

Identification of G551D-CFTR allele *via* AS-PCR of human buccal cells

Matthew R. Cederman^{1,#}, Maggie F. Leff^{1,#}, Megan R. Monforton¹, Ally M. Bauer¹ and Douglas B. Luckie^{1,2,*}

¹Lyman Briggs College; ²Department of Physiology, Michigan State University, East Lansing, MI 48824, USA.

ABSTRACT

The G551D mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) is the third most common allele found in cystic fibrosis patients. This mutation is a single base pair substitution at the 551st codon of the CFTR gene that leads to a substitution of glycine (GGT) by aspartic acid (GAT). DNA detection of such a mutation can be achieved through polymerase chain reaction (PCR) amplification utilizing allele-specific primers. In testing a more reliable approach to a diagnostic assay, an intentional mismatch strategy as described by Hidenobu Yaku's group was applied to the oligonucleotide design in an attempt to decrease the number of false positives encountered and a third nested primer was added to further confirm the amplified region.

KEYWORDS: cystic fibrosis, genetic diagnosis, primer design, intentional mismatch.

INTRODUCTION

Cystic fibrosis diagnostic and therapeutic advances are possible with the help of ongoing research and experimentation. Mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene lead to cystic fibrosis (CF), an autosomal recessive disease [1]. Cystic fibrosis is a multisystem disease that primarily impacts the lungs, pancreas, and liver [1]. The role of the CFTR protein within the human

body is to control the passage of chloride ions across the membrane of epithelial cells [2]. Functional CFTR proteins are incorporated into epithelial cells; as a result when the channel is open chloride ions are transported through the apical membrane followed by the vectorial movement of water. Combined with water, the mucus within the conducting zone of the respiratory tree becomes thin, wet, and is easily swept away by the cilia [3]. The expression of mutated CFTR proteins can result in the partial or complete absence of chloride passage across the cell membrane. In the absence of chloride movement into or out of the cell, water movement is also inhibited, leading to the production of dry, thick mucus [4]. Dehydrated mucus begins to accumulate and prevent cilia from properly removing it. Additionally, airborne particles and bacteria become trapped within the mucus, in turn leading to colonization and further infection [4]. Currently, there is no cure for cystic fibrosis or any treatment that fully restores chloride movement within affected epithelial cells. However, Vertex Pharmaceuticals Inc. has engineered Trikafta and Kalydeco, two drug treatments applied to help reduce the effects of cystic fibrosis in affected patients. Trikafta, a combination of three drugs, elexacaftor, tezacaftor, and ivacaftor, was FDA approved in 2019 and is aimed to help those with at least one copy of the $\Delta F508$ mutation which is present in about 90% of cystic fibrosis patients [5]. Kalydeco is a potentiator aimed specifically at the G551D-CFTR missense mutation. The drug has shown improvements in chloride diffusion and lung function in the G551D mutation and other Class III gating mutations [6].

*Email id: luckie@msu.edu

#Shared first authorship

Among the over one thousand known mutations of the CFTR gene, the G551D mutation is the third most prevalent cystic fibrosis-associated mutation, accounting for 2.3% of cases [7]. G551D is a missense mutation that occurs on exon 11 at the 551st amino acid and is caused by a single base pair substitution from a guanine (G) to adenine (A). Due to this mutation, there is an amino acid change from glycine (GGT) to aspartate (GAT). A missense mutation results from a change in amino acid without impacting other codons and can be either conservative or radical. A conservative missense is a change in amino acids that are similar in polarity. Whereas a radical missense mutation, like that of G551D, occurs from a change in polarity as well, which is more likely to disturb protein function. Although the G551D-CFTR protein is mutated, it is not marked for degradation and is still taken to the cell membrane despite its inability to function properly. Once integrated in the phospholipid bilayer of the cell membrane, nucleotide binding domain one (NBD1) of the CFTR protein is believed to be impaired. This specific mutation is considered a class III, or gating mutation, which results in diminished ATP binding and hydrolysis at NBD1 [8]. Because the protein has the capability to be incorporated into the membrane, unlike class I or II mutations, medication and treatments may have a greater likelihood of being effective in restoring some anion flux to epithelial cells.

Polymerase chain reaction (PCR) is a genomic amplification technique that can be used to identify the presence of specific alleles in a sample of DNA. PCR consists of three stages that are repeated through numerous cycles, overall compounding the total amplified target segments of DNA [9]. PCR primers were designed using the Yaku method. This strategy of primer design utilizes an intentional mismatch at the 3' end in an attempt to reduce the number of false positives obtained from AS-PCR of single polymorphisms [10]. An additional confirmation of the designated amplified region was also employed with a third nested oligonucleotide primer.

MATERIALS AND METHODS

Primer design

Primers were designed using data from prior literature and the cystic fibrosis mutation database. Considering the mutation G551D derives from a single nucleotide

polymorphism (SNP), allele-specific primers were designed. Using the intentional mismatch on the 3' end of the Yaku oligonucleotide for primer design, we implemented a forward primer for wild type 1 (FPWT1): 5'-GGAGAAGGTGGAATCACACTGAGTGGGGG-3' (29 bp). The guanines (G) on the last nucleotides stabilize 3' annealing when binding to the cytosines (C) found on the wild type DNA strand [10]. The forward primer for mutant type 1 (FPMT1) with the sequence 5'-GGAGAAGGTG AATCACACTGAGTGGGGA-3' (29 bp), was designed to destabilize 3' annealing when not presented with the presence of the point mutation at the 551st base pair position on exon 11 for G551D mutant DNA. A third designed forward primer (AFP1) was used as a secondary confirmation of our experiment. It utilized the same universal reverse primer (URP1) as FPWT1 and FPMT1. It was created to amplify a smaller region of the intended target, which yielded a 626 base pair amplification: AFP1 5'-CTCAGAATCTGTGCCCGTATCTTGG-3' (25 bp) [11]. The universal reverse primer (URP1) used was designed to anneal to the DNA strand and amplify a DNA segment of 882 base pairs when used with the FPWT1 and FPMT1 primer. The universal reverse primer has the following sequence: 5'-CCAGGCAATTGCAGGTGCTCAGTTAGGG-3' (28 bp). Forward and reverse primers published by Friedman *et al.* 1991 (referenced as Friedman primers) were used as a control and provided evidence of success in amplification from purified CFTR gene: 5'-CAACTGTGGTTAAAGCAATAGTGT-3' (24 bp) for the forward, and 5'-GCACAGATTCTGAGTAACCATAAT-3' (24 bp) for the reverse [12]. The primer pair used to target the Rz gene of bacteriophage Lambda were: 1Rz1F 5'-GATGTsATGAGCAGAGTCACCGCGAT-3' and 1Rz1R 5'-GAGGGTGAAATAATCCCGTTCAG-3'.

All primers were ordered from Integrated DNA Technologies (IDT). Prior to being determined experimentally, annealing temperatures were calculated using a common equation which approximates the melting temperature of each primer: $T_m = 64.9\text{ }^\circ\text{C} + 41\text{ }^\circ\text{C} \times (\text{number of guanines and cytosines} - 16.4)/n$, where n = the length of primers [13]. Optimal annealing temperatures were initially tested at 3 °C- 5 °C below the melting point. Using this formula, primers were calculated to have the following annealing temperatures: FPWT1

60 °C, FPMT1 59 °C, URPI 59 °C, forward Friedman primer 48 °C, reverse Friedman primer 48 °C and AFP1 58 °C. Based on these, 59 °C was used as our initial overall annealing temperature for PCR testing, excluding the forward and reverse Friedman primers.

Genome purification

Human DNA samples were obtained utilizing a Chelex-100 resin protocol upon wild-type buccal epithelial cheek cells. The Chelex-100 resin was diluted in order to create 10% Chelex solution [14]. A 1.5 mL epithelial cell solution was centrifuged at 14,000 ppm for 5 minutes, separating the supernatant and the cellular pellet. The supernatant was removed and replaced with 500 µL of 10% Chelex solution. The sample was then vortexed until the pellet was resuspended. The sample was then placed in a water bath and incubated for 5 minutes at 56 °C to lyse the cells and then vortexed again to mix well. Heating with a water bath and centrifugation were repeated two additional times. The tube was then placed in a sand bath for 5 minutes at 100 °C. The epithelial cell sample with the 10% Chelex solution was centrifuged one last time at 14,000 ppm for 5 minute to reform the pellet. The final supernatant, containing the wild type human DNA, was removed. The genomic DNA samples were kept at -20 °C until use [14].

Polymerase chain reaction

PCR was regularly conducted with a cocktail containing 5 µL 10x PCR buffer, containing 200 mM Tris-HCl and 500 mM KCl, 10 mM dNTPs (deoxynucleotide triphosphates) (1 µL), 4.5 µg/µL DNA template (1 µL), 1x Taq polymerase (1 µL), 25 nmol forward primer (1 µL), 25 nmol reverse primer (1 µL), 25 mM MgCl₂ (1.5 µL), and 38.5 µL deionized water. The cocktail was placed in a thermocycler and typically amplified through 36 cycles of denaturing, annealing, and elongation. The initial denaturation occurred at 95 °C for 3 min, then 36 cycles were repeated at 95 °C for 30 seconds, then 51-59 °C for 1 min, and 72 °C for 1 minute, followed by a final elongation at 72 °C for 5 minutes. For annealing temperatures, 51 °C was used for Lambda primers and 59 °C for the CFTR primers.

Gel electrophoresis

Gels were created either the day of or two days prior to PCR to analyze DNA band results. The materials included 0.4 g agarose powder (1% agarose gel),

20x lithium borate (LB) buffer (2 mL), 10000x SYBR-safe™ dye (4 µL), and 38 mL deionized (DI) water. The solution was heated until the solutes were dissolved, avoiding boiling. After solutes were dissolved the solution was allowed to cool to the touch and then SYBR-safe dye was added and the mixture was then cast. Samples were loaded into agarose gels and run at 180 V for approximately 25 minutes. The results were analyzed and documented by UV transillumination.

RESULTS

Primer design

Yaku primer design method was applied to test for increased specificity in genetic detection through raised steric hindrance [10]. With successful annealing of the wild-type forward primer (FPWT1) to wild-type DNA, elongation is favored because there is minimal steric hindrance (Figure 1A) [15]. When FPMT1 was used with wild-type DNA, no elongation took place due to steric hindrances among the primer and DNA located on the 3' end (Figure 1B). In order to allow for amplification URPI was utilized to provide reverse elongation (Figure 1E) [16]. A nested third forward designed primer (AFP1) was implemented as a secondary confirmation. With this combination of FPWT1 followed by AFP1, further amplification occurred because AFP1 was nested between the two designed primers: FPWT1 and URPI (Figure 2).

PCR controls

As a first step in establishing a reliable PCR diagnostic assay to positively identify the G551D-CFTR missense mutation, several controls were employed. One PCR control utilized purified genome from the Lambda virus as a template versus established primers. Once Lambda DNA PCR analysis was concluded, the products were analyzed through gel electrophoresis. The resulting Lambda PCR products were visualized in lanes 2-6 of the gel (Figure 3A). The analysis calculated a molecular size of 367 base pairs for the amplified segment of the Rz1 gene in lane 6, supportive of successful target amplification of 395 base pairs (Figure 3B).

Purified human DNA control

To isolate wildtype human DNA, a Chelex protocol was used to extract DNA. A second PCR control regularly utilized primers published by Friedman *et al.* and verified the genome and CFTR gene to

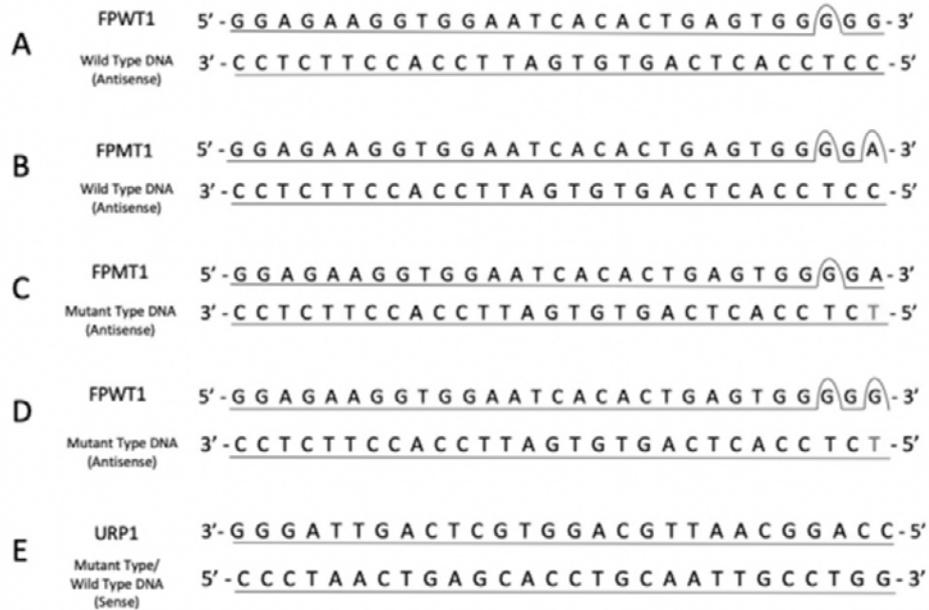


Figure 1. Designed primers for PCR testing. The Yaku method was applied when creating forward primers for the antisense strand of DNA to detect the G551D-CFTR mutation. The method is designed to reduce improper annealing between primers and target DNA sequences by increasing steric hindrance through an intentional mismatch. These mismatches are located at the third to last nucleotide, and G551D mutation at the final nucleotide in the primer sequence. (A) When using the wild type forward primer (FPWT1) with wild type DNA, the result is proper elongation of the DNA. Although there is a mismatch on the third to last base pair, it is hypothesized to not provide enough steric hindrance to inhibit Taq polymerase from binding to the sites following the “G” to “T” mismatch and will allow for DNA elongation. (B) When using the mutant type forward primer (FPMT1) with wild type DNA, elongation will be inhibited due to the mismatch of “G” to “T” on the third to last base pair and “G” to “A” on the final base pair in the sequence which creates enough steric hindrance to inhibit the mutant primer from annealing and properly elongating the DNA. (C) When FPMT1 is used with mutant type DNA, it will bind and properly elongate the DNA sequence. (D) The use of FPWT1 with mutant type DNA will create steric hindrance that will not allow for the Taq polymerase to properly bind and elongate the DNA. (E) The universal reverse primer (URP1) is oriented from 3’ to 5’ in order to anneal and elongate the sense strand of the DNA in a reverse direction. When designing the reverse primer, there was no need for intentional mismatch because there is no mutation at this site, so normal base pair matches were used instead. The URP1 is a common reverse primer.

be intact. Products amplified from purified human DNA were loaded into wells 2-5 with varying concentrations of resulting PCR DNA to DI water, with a 1 kb+ DNA ladder as a molecular weight marker. Additionally, a sample of Lambda DNA was placed into well 6 as a control (Figure 4A). Although only a faint band appeared in lane 2, semi-log analysis indicated the molecular size was 426 bp, with expected amplification of 425 bp (Figure 4B).

Nested confirmation

PCR amplification using FPWT1 and URP1 with wild type DNA was analyzed through gel electrophoresis in a 1% agarose gel (Figure 5A).

Analyzing DNA migration, lane 4 DNA band was calculated to be 822 base pairs, supportive of the target amplification of 882 base pairs (Figure 5B). A nested third forward designed primer (AFP1) was implemented as a secondary confirmation. The annealing temperature of AFP1 was found to be 61.1 °C, as optimal for amplification of the target sequence (Figure 6A). A semi-log plot using the 1 kb+ DNA ladder migration distance in lane 1 was created in order to determine the molecular size of the unknown DNA bands. Lane 3 was calculated to have an amplification length of 662 base pairs, with a targeted amplification length of 631 base pairs (Figure 6B).

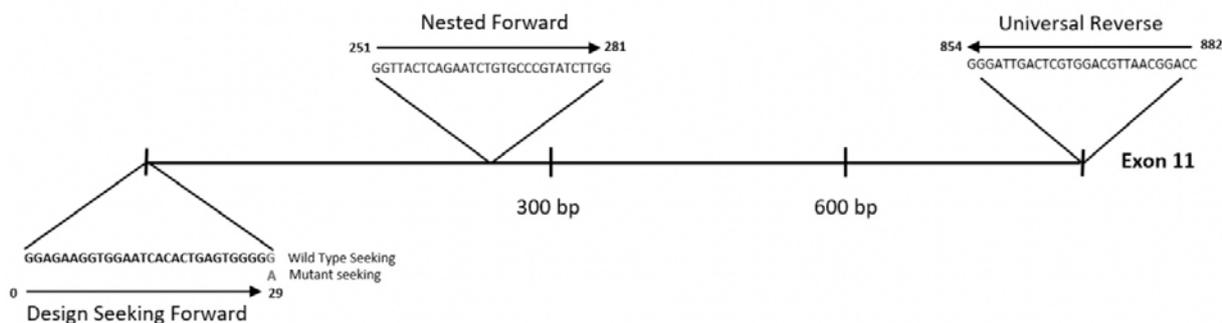


Figure 2. CFTR gene annealing sites and sequences of designed primers. Diagrammed at each indicated primer name is an arrow indicating the elongation direction, and the nucleotide binding locations, and distances. Both designed forward primers anneal in the same location at the same length, despite the difference in the last nucleotide of their sequence. The universal reverse primer elongates in the opposite direction to allow for specific amplification of the target region of the CFTR gene on exon 11, in which its sequence is observed in the 3' to 5' direction. The nested primer location was intentionally placed between the designed forward and universal reverse to allow for secondary confirmation of the original amplification.

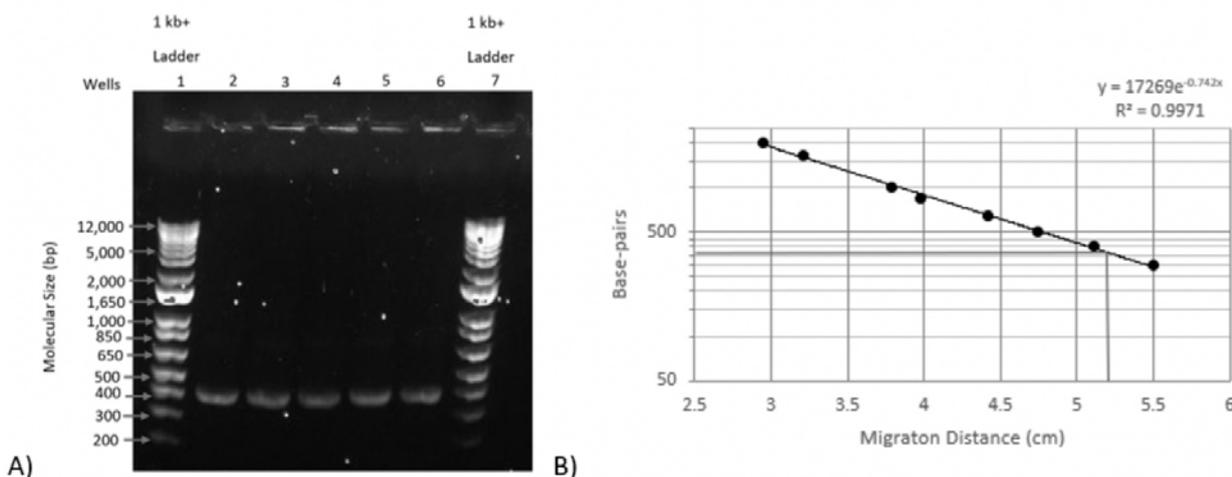


Figure 3. Control amplification results of lambda genome gel electrophoresis. **(A)** Lanes 1 & 7 were loaded with 1 kb+ ladder. Lanes 2 through 6 were loaded with PCR amplification products. The initial denaturation occurred at 95 °C for 3 min, and was followed by 36 cycles of denaturation at 95 °C for 30 seconds, annealing at 56 °C for 1 minute, and elongation at 72 °C for 1 minute, followed by a final elongation at 72 °C for 5 minutes. The resulting DNA bands were analyzed by 1% agarose gel at 200 V for 20 minutes. **(B)** Analysis of length of PCR products using semi log comparison to MW markers. Using the migration distance of the 1 kb+ ladder in well 1, in centimeters, at each distinguished DNA marker, a semi-log plot was created, and exponential trendline was applied. The equation of the trendline $y = 17269e^{-0.742x}$, calculated the base pair length of the fragment to be approximately 367 bp; band was expected to be 395 bp.

Simultaneous primer utilization

The use of the AFP1 in addition to PCR was intended as a secondary confirmation. Due to the difference in the annealing temperatures of the two forward primers, FPWT1 and AFP1, it was not ideal to utilize both forward primers simultaneously. This was

experimentally determined through the combination of primers in a PCR reaction with an adjusted annealing temperature of 60.8 °C and 61.1 °C, which was within the annealing range of FPWT1 but above the range of AFP1. The 1% agarose gel analyzed through electrophoresis resulted in the absence of DNA amplification (Figure 7).

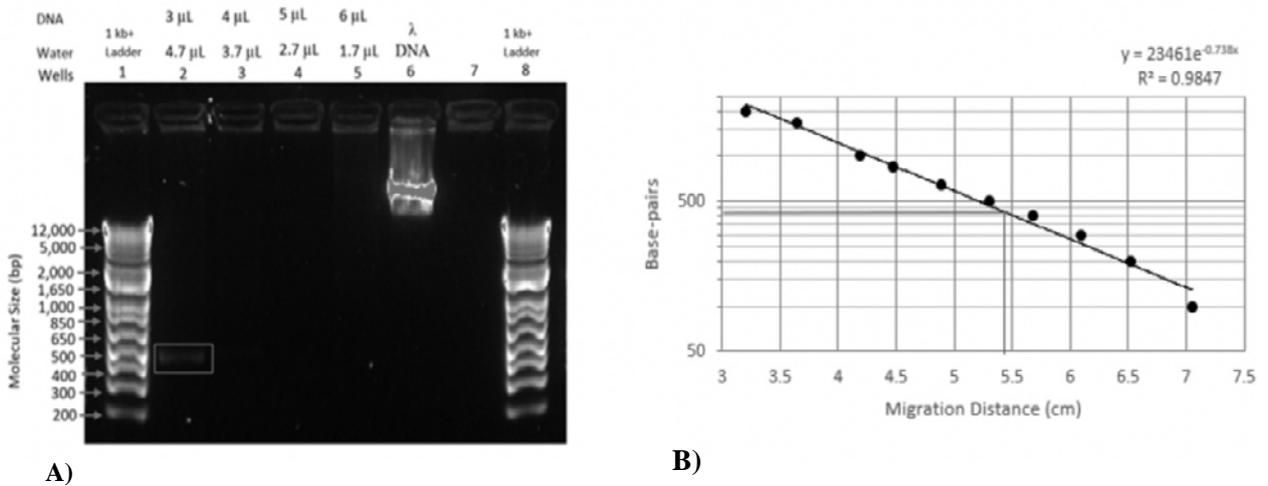


Figure 4. Amplification of purified human DNA utilizing Friedman primers. **(A)** Lanes 1 & 8 were loaded with 1 kb+ ladder. Lanes 2-5 were loaded with PCR amplified products. Lane 6 contained lambda genomic DNA. Denaturation occurred at 95 °C for 30 seconds, and was followed by annealing at 50.8 °C for 1 minute, then elongation at 72 °C for 1 minute 30 seconds. Afterwards, final elongation occurred at 72 °C for 7 minutes. Variations in PCR product loading concentrations were tested to determine optimal ratios. Lane 2 was observed to produce the most visible band. The gel was run at 180 V for 22 minutes. **(B)** Analysis of length of PCR products using semi-log comparison to MW markers. Using the migration distance of the 1 kb+ ladder in lane 1 and the band in lane 2, a semi-log plot was created. The trendline $y = 23461e^{-0.738x}$, calculated the base pair length of the fragment to be approximately 426 bp; the amplified segment was expected to be 425 bp.

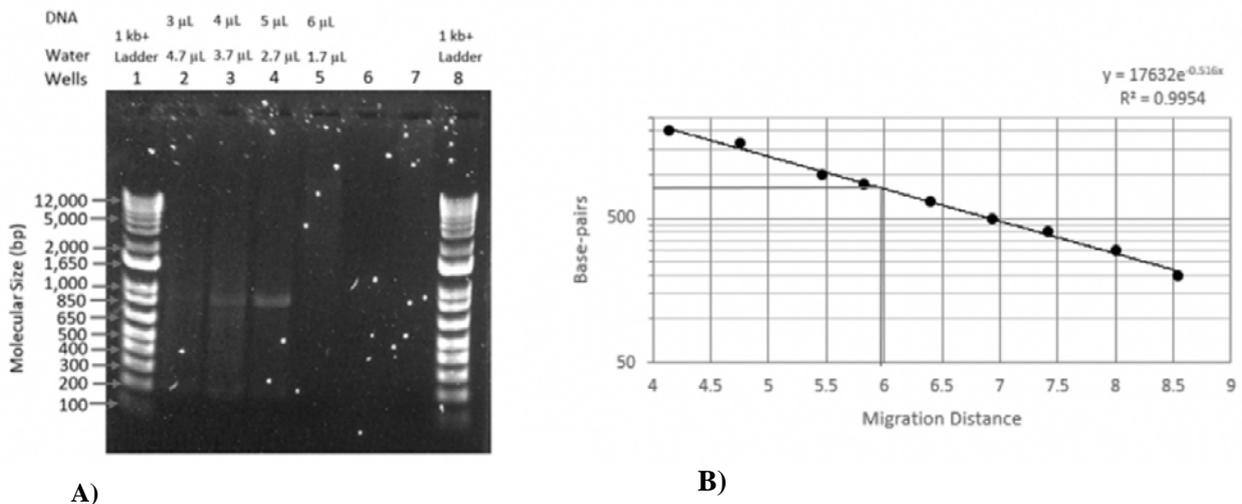


Figure 5. Implementing G551D-CFTR detection with FPWT1. **(A)** Lane 1 & 8 contained 1 kb+ ladder. Lanes 2-5 were loaded with PCR amplified products at increasing concentrations. Denaturation occurred at 95 °C for 30 seconds, and was followed by annealing at 62 °C for 1 minute, then elongation at 72 °C for 1 minute 30 seconds. Afterwards, final elongation occurred at 72 °C for 7 minutes. The resulting gel visualized DNA bands. Lane 4 was observed to produce the most visible band. **(B)** Analysis of length of PCR products using semi-log comparison to MW markers. A semi-log plot compared the migration distance of 1 kb+ DNA ladder bands versus that of lane 4. The trendline equation of $y = 17632e^{-0.516x}$ calculated the amplification length of the DNA band in lane 4 to be approximately 822 bp. The primers were designed to amplify a segment of 882 bp.

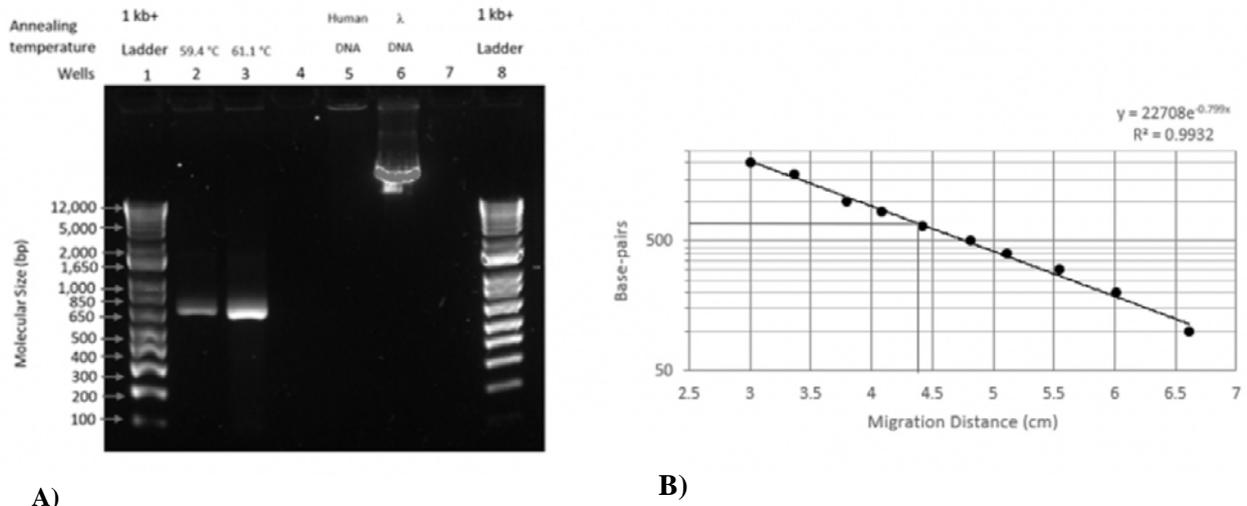


Figure 6. Amplification using the nested primer (AFP1) versus wild-type PCR products. **(A)** Lanes 1 & 8 were loaded with 1 kb+ ladder. Lanes 2 and 3 contained identical PCR reactants except for an alteration in the annealing temperature. The cycling denaturation occurred at 95 °C for 45 seconds, and was followed by annealing of the AFP1 and URPI at indicated temperatures for 1 minute and 30 seconds, and elongation at 72 °C for 1 minute and 30 seconds, with a final elongation at 72 °C for 7 minutes. **(B)** Analysis of length of PCR products using semi-log comparison to MW markers. The semi-log plot was based upon the 1 kb+ ladder in lane 1 vs the migration distance of the DNA band in lane 3. The trendline $y = 22708e^{-0.799x}$ calculated the DNA band length in lane 3 to be approximately 662 bp. The band was expected to be 631 bp.

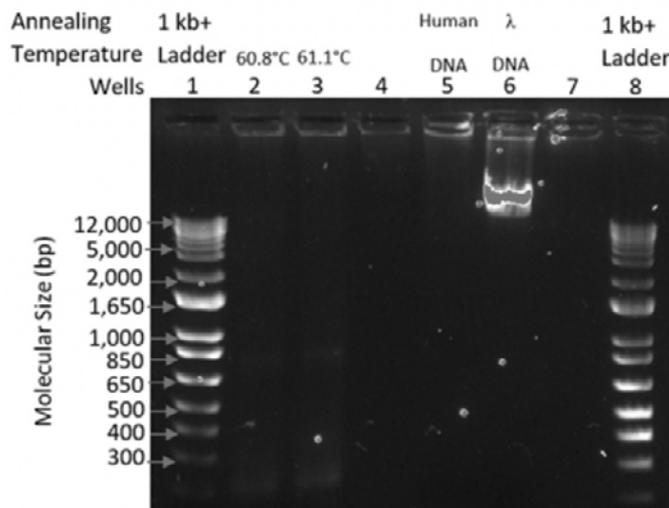


Figure 7. PCR amplification with two forward primers, FPWT1 and AFP1, utilized simultaneously. The nested forward primer AFP1 was used in conjunction with forward primer, FPWT1 versus common reverse primer URPI. Lanes 1 & 8 were loaded with 1 kb+ DNA ladder. Lanes 2 and 3 were loaded with identical PCR reaction products that only varied by annealing temperatures: 60.8 °C and 61.1 °C, respectively. During thermocycling, the denaturation occurred at 95 °C for 45 seconds, and was followed by annealing of the AFP1 and URPI at the indicated temperatures for 1 minute and 30 seconds, and followed by elongation at 72 °C for 1 minute and 30 seconds. The PCR cycles were concluded by a final elongation at 72 °C for 7 minutes. Lanes 5 and 6 were loaded with human genomic DNA and λ genomic DNA samples, respectively, as controls. The 1% agarose gel was run at 180 V for 21 minutes and then visualized.

DISCUSSION

Experimental design

The implementation of the Yaku method, the intentional mismatch located on the third base pair of the 3' end, and a site specific mismatch on the first base from the 3' end of our designed forward primers (107,815 bp to 107,844 bp in exon 11) were designed to reduce false positives in an assay for the detection of G551D-CFTR [10]. This strategy was tested to increase the confidence and specificity of the genomic detection of SNPs, like G551D-CFTR. Theoretically the specificity was to be increased due to the steric hindrance between the 3' end of the forward primers and the DNA template, resulting in the inability for Taq polymerase to elongate an unspecified DNA strand [17].

Control standards

Two controls were regularly employed to support the integrity of the methodology and genomic template used. The Lambda PCR control test regularly resulted in DNA amplification of a band calculated to be approximately 367 bp (expected 395 bp) and thus was used as the foundation to adjust temperatures and concentrations as needed for experimentation. Wild-type seeking PCR primers tested and published by Friedman *et al.* 1991 were a secondary routine control used (referred to as Friedman primers, FPWT1, FPMT1). As a result of successful annealing of FPWT1 to isolated human DNA, a band of approximately 822 bp was visualized in lane 4 (Figure 5A). This was indicative of success given the primers were designed to amplify a segment of 882 bp. In a competitive test, the combination of AFP1 & FPWT1 versus URP1 primers, with human purified DNA as a template, resulted in no amplification. This was attributed to the inability for the two primers to anneal at the same time given the primers' required temperatures and their competition for template DNA.

Primer results

After the first two experimental controls yielded successful results, experimental procedures were focused on amplification of G551D-CFTR. In the G551D experiments, PCR and gel electrophoresis yielded products that also suggested proper amplification had occurred using FPWT1 and AFP1 primers with wild-type DNA template, as well as

no amplification using FPMT1 with wild-type DNA. These findings supported the view that the Yaku sterical hindrance strategy was successful in increasing selective amplification.

CONCLUSIONS

In this study the experimental findings were supportive of the Hidenobu Yaku research group's finding that intentional mismatches can aid in primer design for SNPs. Yet, in future studies, there are additional aspects that will be focused upon to ensure this G551D-CFTR AS-PCR detection is reliable. This includes PCR testing with G551D-mutant template DNA, proper combination of forward and reverse primers with AFP1, as well as the possible utilization of the restriction enzyme PmeI as another verification method. Further testing and adjustments to concentrations and annealing temperatures of primers must take place in order to confirm conclusive results. Upon successful and conclusive results, this AS-PCR protocol can provide additional experimental support for strategies to target SNPs when using oligonucleotide-based assays.

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CONFLICT OF INTEREST STATEMENT

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria), or non-financial interest (such as personal or professional) in the subject matter or materials discussed in this manuscript.

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