DETECTION OF MULTIPLE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) MUTATIONS USING HIGH RESOLUTION THERMAL MELT ANALYSIS ON A MICROFLUIDIC CHIP

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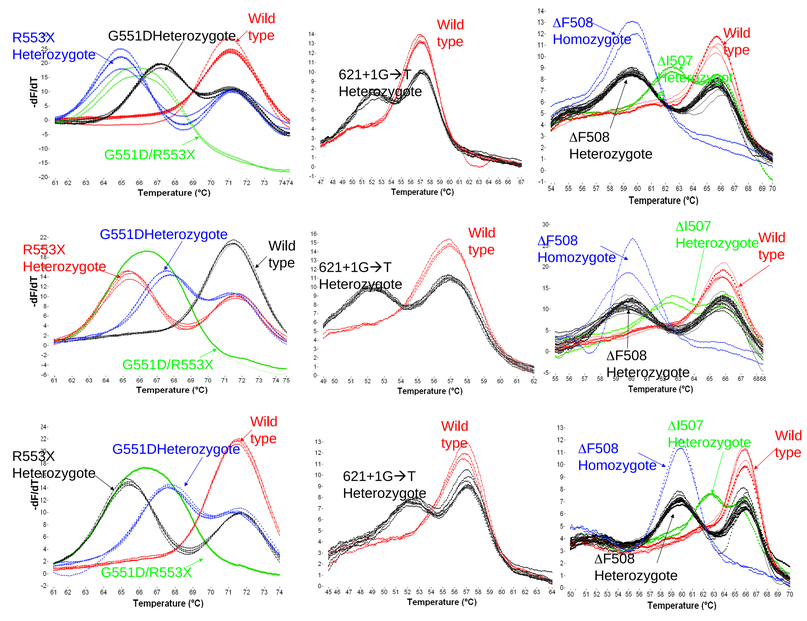
Abstract

Cystic Fibrosis is an autosomal recessive genetic disease that is caused by mutations in the CFTR gene. The current method of detection of this disease is via a positive sweat chloride test followed by sequencing of the 23 mutations recommended by the American College of Medical Genetics which is very costly and time consuming. We have utilized an unlabeled probe assay followed by high resolution thermal melt analysis to distinguish genotypes by differences in melting temperature using a saturating double stranded DNA binding dye (LC Green Plus). This method utilizes PCR primers and a 3' phosphate-modified probe that covers the mutation of interest. As the DNA is denatured, the intercalating dye is released from the helix and becomes non-fluorescent in a sequence dependent manner. Differences in the probe melting temperatures indicate the presence or absence of a mutation. We have developed a microfluidic lab-on-a-chip system capable of differentiating homozygous, heterozygous and compound heterozygous mutants of all 23 CFTR ACMG mutations.

Experiment: Reproducibility CFTR genotyping using unlabeled probes Three of the 23 CFTR variants were analyzed in multiple Human gDNA samples (Coriell), representing heterozygous, homozygous, and wild type genotypes. Asymmetric amplification was carried out in the presence of a 3'-blocked probe covering the mutation of interest and LC Green Plus, followed by probe melting analysis, in an LC480 instrument (Roche). Samples were run in duplicate wells and multiple runs (3) were conducted on different days.

Results: Reproducibility CFTR genotyping using High Resolution Thermal Melt Analysis (HRTm)

Figure 1, below, shows –dF/dT vs temperature plots of multiple gDNA samples for each of three assays and three runs. Genotypes representing ΔF508 heterozygote and homozygote were accurately and reproducibly distinguished from wild type. Two heterozygote genotypes (G551D and R553X) were correctly distinguished from wild type and a double heterozygote (G551D/R553X). The heterozygote genotype representing Exon 4 (621+1G→T) was successfully identified from wild type.



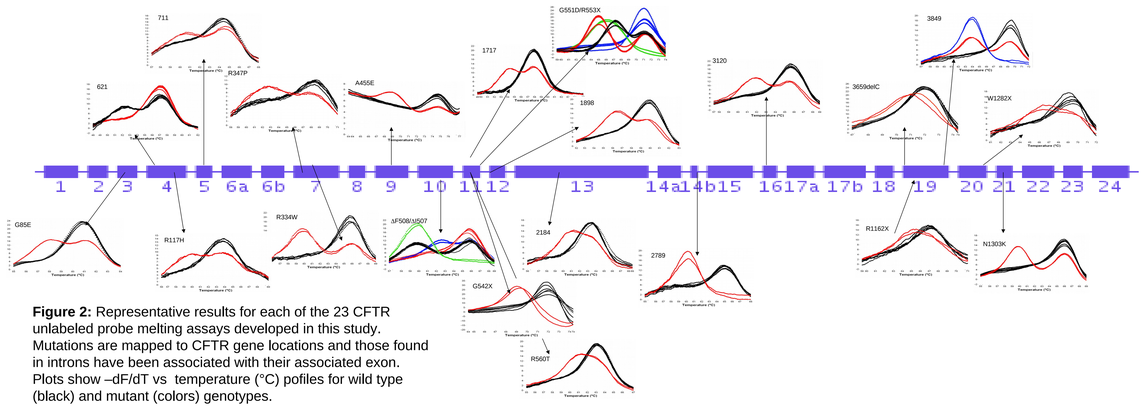


Figure 2: Representative results for each of the 23 CFTR unlabeled probe melting assays developed in this study. Mutations are mapped to CFTR gene locations and those found in introns have been associated with their associated exon. Plots show –dF/dT vs temperature (°C) pofiles for wild type (black) and mutant (colors) genotypes.

Experiment: Microfluidic PCR and High Resolution Thermal Melt Analysis

The microfluidic chip, Figure 3, consisted of two bonded quartz plates; one piece with etched channels, the other piece with nine metal traces and through holes to serve as reagent reservoirs. A capillary inserted perpendicular to the plates allowed samples to be introduced into the channels directly from a microtiter plate by pressure driven flow. Primers and LC Green Plus introduced via the capillary were mixed with Taq polymerase and dNTPs stored on the chip. The mixture was then split into 8 parallel channels each of which was supplied with a different DNA sample, for a total reaction volume of 15 nL. Varying power levels were applied to the metal traces to rapidly cycle the temperature of the samples flowing through the amplification region. Thermal melting of the amplified DNA was accomplished by means of a Peltier device, capable of linear heating rates from 0.1 to 1.0 ºC, located under the detection zone. Laser-induced fluorescence was used to measure decrease in LC Green plus fluorescence during DNA thermal melting as samples passed through the detection zone.

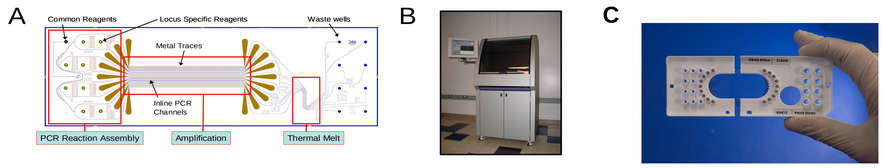
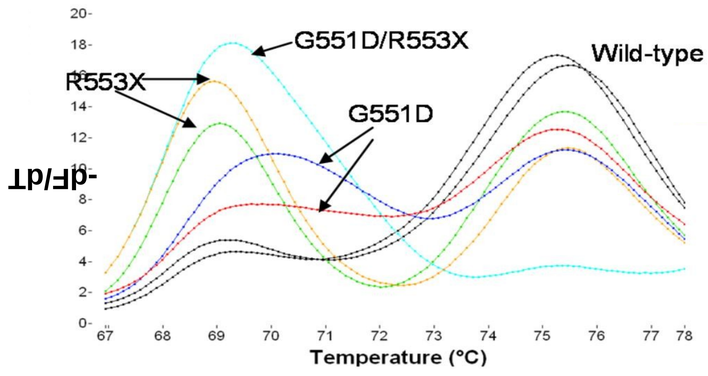


Figure 3: A schematic of the microfluidic chip design for integrated PCR and thermal melting (A), the instrument (B), and a scale photo of PCR-T_m chip (C).

Results: Microfluidic PCR and High Resolution Thermal Melt Analysis

The unlabeled probe assay for mutations G551D and R553X of CFTR Exon 11 was run on the microfluidic device. In this assay a 93 bp PCR product is generated in the presence of LC Green Plus and a single, unlabeled probe is able to distinguish both heterozygous mutations and the double heterozygous mutation from the wild type genotype. A total of 7 different DNA samples, plus a no-template control were run on the microfluidic chip. The results in Figure 4 below display four melting profiles resulting from the 7 samples and demonstrate the ability to differentiate wild type, both single mutations and the double mutation in Exon 11.



Experiment: Genotyping Blind Study

PCR reactions for the 23 ACOG variants were analyzed against 10 anonymized gDNA samples representing 10 different patients and 1 control gDNA sample. Immediately after PCR, thermal melting analysis was performed on all samples using the LC480 (Roche) at 1°C/s.

Results: Genotyping Blind Study

- 210 gDNA samples genotyped
- 1/1 Homozygote correctly called
- 13/14 Heterozygotes correctly called (1 false negative)
- 194/195 Wild-types correctly called (1 false positive)

Figure 5 below shows results of 6 easily genotyped assays (A-F). The plots are the negative first derivative of fluorescence (-dF/dT) with respect to temperature (°C).

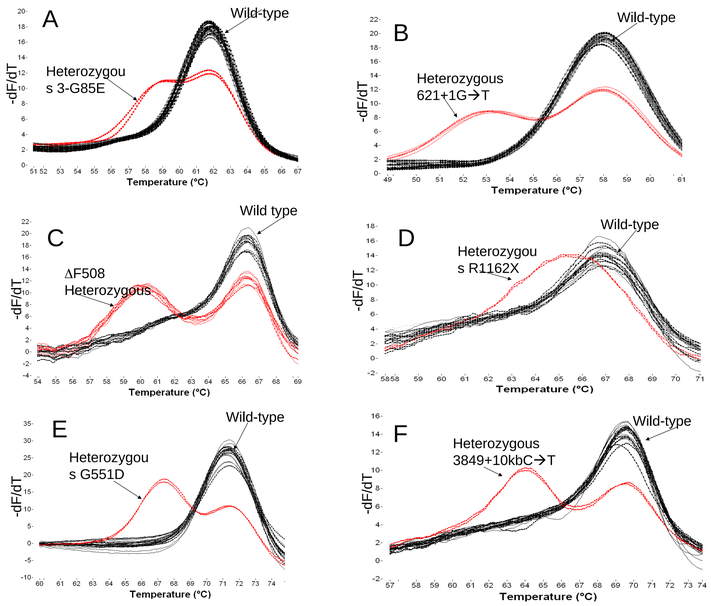


Figure 6, below, shows the negative first derivative of fluorescence with respect to temperature (ºC) plots for two assays that were miscalled in the blind study. The graphs represent assays that are not clearly discriminate from the wild type (G) or Tm shift is not easily differentiated from wild type (H).