



Identification of the W1282X-CFTR SNP Mutation in CF Patient Cells via AS-PCR

SunYoung You

Michigan State University

Mallory Bergmann

Michigan State University

Jessica Lessel

Michigan State University

Makayla Hart

Michigan State University

Alexandria Belyue

Michigan State University

Eva Conley

Michigan State University

Alexa Baker

Michigan State University

Sydney Nguyen

Michigan State University

Laurence Newmeyer

Michigan State University

Joseph Romanelli

Michigan State University

Douglas Luckie

Michigan State University

ABSTRACT

The W1282X cystic fibrosis transmembrane conductance regulator (CFTR) gene mutation causes a severe form of cystic fibrosis which accounts for 1.2% of the CF worldwide. This single nucleotide polymorphism (SNP) CFTR mutation is located on exon 20, consisting of a change from guanine to adenine at the 3846th base pair, creating a premature stop codon. The purpose of this study was to develop a polymerase chain reaction (PCR) based diagnostic assay to identify the W1282X mutation using allele-specific primers. We hypothesized that the Yaku method,

which introduces an additional intentional base pair mismatch, would reduce non-specific binding in allele-specific PCR (AS-PCR). To increase accuracy, we also employed a “nesting” amplification approach. A published reverse primer (PRP1) and a universal forward primer (UFP1) were designed to first amplify a 718 bp “template” region. After which, a published forward primer (PFP1) and Yaku reverse primers (RPMT1 and RPWT1) were paired to produce a 316 bp region diagnostic of the presence of either mutant W1282X-CFTR or wild-type CFTR in DNA samples. PCR products were analyzed via agarose gel electrophoresis, revealing expected bands of approximately 316 base pairs, supportive of successful W1282X detection with Yaku primers.

Keywords: cystic fibrosis, genetic diagnosis, SNP, PCR, gel electrophoresis.

INTRODUCTION

Cystic Fibrosis (CF) is an autosomal recessive disorder distinguished by the buildup of thick mucus in the lungs, digestive tract, and salty sweat from sweat glands [1]. There are thousands of documented mutations within the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene known to cause CF [2]. Typically, a healthy, non-mutated CFTR gene produces a CFTR protein that functions as a chloride ion channel, transporting chloride across epithelial cells [2]. A mutated CFTR gene, however, produces a misfolded, defective CFTR protein or fails to produce the protein entirely. Consequently, a buildup of thick mucus due to high chloride ion concentration, occurs within the pancreas and lungs, leading to persistent lung infections in CF patients [3]. Treatments for CF include but are not limited to antibiotics such as tobramycin or penicillin, postural drainage, physiotherapy, oral pancreatic enzymes, and more recently, correctors and potentiators [4, 5]. Sweat tests are commonly used to diagnose CF due to high levels of sodium and chloride in patients’ sweat [4]. In addition, polymerase chain reaction is used to diagnose CF, particularly in infants and adolescents, improving quality of life through early therapeutic and medicinal treatment [6]. Polymerase chain reaction (PCR) is a technique developed to amplify specific DNA sequences [7]. The PCR process replicates a segment of genomic DNA utilizing forward and reverse primers which mark the ends of the desired region of amplification [8]. In a single thermal cycle of PCR, the sample is heated up to 95°C to denature and separate dsDNA in lieu of the role of helicase used in vitro DNA replication, breaking the hydrogen bonds holding the DNA strands together [9]. The temperature then drops to around 50-60°C, allowing primers to anneal to complementary DNA sequence sites by forming hydrogen bonds to single strand DNA template [9]. In the final step, the temperature rises to 72°C to enable a thermostable DNA polymerase to replicate the segment of DNA between primers [9]. When the cycle is repeated multiple times, the exponential DNA replication produces large amounts of target DNA (i.e. 30 cycles yield over 1 billion copies) [10]. Gel electrophoresis is frequently performed to visualize and analyze the PCR products [11]. Since DNA is negatively charged as a result of the presence of phosphate groups, an applied electrical field moves DNA fragments through the gel matrix separating them by size [12].

This study focused on designing a PCR diagnostic assay for the W1282X mutation, a nonsense mutation that causes CF [13]. The W1282X-CFTR mutation is prominent in those who are of Ashkenazi Jewish descent and the second most common CF-mutation in the general population [13, 14]. The W1282X mutation is caused by a single nucleotide point or polymorphism (SNP), leading to a transition of guanine to adenine, at the 3846th base pair in exon 20. The codon TGG is changed to a stop codon, TGA, causing translation to terminate prematurely [15].

This study also aimed to investigate if the Yaku, and other methods, could improve the identification of SNPs via PCR. Implementing the additional intentional base pair mismatches at the 3' end of the primer has been found by Yaku et al to reduce non-specific binding and amplification in AS-PCR versus SNPs [16]. This better permits traditional PCR techniques to be used for effective detection of the point mutations by preventing PCR primers from binding to inappropriate non-complementary template sequences [16].

METHODS

Source of Materials

Unless otherwise indicated, chemicals were obtained from Sigma-Aldrich. Oligonucleotide DNA primers for polymerase chain reaction were purchased from IDT. Taq Polymerase enzyme and buffer (10X) were obtained from Syd Labs. The 100 bp DNA MW ladder and agarose were obtained from Gold Biotechnology. A 1 kb DNA Ladder and SYBR-Safe™ DNA gel stain were sourced from Invitrogen. A gel loading dye was sourced from New England Biolabs. A 20X LB (lithium borate) electrophoresis buffer was obtained from Faster Better Media. IB3-1/S9 cells were purchased from ATCC. Wild-type buccal cells were obtained from human subjects.

DNA Purification

Chelex-100 resin was used to extract genomic DNA from cell samples. Each sample of cells was first concentrated by centrifuging 1.5 mL at 14,000 rpm for 2 minutes until a cell pellet was formed. The supernatant was removed and 500 µL of 10% Chelex-100 resin solution was added cell pellet and vortexed until the pellet was resuspended. Each sample was incubated at 56°C for 10 minutes and vortexed for 5 seconds every 5 minutes. After 10 minutes, the sample was incubated at 95°C for 5 minutes and the solution was centrifuged at 14,000 rpm one final time for 5 minutes to form a pellet of resin. The supernatant containing purified DNA was separated from the resin with cell debris and stored at -20°C until use. To measure nucleic acid concentration and purity of DNA samples, a 260/280 analysis was performed with a Thermo Scientific NanoDrop Lite - UV Spectrophotometer.

Cell Culture

S9/IB3 is a stable cell line with a W1282X-CFTR allele. S9 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/mL penicillin, and 10 µg/mL streptomycin.

Primers

Allele-specific primers were designed following the Yaku method of introducing intentional base pair mismatches on the third nucleotide from the 3' end [16]. Two reverse allele-specific primers, RPWT1 and RPMT1, were designed, along with a universal forward primer (UFP1). RPWT1 was a wild-type seeking allele-specific reverse primer: 5'-ATCACTCCAAAGGCTTT CATC-3' (21 bp)—cytosine (C) on the last nucleotide at 3' end annealed to guanine (G) on the wild-type DNA sequence. RPMT1 was a mutant seeking allele-specific reverse primer: 5'-ATCACTCCAAAGGCTTTCA~~TT~~-3' (21bp)—thymine (T) on the last nucleotide at 3' end annealed to adenine (A) on SNP mutation site at 3846th base pair on exon 20 of W1282X mutant CFTR gene sequence. The third nucleotide from the 3' end in both reverse primers was designed to never anneal to both mutant or wild-type strains to create two intentional mismatches [16]. In both primers, a cytosine (C) was replaced with adenine (A) so as to not anneal to guanine (G).

UFP1 was a universal forward primer with the sequence of 5'-TACTCATTCACTGAGGA GTGG - 3' (21bp), which was designed to anneal at a site 718 base pairs upstream.

As a PCR-positive control, the Rz gene of the Lambda bacteriophage genome was amplified using Rz gene primers. 1Rz1F was a Rz gene forward primer: 5'-GATGTATGAGCAGAGTCA CCGCGAT-3' (25bp) and 1Rz1R was a Rz gene reverse primer: 5'-GAGGGTGAATAATCCC GTTCAG-3' (23bp) [17]. As another positive control, published primers were used. PFP1-outer was a published forward primer: 5'-GGTCAGGATTGAAAGTGTGCA-3' (21bp) and PRP1-outer was a published reverse primer: 5'-CTATGAGAAAAGTCACTGGA-3' (21bp) [18]. PFP1-inner was another published forward primer: 5'-TACCTATATGTCACAGAAGT-3' (20bp) and PRP1-inner was another published reverse primer: 5'-GTACAAGTATCAAA TAGCAG-3' (20bp) [18]. The annealing temperatures for the primers were calculated using the theoretical melting temperature formula:

$$T_m = 64.9^{\circ}\text{C} + 41^{\circ}\text{C} \times (\text{number of G's and C's} - 16.4) / N$$

where N was the length of the primer [20]. The calculated melting temperatures for primers were RPWT1- 50.45°C, RPMT1- 48.50°C, UFP1- 49.73°C, IRz1F- 59.32°C, IRz1R- 55.27°C, PFP1 52.40°C, PRP1 50.45°C. Initial annealing temperatures were tested at 3-5°C below the melting point. Due to this, an annealing temperature of 3°C below the UFP1, 46°C, was first used for all primers excluding the Rz gene and published primers which were 51°C and 50°C, respectively.

Polymerase Chain Reaction

For each individual test, ingredients for PCR included 5µL 10x PCR buffer (containing 200mM Tris-HCl and 500mM KCl), 1µL 10mM dNTPs (deoxynucleotide triphosphates), 1µL of 1µg/ µl DNA template, 1µL of 5U/µl *Taq* polymerase, 1µL 100µM forward primer, 1µL 100µM reverse primer, 1.5µL 25 mM MgCl₂, and 38.5µL dH₂O (deionized water). PCR cocktails were incubated in a Bio Rad T100 thermocycler. PCR protocols varied where indicated but generally had an initial denaturation at 95°C for 3 minutes, then cycled for 30 times of denaturation at 95°C for 30 sec, varying annealing temps for 1 min, and extension at 72°C for 1 min, then followed by a final extended elongation at 72°C for 5 min. The annealing temperature of 53°C was used for published Rz gene primers versus lambda viral genome [17]. For the published primers, Drury et al protocols were followed [18].

Nested Polymerase Chain Reaction

To increase specificity, a two-step amplification process was performed. The first-run PCR consisted of the designed UFP1 paired with PRP1 from Drury, *et al*. The second-run PCR utilized PFP1 and RPMT1 primers. First-run PCR cocktails consisted of template DNA of either purified wild-type CFTR genomic DNA extracted from buccal cells or isolated mutant W1282X-CFTR genomic DNA from S9 cells. After first-run amplification, 5µL of the first-run PCR products were added to the second-run cocktail mixture, serving as DNA template. Unless indicated otherwise, first-run PCR started with an initial denaturation at 95°C for 12 minutes followed by 30 PCR cycles comprised of denaturation at 95°C for 30 seconds, annealing temperature for 30 seconds, and elongation at 72°C for 45 seconds followed by a final elongation at 72°C for 12 minutes. The second-run PCR began with an initial denaturation at 95°C for 12 minutes, followed by 35 PCR cycles comprised of denaturing at 95°C for 20 seconds, annealing for 45 seconds at a

temperature gradient for the mutant target, and 46 °C for the wild-type target, elongation at 72°C for 45 seconds, completed with a final elongation at 72°C for 12 minutes.

Gel Electrophoresis

A 1% agarose gel was used to perform gel electrophoresis. The materials included 0.5g of agarose, 2.5 ml of LB 20x (5mM lithium, 10mM borate) buffer, 47.5 ml of dH₂O, and 5μL of 10,000x SYBR-Safe™ dye. The mixture of dH₂O, 20x LB buffer, and agarose powder was warmed in the microwave until dissolved. After the mixture was cooled, SYBR-Safe™ dye was added, and the gel cast. A 350 ml volume of 1X LB buffer solution was made by mixing 17.5 ml of 20x LB buffer with 332.5 ml of dH₂O and poured into the electrophoresis chamber to submerge the gel. 6X loading dye containing 30% glycerol, 0.25% (0.025g/10ml) bromophenol blue, and 0.25% (0.025g/10ml) xylene cyanol was used. The gel electrophoresis was run at 150V for 30 minutes. The gel was analyzed and photographed under UV light.

Data Analysis

The data for gel electrophoresis was analyzed by comparing the migration of bands in the molecular weight ladder to those of PCR products using a semi-log plot. The gel electrophoresis was analyzed using the migration distance of the bands to calculate base-pair length. For the molecular weight ladder, the graph compared the distance traveled, well to product, on the x-axis while a PCR band's known size was on the y-axis, exponentially. A trendline was added and R² value was obtained. The trendline equation was used to calculate PCR product base-pair lengths.

RESULTS

In this study, a novel strategy was applied to diagnose SNP mutations. To detect the presence of W1282X allele of CFTR in samples by implementing the Yaku method, a PCR primer seeking wild-type CFTR gene sequence (RPWT1) was specifically designed to have an additional intentional mismatch by placing a cytosine nucleotide at 3' end and similarly for the PCR primer for mutant type (RPMT1) to have a thymine nucleotide at 3' end (Figure 1). Thus, in this AS-PCR assay, RPWT1 was designed to only anneal to wild-type DNA and RPMT1 to only anneal to mutant-type DNA in the experiment (Figure 2).

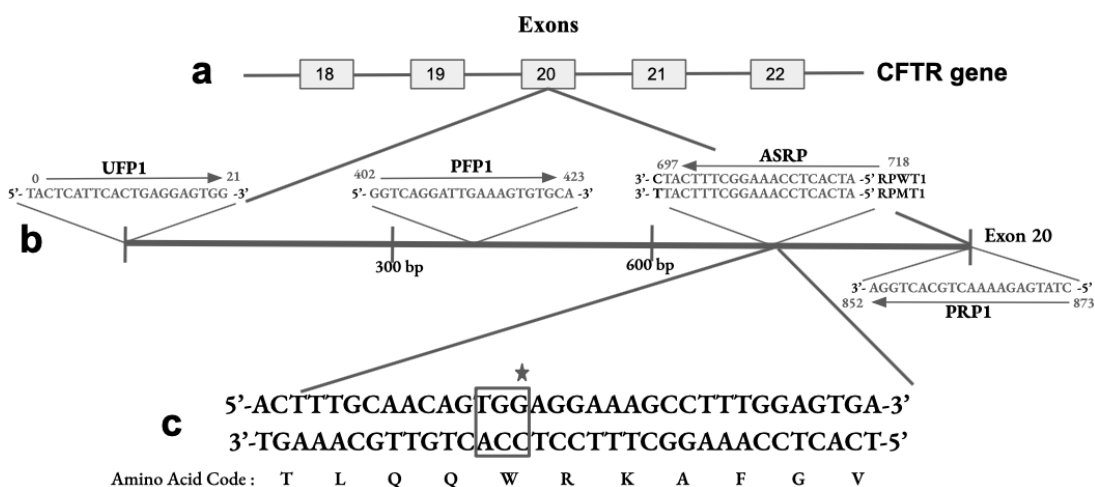


Figure 1: CFTR gene region containing the W128X mutation targeted by primer annealing sites.

(a) Represents a portion of CFTR gene region on chromosome 7 including exons 18 through 22. **(b)** Displays expanded view of exon 20. Published forward (PFP1) and reverse (PRP1) primers anneal to two locations—one from bp 402 to 423, another from bp 852 to 873—on exon 20 bordering the mutation point. The arrows shown alongside the primer sequences indicate elongation direction, and the numbers at the front and back of the arrows show the base pair position in exon 20. Yaku allele-specific reverse primers (ASRP) for wild type (RPWT1) and mutant CFTR (RPMT1) anneal to the same location with differences in sequence at the first and third nucleotide of the 3'-end. UFP1 is used as a universal forward primer. **(c)** Presents the wild-type DNA sequence which includes the W1282X mutation site shown in a box. The letters below the DNA sequence indicate the code of each amino acid for every codon, and the code W is where single nucleotide polymorphism (SNP) point mutation resulted in altering TGG to TGA.

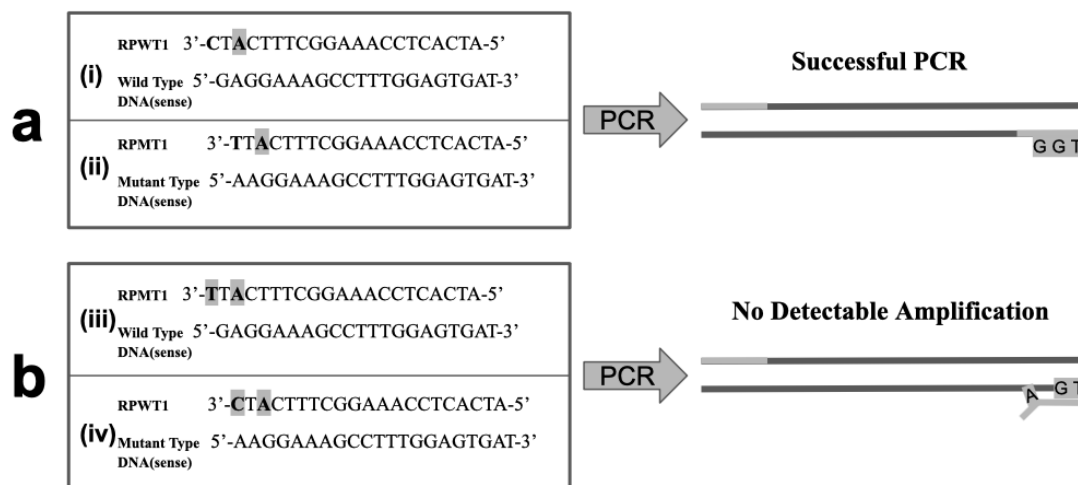


Figure 2: Design of Yaku allele-specific annealing and amplification during PCR.

The Hidenobu Yaku method was applied in the design of allele-specific primers for a PCR-based diagnostic assay. To reduce non-specific binding, in addition to acute mismatches that will occur at the mutation site, where the matching complement is C for wild type and T for mutant type, an additional intentional chronic mismatch was also introduced at the third nucleotide near the 3'-end. **a.** When Yaku primers attempt to anneal to the appropriate target genome the single mismatch does not prevent annealing and extension during PCR. In (i) and (ii) reverse primers that seek wild-type (RPWT1) and mutant (RPMT1) CFTR target sequences lead to successful PCR extension when facing the appropriate wild-type and mutant DNA sequences, respectively. **b.** Conversely, when Yaku primers attempt to anneal to the inappropriate target genome, the double-mismatch in fact interrupts annealing at 3'-end base positions #1-2-3 via significant steric hindrance and leads to no detectable amplification during PCR. In (iii) and (iv) when mutant-seeking primer (RPMT1) faces wild-type DNA template, and wild-type seeking primer (RPWT1) encounters mutant-type CFTR sequence, the amplification fails.

DNA Template: Purification of Buccal and S9 Cell DNA

Human genomic DNA was extracted and purified from buccal and S9 cells using a Chelex-100 resin protocol. Purified human genomic DNA samples were analyzed via UV spectrophotometry. Samples with an absorbance 260nm/280nm ratio between 1.75-1.79 were found and deemed appropriate for use in the described PCR studies.

PCR Controls: Amplification of Lambda Viral Genome with Control Primers

PCR controls were routinely used to assess the status of the reagents of PCR and the integrity of the CFTR locus in the purified template. The Rz gene of lambda virus template DNA was amplified by PCR using an annealing temperature at 53°C with Rz forward and Rz reverse primer sequences published in the literature [21]. PCR followed by gel electrophoresis yielded expected products (Figure 3a). A semi-log plot was used to examine the size versus migration distance of the PCR products. The X-axis represents the distance between the well and bands and the Y-axis represents the known molecular size. The R^2 value was 0.9983 and the trendline equation $y = 12165e^{-0.965x}$ (Figure 3b). The lambda distance traveled was 3.76cm and the calculated molecular size was 324bp. The expected band length was 395bp.

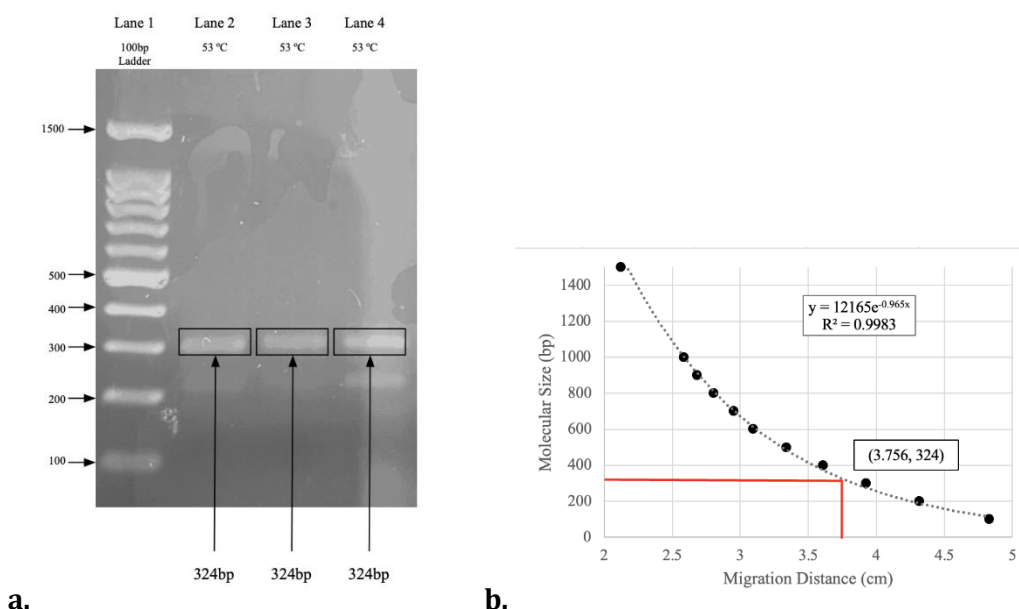


Figure 3: Control PCR studies using published RZ gene primers.

Rz gene segment was successfully amplified from commercially purified lambda viral template DNA using control published primers. **a.** MW 100bp ladder was present in Lane 1, Lanes 2-4 contained lambda PCR products using Rz1F and Rz1R primers versus lambda template. **b.** Determination of band size of designed Rz primer PCR product using migration distance. A semi-log plot was created using the 100bp ladder in Lane 1 which yielded the trendline equation of $y = 12165e^{-0.965x}$ and an R^2 value of 0.9983. Using the trendline equation, the calculated length of the bands in Lanes 2-4 is 324bp. The expected band length for the Rz gene primer PCR product is 395bp.

Yaku WT Primers: Amplification of Wild-type CFTR Template

Two AS-PCR primers were designed with the Yaku strategy to detect W1282X mutation. The Yaku primers were first evaluated versus human wild-type CFTR genome template. PCR products were amplified at a gradient annealing temperature of 48°C-51°C. Products were visualized by electrophoresis (Figure 4a). The gel visualized the products of a nested protocol PCR. The first-run contained UFP1 vs PFP1 PCR primers and the second-run contained PFP1 vs RPWT1 primers. The semi-log plot analysis yielded a trendline with an R^2 value of 0.9193 and

a $y = 12165e^{-0.965x}$ equation (Figure 4b). The distance between the well and the band of the product was 8.35cm. The calculated band length was 313bp (expected 316bp).

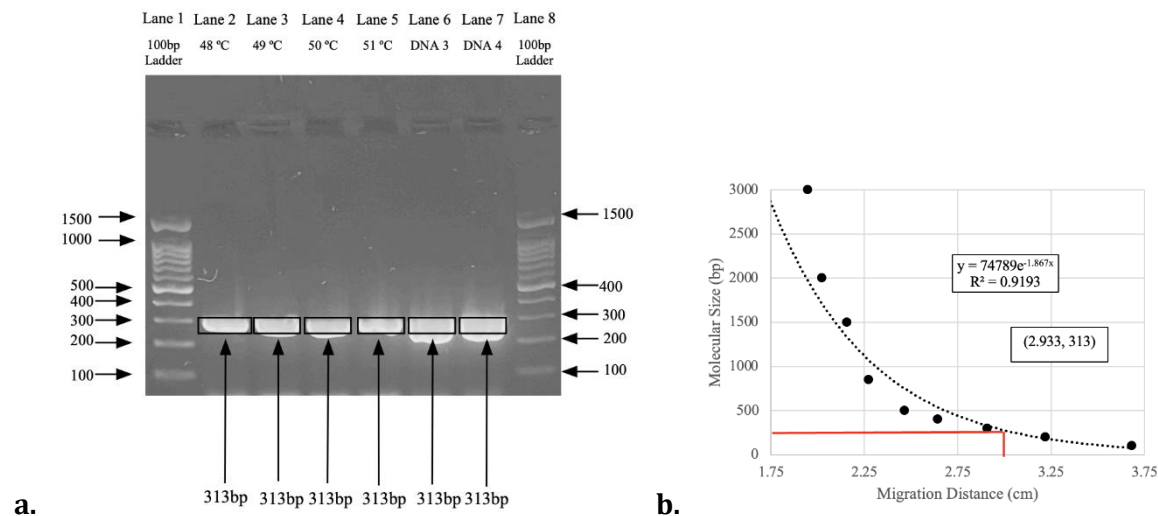


Figure 4: Designed 'Yaku primers' versus wild-type CFTR template in PCR.

PCR primers designed with the Yaku method were tested against human wild-type CFTR genome coupled with Drury et al nested protocols. **a.** PFP1 vs. RPWT1 coupled with an aliquoted nested product containing UFP1 versus PFP1. A 100bp ladder was present in Lane 1. Lanes 2-5 contained WT PCR products amplified using the nested protocol. Lane 2 PCR sample had an annealing temperature of 48°C, Lane 3 had 49°C, Lane 4 had 50°C, Lane 5 had 51°C. Lanes 6 & 7 contained different concentrations of DNA; Lane 6 with 3μL and Lane 7 with 4μL. Lane 8 contained a 100bp ladder. **b.** Determination of band size of designed Yaku primer (RPWT1) PCR product using migration distance. A semi-log plot created using the 100bp ladder in Lane 8 yielded the trendline equation of $y = 74789e^{-1.867x}$ and an R^2 value of 0.9193. Using the trendline equation, the calculated length of the bands in Lanes 2-7 is 313bp. The expected band length for the designed Yaku primer PCR product is 316bp.

Yaku MT Primers: Amplification of Mutant W1282X CFTR Template

PCR primers designed with the Yaku strategy were evaluated when facing mutant W1282X-CFTR genome template. PCR products were amplified with an annealing temperature gradient of 48°C-51°C. Expected products were yielded from PCR followed by gel electrophoresis. The PCR products containing first-run UFP1 vs PFP1 primers, followed by second-run PFP1 vs. RPMT1, were visualized in the gel (Figure 5a). A semi-log plot was used to examine the band size versus the migration distance. The semi-log plot analysis yielded a trendline with an R^2 value of 0.9967 and a $y = 8263.73e^{-1.17x}$ equation (Figure 5b). The distance between the well and the band of the product was 2.81cm and the calculated molecular size was 308bp. The expected band length was 316bp.

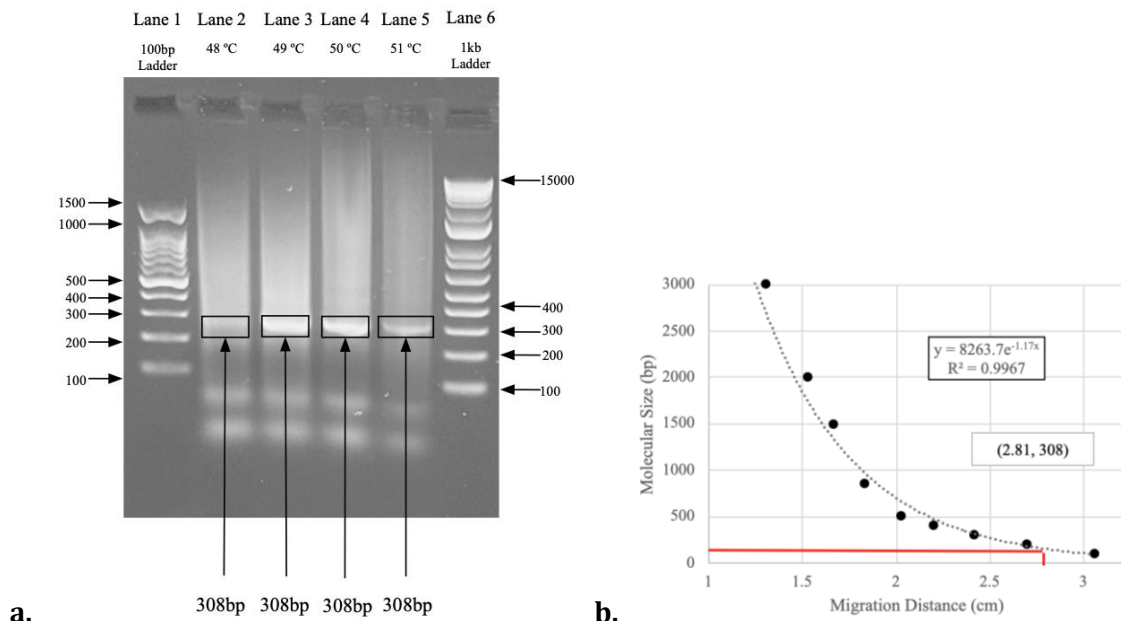


Figure 5: Designed 'Yaku primers' versus mutant-type CFTR template during PCR.

PCR primers designed with the Yaku method were tested against human mutant-type CFTR genome coupled with Drury et al nested protocols. **a.** PFP1 vs. RPMT1 coupled with aliquoted nested product containing UFP1 versus PFP1. A 100bp ladder was present in Lane 1. Lane 2-5 contained MT PCR product that was amplified using the nested protocol. Lane 2 PCR sample had an annealing temperature of 48°C, Lane 3 had 49°C, Lane 4 had 50°C, and Lane 5 had 51°C. Lane 6 contained 1kb ladder. **b.** Determination of band size of designed Yaku primer (RPMT1) PCR product using migration distance. A semi-log plot was created using the 1kb ladder in Lane 6 which yielded the trendline equation of $y = 8263.73e^{-1.17x}$ and an R^2 value of 0.9967. Using the trendline equation, the calculated length of the bands in Lanes 2-5 is 308bp. The expected band length for the designed Yaku primer PCR product is 316bp.

CONCLUSION

Cystic fibrosis, or CF, is an autosomal recessive disease caused by a defect in the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) protein [1]. Alteration of the expressed CFTR protein impairs chloride conductance in the epithelial tissues, including the lungs, gastrointestinal tract, and reproductive organs [13]. The W1282X mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene causes a severe form of cystic fibrosis which accounts for 1.2% of the CF worldwide [15]. W1282X is a single nucleotide polymorphism (SNP) of CFTR located on exon 20, consisting of a change from guanine to adenine at the 3846th base pair, creating a premature stop codon (TGA). The destabilization of its mRNA as well as truncation of the expressed W1282X-CFTR, greatly reduces the presence of any fully functional CFTR proteins in epithelial cells, leading to CF [22].

The use of polymerase chain reaction, or PCR, permits the design and application of allele-specific DNA primers that distinguish wild-type gene sequences from mutant ones allowing for the diagnosis of cystic fibrosis [18]. PCR primer designs based on the Yaku method allow for the reduction of non-specific binding leading to false positive results [16]. The Yaku method

uses DNA primers that intentionally form one additional mismatched pairing with the template DNA [16]. These primers are capable of detecting SNPs with fewer pseudo-positive results.

It was hypothesized that the Yaku method combined with nesting primer approaches could provide a more precise and accurate identification and diagnosis strategy when applied to the W1282X-CFTR single base (SNP) mutation.

Original Experimental Design

The purpose of this study was to develop a PCR-based diagnostic assay to identify the W1282X-CFTR mutation using allele-specific primers. We hypothesized that the Yaku method, which introduces an additional intentional base pair mismatch at the 3' end, would reduce non-specific binding in allele-specific PCR (AS-PCR). In our AS-PCR assay, we used a two-step method.

The first-run PCR employed a "nesting" amplification approach, utilizing a published reverse primer (PRP1) and a universal forward primer (UFP1) designed to amplify a 718 bp "template" region from the CFTR gene. After which, in a second-run PCR, a published forward primer (PFP1) and Yaku-implemented reverse primers (RPMT1 and RPWT1) were paired to produce a 316 bp region from the template region when facing either mutant W1282X-CFTR or wild-type CFTR DNA samples. Wild-type seeking primers would only produce a band when facing DNA template which was wild-type at the 1282 locus, and mutant-seeking primers would only amplify successfully from mutant W1282X template DNA [18].

Prior research has been done to detect the W1282X-CFTR mutation using traditional PCR and has been successful [19]. By performing purification of buccal and S9 cell DNA, and using PCR methods implementing nesting and the Yaku method, it was expected detection of the W1282X mutation through gel electrophoresis may better reduce false positive results.

Ultimate Findings

As a control, a segment of the Rz gene of lambda virus bacteriophage genome was amplified via PCR with established primers to provide regular checks that could ensure that reagents and equipment continued to operate optimally. The strong amplification of a 395 base pair segment repeatably supported the success of this control.

The successful purification of genomic DNA from buccal and S9 cell genomic DNA was supported by visualized in gel electrophoresis and absorbance in UV spectrophotometry to detect DNA and RNA content. The 260nm/280nm absorbance ratio of nearly 1.8 was supportive of good purity in the extracted genome.

An implied control also evaluated the quality of isolated and purified wild-type genomic DNA from human buccal cells. Successful PCR utilizing published primers from Drury et al [18], that targeted the wild-type CFTR gene, regularly supported the integrity of the human CFTR gene.

Lastly, the nesting + Yaku strategy was developed and studied. As designed, PCR products analyzed via agarose gel electrophoresis, revealed expected bands of approximately 316 base pairs, supportive of successful W1282X detection after a nesting PCR approach was followed by the application of the appropriate Yaku primers.

Optimization Experiments

We will next complete optimization tests. These experiments will determine the optimal conditions to use when Yaku primers face inappropriate targets. For example, when wild-type seeking primers will face mutant DNA loci, and vice versa [14]. To further this research to improve diagnostics, other methods of PCR amplification can also be used. An example is the application of PCR-driven site-directed mutagenesis, which creates a specific DNA sequence change in the PCR products which can aid diagnosis [20].

Future Directions

The research conducted in this study supports the findings of Yaku and colleagues. Further research to verify the specificity of this assay may lead to improved diagnostic testing in clinical settings to identify genetic mutations, such as W1282X.

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Conflicts of Interest: The authors declare no conflict of interest.

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