8: Display plot for Sample ID #26.

Phase 3:

Progressing onto phase 3, the REPLI-g Single Cell Kit for amplification was

chosen for continued use throughout this project. First, the Single Cell Lysis method

needed to be tested to observe if it works concurrently with the REPLI-g Single Cell Kit.

Therefore, 2 more replicates from each cell line were used to test whether or not the

single cell lysis protocol will work successfully with the REPLI-g Amplification system

instead of the lysis method recommended by Qiagen for the REPLI-g kit. It was observed

that the lysates worked when they underwent the REPLI-g amplification, the Amplidex

PCR and the CE. This step is necessary because the arrested embryos, used later in phase

3, are stored as lysates.

Use of Arrested Embryos with Methodology

The next step was to involve the use of arrested embryos from patients seeking

IVF for FXS. These embryos were treated identically to the cell lines. The samples were

previously lysed, therefore the first step involved undergoing the REPLI-g amplification.

The samples then underwent the Amplidex PCR and ended with CE on the 3730xl DNA

Analyzer. 10 out of 12 of the samples produced reliable results. Figure 9 exhibits the

display plot for one of the arrested embryo samples that displayed good signal intensity.
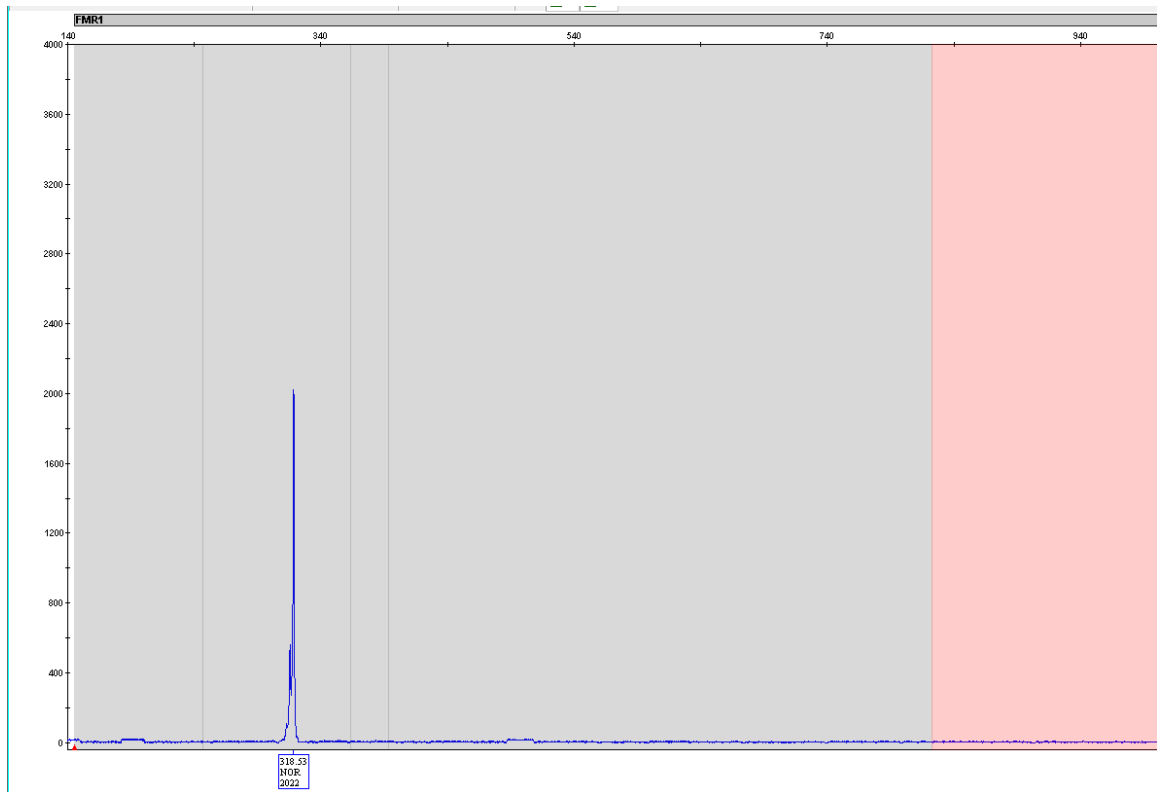
Figure 9: *Display plot for arrested embryo 2 replicate C.*

Two samples did not produce any signal to be detected by the CE. A question

arose if those two samples had any commonalities between them. During this time the

samples underwent genotyping to determine the gender of each; while simultaneously

comparing the results to the embryo quality. Two different gender assays were used to

confirm the proper call for each arrested embryo. Figure 10A presents the gender assay

results for the assay AMELXY. If the samples exhibited the VIC probe with a blue dot,

then the samples were female. If the samples exhibited the FAM probe with a red dot,

then the samples were determined as male. There are some samples that showed up as heterozygous for both and labeled with a green dot. Those were determined to obtain over amplified male gDNA. In figure 10B, the results for the ANAACKX gender assay differ from AMELXY assay. For ANAACKX, the female samples are labeled red for the FAM probe and the male samples are labeled green for being heterozygous for both VIC and FAM. There were some male samples again that over amplified again and those are labeled blue for the VIC probe. Table 4 displays the gender results for each arrested embryo used compared to the embryo quality. The embryo quality is defined as the cell stage of an embryo when it arrested.

Table 4*: Demographics of arrested embryos.*

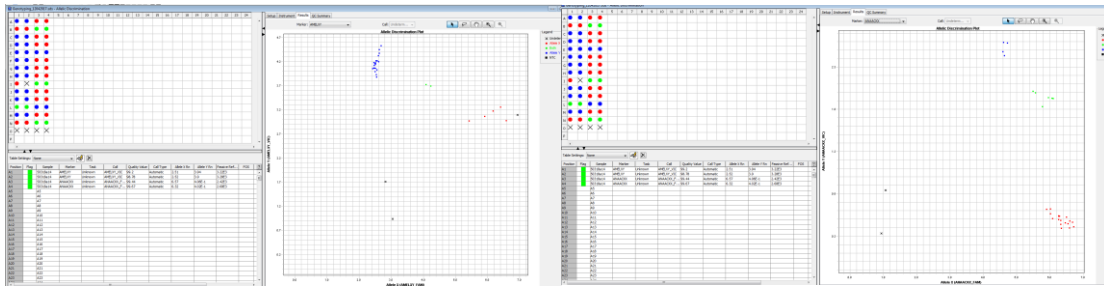| Sample ID	Embryo Quality	Gender	Repeat Size
50318a14	5 cell	Female	32;38
55138a1	Morula	Male	30
55138a23	6 cell	Female	32;39
55970a7	8 cell	Female	31;60
55970a10	8 cell	Male	30
55970a12	Morula	Female	30;31
42619a3	8 cell	Female	30;30
42619a8	Morula	Female	x
42619a9	8 cell	Male	x
49053a6	Morula	Female	28;29
49053a7	8 cell	Female	28;28
49053a8	5 cell	Male	29

Figure 10A: *Genotyping results for gender assay AMELXY.*   **Figure 10B**: *Genotyping results for gender assay ANAACKX.*

Phase 4:

Use of TE biopsies with Methodology

      Once retrieved from long-term cryo-storage, the whole embryos were biopsied into small clinically approved sizes. It was unclear how many cells were in each biopsy, but the operator attempted to make each biopsy around the same size. The use of biopsies from whole embryos marks the final stage of the project. These samples are identical to the samples that would be run using this methodology if it were to become clinically implemented. The whole embryos that were biopsies can be found in table 5.

Table 5: *The discarded aneuploid whole embryos used for the final phase.*

Embryo #	Ploidy Status	PCR 24 Results	FX Affected?	Gender
1	Aneuploid	47,XX,-1,+3,+10	yes	F
2	Aneuploid	47,XX,+10	no	F
5	Aneuploid	47,XY,+2	yes	M
4	Aneuploid	47,XXX	Inconclusive (XXX)	F
3	Aneuploid	45,XX,-9	no	F

      The CCS method of these embryos at the time of their fertilization was PCR 24, which is a qPCR based method for aneuploidy screening. Therefore, simultaneously the

DNA from the biopsies of these embryos was also run on NGS to compare the CCS

results. Below in figure 12, are the chromosome copy number plots for both qPCR and

NGS. The qPCR method displays the chromosome number along the x-axis and the

chromosome copy number along the y-axis. Each number, meaning 0, 1, 2, or 3, has a

threshold that needs to be reached in order for the algorithm to detect it. For the example

in figure 11A, this karyotype was determined as 45, XX, -9. Comparatively, the NGS

algorithms are a bit different. They display each amplicon and its copy number for each

chromosome. Along the x-axis is the chromosome number and along the y-axis is the

copy number. In figure 11B, there is an extra plot for NGS analysis that exhibits the

alternative allele fraction for each amplicon as well. Allele fraction can be defined as the

percentage of a sample represented by an allele.
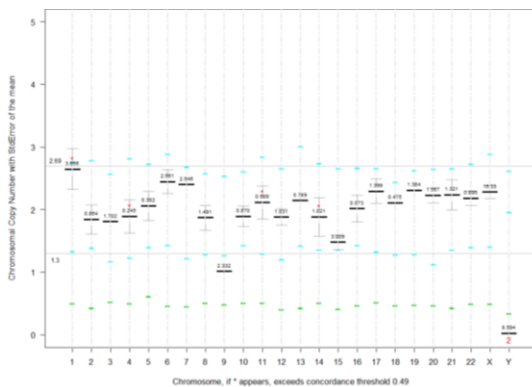
Figure 11A: *qPCR chromosome copy number plot.*

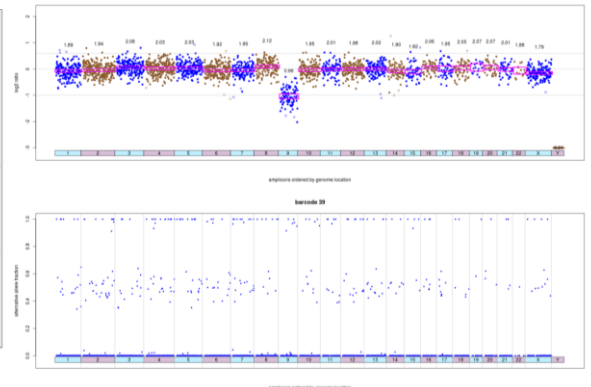*Figure 11B*: *NGS chromosome copy number plot.*

After running the REPLI-g amplification, the Amplidex PCR, and the CE, the

results for the biopsies were as expected. In figure 12, the display plot presents a peak

within the normal range and a peak within the premutation range for this female embryo.

This plot exhibits the accuracy of our method because the peaks show quality signal

intensity.

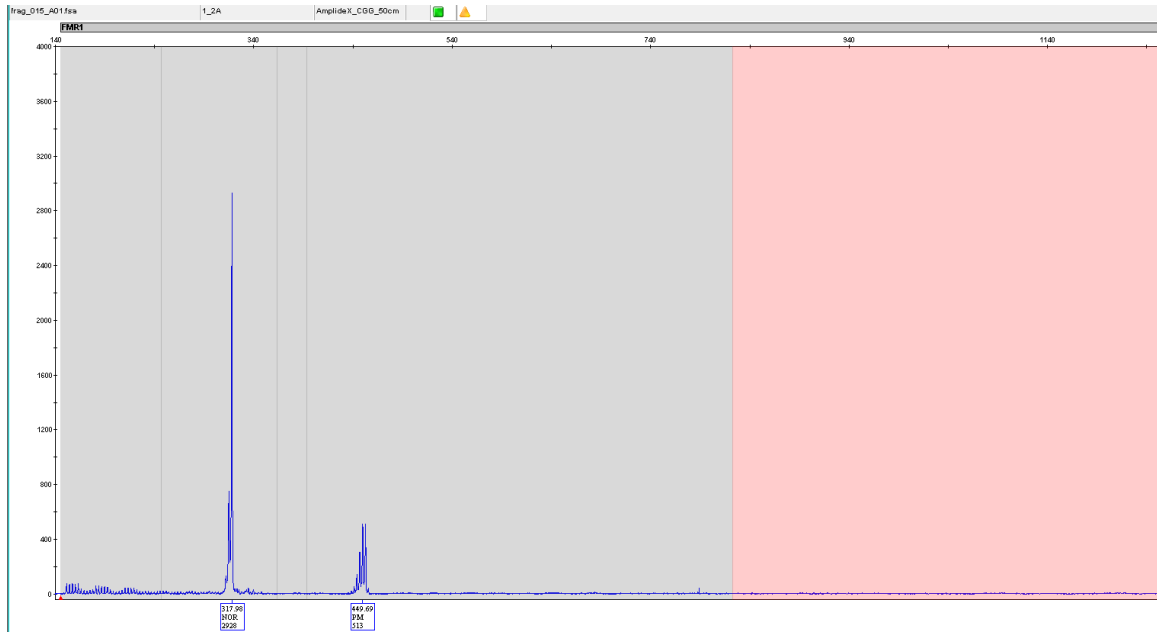***Figure 12:*** *Display plot for embryo 1*

Overall, table 6 briefly displays a comparison of demographics of the aneuploid whole embryos used. These demographics include NexCCS (NGS based CCS), which proved to be a more sensitive method for CCS, and the inheritance patterns of their alleles. Data within the highlighted light blue section of the table was determined throughout the last phase of this project.

Table 6: Overall comparison of the aneuploid whole embryos used

Embryo #	Gender	PCR 24 Results	NexCCS Results	FX Affected?	CGG Repeats	Maternal Repeat Size	Paternal Repeat Size
1 - A	F	47,XX,-1,+3,+10	46,XX,-1,+10	yes	29;74	29;58	29
1 - B	F	47,XX,-1,+3,+10	46,XX,-1,+10	yes	29;74		
2 - A	F	47,XX,+10	46,XX	no	29;29		
2 - B	F	47,XX,+10	46,XX	no	29;29		
3 - A	F	45,XX,-9	45,XX,-9	no	32;36	32;149	36
3 - B	F	45,XX,-9	45,XX,-9	no	32;36		
4 - A	F	47,XXX	46,XX	Inconclusive (XXX)	32;37		
4 - B	F	47,XXX	46,XX	Inconclusive (XXX)	32;37		
5 - A	M	47,XY,+2	46,XY	yes	>200	31;99	Needs Testing
5 - B	M	47,XY,+2	46,XY	yes	>200		

DISCUSSION

In this study, the development and validation of a method to detect FX CGG repeats within the FMR1 gene of embryo TE biopsies has progressed remarkably for reproductive competence within IVF patients seeking SGD testing for FXS. Proper validation began with the use of gDNA from samples with known repeat sizes. The gDNA was isolated from different sample types; such as blood, buccal swabs, and villi. It was determined that gDNA isolated from multiple samples types can undergo this methodology and will produce desirable results.

Finding an amplification method is necessary in this study because the small input of DNA provided from the limited number of cells in a TE biopsy cannot be detected without it. Four different amplification methods were tested to amplify the minute amounts of initial DNA. The first method, using the GenomePlex WGA4 Amplification kit, did not produce any results when the CE was run. No peaks were detected during the

analysis because the GenomePlex WGA4 amplification method involves random primer annealing. It is uncontrollable which region gets amplified. Additionally, there is only about a 60% coverage for WGA products and allele drop out is bound to occur. The amplicon produced by WGA is only around 400-500 base pairs; therefore, if the FMR1 amplicon falls outside this range it will not be detected by the CE. Because the FMR1 amplicon can consist of over 200 CGG repeats at times, the expected amplicon would be over 600 base pairs which would not work for this particular method. The same scenario occur for method number 2 using the SurePlex DNA Amplification system. No peaks were detected using the method as well for similar reasons as previously discussed.

The next tested method involved a targeted pre-amplification of the FMR1 gene with uniquely made primers. A lot of trouble shooting occurred for this methodology. Validation tests needed to be done to ensure the primers were outside the desired amplicon region before beginning. NGS was one of those validation steps and figure 5A and 5B confirm that our FMR1 primer is outside the FMR1 region. However, the signal intensity of the peak is very low, unlike the signal intensity for peaks of gDNA. Additionally, multiple tests needed to be run to determine how many cycles were necessary for this pre-amplification step. It took 42 cycles for a peak to be detected after running the CE. Nevertheless, a peak was detected only once for the practice samples of the aneuploid control cell line 1359. Use of this kit was attempted on the samples picked from the FX cell lines. It resulted in the presence of too many peaks being displayed. This could be the result of too much amplification, contamination, or the presence of more than 6 cells initally. It was disappointing this method did not produce desirable results because this protocol is done concurrently with the present CCS method for embryo TE

biopsies. Therefore, no additional biopsies will be needed from embryos that elect to undergo SGD testing for FXS.

Lastly, the REPLI-g Single Cell Kit for amplification was tested. Although this method is also a WGA method, it is additionally an MDA method. MDA methods are very effective when the production of long fragments is necessary. Each enzyme remains bound and continues replication of a single template for up to 1,000 kilo bases. The specific enzyme used is Phi29 DNA polymerase, which has very low error rates due to its 1000 fold higher fidelity than taqDNA polymerase. There are fewer enzyme binding events; therefore, less opportunities for certain templates to be over or under-represented. The high affinity for use on small starting material and good processing is necessary for generating the longer fragments. There are also only 1-2 steps during the preparation of the library, which reduces the chance DNA loss by having too many intermediate steps. The REPLI-g Single Cell Kit for amplification produced desirable results, as seen in figure 4. For extra confirmation of the ability of this method, 48 samples were collected and de-identified. Knowing which cell line each sample came from causes some bias when conducting the data analysis. Blinding the samples helps remove that bias from the study. This method was chosen to advance onto phase 3 to see if it could work on more realistic samples; such as arrested embryos and biopsies of whole embryos.

To begin phase 3, additional tests were conducted to ensure the single cell lysis method works concordantly with the REPLI-g amplification method. After many test runs, it was observed that it worked accurately. These tests were done because the arrested embryos used in this phase were already lysed using that method. Unfortunately, because the embryos arrested before the blastocyst stage, there was no PGD for FX

conducted. Therefore, when the testing for this project occurred, there was no way to confirm our results. However, the parental repeat sizes were determined and used to observe which allele was inherited by each embryo. 10 out of the 12 arrested embryos used produced good results after going through the developed methodology. The 2 samples that did not work produced no signal when undergoing the CE. After conducting the genotyping for gender and observing embryo quality, there were no commonalities between the 2 samples that did not produce any signal.

Whole embryos were then biopsied to provide this project with the most realistic sample type to test the methodology. 2 replicates from each of the 5 biopsied aneuploid whole embryos were used to confirm the use of this methodology on clinical samples. Because these samples are discarded aneuploid whole embryos, they underwent PGD. The standard CCS method used for these embryos was PCR24, a qPCR based method of CCS for aneuploidy screening. Currently, the standard method for CCS testing changed to NexCCS, a NGS based method. A comparison was done to observe the differences between the 2 methods, seen in table 6. Fortunately for this clinic, the current method of CCS using NexCCS is the more sensitive method. After review of the FX results for the biopsies, in figure 12, the correct number of peaks and repeat sizes were determined for each of the samples. It gave great confidence that this method may be clinically implemented in the future.

Overall, there were many validation and preparatory steps taken to ensure this methodology works accurately. Further investigation of validating this platform could involve de-identifying the discarded aneuploid whole embryo biopsies to confirm the CGG repeat sizes and the sensitivity of the developed method. More experiments with a

larger sample size are necessary before transitioning this methodology to be clinically implemented.

The developed method can aid female patients who seek IVF treatment for SGD testing of FX in many different ways. Women who inherited a premutation FX allele are at risk for FXPOI and can benefit greatly by this new method. These patients may have difficulty reproducing because of FXPOI; but, using this method they can ensure that their affected allele does not get transmitted to their offspring. Similarly, confirming no expansion has occurred if the affected allele was transmitted. Another way this method benefits female patients is if they are of advanced maternal age. Women of advanced maternal age also have difficulty reproducing because they may not have the ability produce embryos that survive through to the blastocyst stage. There is a higher rate of aneuploidy that is present in the eggs of women of advanced maternal age. Now, factor in the chance that these older women also have a premutation FMR1 allele. They would not be able to produce many embryos; while also needing to worry about transmitting their affected FMR1 allele to their offspring. If a woman is a carrier of a premutation FMR1 allele has two embryos available for transfer, where one is euploid but inherited her FX affected allele and the other is aneuploid, this newly developed test can help. Instead of discarding the euploid embryo just because it inherited the affected X chromosome, this new test can determine the exact number of CGG repeats present on that affected X chromosome. Therefore, if little to no expansion occurred that embryo could be a candidate to be transferred and save the embryo from being discarded.

Physicians can also benefit from this developed method because they can provide similar patients with proper counseling needed to go through the strenuous process of

IVF. Transferring embryos with FMR1 premutation alleles will encompass more diligent genetic counseling and detailed consents. This newly developed method to detect FX CGG repeats within the FMR1 gene of embryo TE biopsies can help save many embryos that may be suitable for transfer from being discarded; while simultaneously further the field of reproductive competence.

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