The LightTyper™: High-Throughput Genotyping Using Fluorescent Melting Curve Analysis

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ABSTRACT

Instrumentation, chemistry, and software for high-throughput genotyping using fluorescent melting curves are described. The LightTyper™ system provides post-amplification genotyping within 10 min using samples in 96- or 384-well microplate formats. The system is homogenous because all reagents are added at the beginning of the reaction and there is no sample manipulation between amplification and genotyping. High-resolution melting curves are achieved by slow and steady heating. As samples are heated, panels of blue light-emitting diodes excite the probes, and fluorescence emission is acquired with a cooled charge-coupled device camera. A variety of probe chemistries are compatible for genotyping on the LightTyper, including dsDNA dyes, single-labeled probes, and fluorescence resonance energy transfer systems. Genotyping is performed automatically, and each sample is given a score reflecting the similarity of the genotype to the standards provided. Standard genotypes can be selected from within the run or imported from other files. Samples and genotypes can be grouped to allow multiple-allele detection on one or many samples. The utility of the LightTyper is illustrated by genotyping samples for the Factor V Leiden mutation and for mutations in the CFTR gene.

INTRODUCTION

Highly parallel and rapid methods for DNA sequence analysis are important to science and medicine (1). The applications of these methods include finding cancer-related genes, associating gene variants with drug response, determining genetic linkages, understanding molecular evolution, and developing diagnostic tests.

There are many methods available for detecting DNA variants, such as direct (2) and indirect sequencing (3,4), allele-specific amplification (5,6), single-base extension (7), oligonucleotide arrays (8,9), and fluorescent probes (10). Se-

quencing is necessary for initially defining a new region of DNA; however, less expensive technologies that can rapidly and accurately re-sequence DNA are becoming more commonly used in the post-genomic era.

Clinical laboratories often use homogenous genotyping systems because they reduce labor time, risk of contamination, and clerical errors (10). Fluorescent melting curve analysis provides homogenous genotyping and has been used with hybridization probes (11–14), quenching probes (15,16), and GC-tail primers (17). Other chemistries such as exonuclease probes (18,19) and hairpin primer (20,21) and probe (22,23) systems do not use melting curve analysis but can still homogeneously genotype by color multiplexing allele-specific oligonucleotides.

Here we present the LightTyper™ for genotyping using fluorescent melting curve analysis. Fluorescent melting curves are generated with hybridization probes and SimpleProbe™ (Idaho Technology, Salt Lake City, UT, USA) chemistry (Figure 1). Hybridization probe systems produce fluorescence resonance energy transfer (FRET) when two single-labeled oligonucleotides adjacently anneal to complementary target (12). The “anchor” probe is designed to be more thermally stable than the genotyping probe, which overlies a site of sequence variance. A decrease in FRET is observed during slow heating, as the genotyping probe melts and the donor and acceptor fluorophores are physically separated. In contrast, the SimpleProbe system consists only of a single-labeled genotyping probe designed to increase in fluorescence upon target hybridization. Similar to hybridization probes, SimpleProbes show a decrease in fluorescence as the genotyping probe is melted from its target strand. In both systems, genotypes are determined from their characteristic DNA duplex melting profile.

The LightTyper is a high-resolution, high-throughput fluorescent melting curve analysis instrument designed to meet the demands of both clinical diagnostics and research. The information presented is on alpha production prototypes.

MATERIALS AND METHODS

Hardware

The LightTyper instruments were fitted with either a 96- or 384-well aluminum heat block. The heat block was powered by a 56-W heater and controlled by a Platinum Wire Resistive Thermal Device (PT100). Fluorescence excitation was done using two light-emitting diode (LED) boards, each consisting of 61 blue 5-mm LEDs and a custom optic filter (model 475DF40; Omega Optical, Brattleboro, VT, USA). A 12-bit, monochrome cooled charge-coupled device (CCD) digital camera (Photometrics CoolSNAPcTM Monochrome; Roper Scientific Photometrics, Tuscon, AZ, USA) was used for fluorescence acquisition. The CCD was equipped with custom exchangeable optic filters (models 510LP and 600LP; Omega Optical) to accommodate different probe systems. The 510LP filter is compatible for use with dyes having absorbance of approximately 490 nm and emission of approximately 520 nm (e.g., SYBR[®] Green I and fluorescein dyes). The 600LP filter is compatible for use with FRET systems using acceptors with emission greater than 600 nm (e.g., CyTM dyes). Other components included a stepper motor for automatic tray loading and ejection and a bar code reader for easy sample tracking.

Software

A software package was developed for controlling the image processing, analyzing fluorescent melting curves, and automatic genotyping. Before melting the samples, the software preprocesses the CCD images of the microplate. These images are used to calibrate the exposure time of the camera. They are also used to precisely determine the position of the wells on the microplate. During operation, temperature increases from a programmable start temperature to an end temperature. The CCD images of the microplate are acquired during the heating process. Over regular temperature intervals, many images are averaged to obtain one average image of fluorescence. To obtain the fluorescence data values, the pixels corresponding to each microplate well are averaged. Melting curves are con-

structed for each sample by plotting the fluorescence values for that well versus temperature. The T_m for a DNA duplex is the maximum on a plot of the derivative of fluorescence with respect to temperature (dF/dT) versus temperature.

Genotyping can be performed with user-defined standards designated within the run or imported from other runs. In addition, samples of the same genotype can be automatically grouped together based on the similarity of their melting profiles, allowing classifications to be made even without designated standards. Groups with different genotypes are created by clustering together sets of melting curves that are more similar to each other than they are to the curves in other clusters. After the genotype clusters have been found, the median melting curve of each cluster is computed. This median becomes the standard melting curve for that cluster. The software has been developed to discriminate up to seven groups of sample genotypes within a run.

Using the standards, the software generates scores by comparing each sample to each standard. Genotype scores are normalized between 0 and 1. The score, a measure of stringency, is a decreasing function of the distance between the sample and standard. If the sample and standard have identical melting curves, then the score is 1. If the sample and standard are dissimilar, then the score is closer to 0. The default score threshold is 0.7. The resolution, a measure of specificity, is the difference between the best score and the next best score. The default resolution threshold is 0.1. Genotypes are automatically assigned for samples when both the score and resolution thresholds are attained. A sample genotype is designated unknown if the genotype score or resolution is less than the user-specified thresholds. A manual override can be used to change standards or genotype calls.

Chemistry

Temperature uniformity. Complementary probe/target oligonucleotides, designed to melt at low ($T_m \approx 43^\circ\text{C}$), intermediate ($T_m \approx 53^\circ\text{C}$), and high ($T_m \approx 79^\circ\text{C}$) temperatures, were analyzed to assess temperature uniformity across five 96-well and five 384-well LightTyper prototypes. Synthetic templates were used to control for effects on T_m that can be produced by variability in PCR amplification. SimpleProbes and unlabeled templates were received from IT BioChem (Idaho Technology). The low-temperature system consisted of a SimpleProbe (5'-ATTTCAGGATTACACCA-M-F-3'; M designates a modified base, and F designates a fluorescent dye) and complementary target (5'-ACAGTC-TTTGGTGTAACCCTGAAATAAAAA-AGCGATG-3') mismatched (underlined) at a single base. The intermediate-temperature system consisted of a SimpleProbe (5'-TTTATTTTCAGGATTACACCAAAG-M-F-3') and the complementary target used in the low-temperature system. In the high-temperature system, the SimpleProbe (5'-TGAGCGTGACGTT-

Figure 1. Genotyping systems. In hybridization probe chemistry, FRET occurs between a donor probe (fluorescein, $\bullet$) and an acceptor probe (LCRed640 or LCRed705, $\blacklozenge$) as they hybridize adjacently to a single-stranded target. During heating, fluorescence decreases as the genotyping probe melts and is physically separated from the more stable “anchor” probe. In SimpleProbe chemistry, a fluorescein ($\bullet$) dye conjugated to a single genotyping probe increases in fluorescence when annealed to its complementary target. Genotyping is performed in both systems by determining the probe:target duplex T_m of the genotyping probe as it melts from a site of variance ($\underline{\hspace{0.5em}}$).

Product Application Focus

GCCTGAGCCGTGCG-M-F-3') and target (5'-CATCGC-ACGGCTCAGGCAACGTACGCTACCAT-3') were perfectly matched.

Each 10- μ L reaction consisted of 1 $\times$ PCR buffer with 3 mM MgCl₂ (Idaho Technology), 0.2 μ M SimpleProbe, and 0.1 μ M template. Reactions were loaded into 96- and 384-well plates (MJ Research, Waltham, MA, USA) and overlaid with 15 or 10 μ L mineral oil, respectively. Using a PCR thermal block (GeneAmp[®] PCR System 9700; Applied Biosystems, Foster City, CA, USA), the reaction plates were heated for 2 min at 95°C and then cooled for 2 min at 20°C for probe/target annealing. Plates were then loaded into the LightTyper and heated at a rate of 0.1°C/s from 35° to 90°C. Fluorescence of the SimpleProbes was continuously monitored through a 510LP filter.

Differences in T_m s from well to well were analyzed for each plate and compared between instruments using two-way mixed ANOVA (PROC MIXED; SAS[®], Cary, NC, USA). The explanatory variables in the analysis were instrument type (96- or 384-well), T_m (low, medium, and high) and machine (1–5 for both types). The machine effect was treated as a random variable. Since the cv estimate for a 384-well plate is based on four times as many values than for a 96-well plate, the values for the former instrument type were assigned a weight of 4 and the values for the latter instrument type were assigned a weight of 1.

Factor V Leiden. The Factor V Leiden mutation (G1691A) was detected using a commercially available hybridization probe kit (Roche Applied Science, Mannheim, Germany). A total of 50 ng genomic DNA was used for each wild-type, heterozygous, and mutant control. Reagents were loaded manually into 96- (GeneMate, Kaysville, UT, USA) and 384-well plates and overlaid with mineral oil as described above.

Amplification was performed in a GeneAmp PCR System 9700. The PCR protocol consisted of a denaturation step at 95°C for 30 s, followed by 45 cycles of amplification at 95°C for 5 s, 55°C for 10 s, and 72°C for 10 s. Samples were immediately cooled at 40°C for 30 s after a final denaturation at 95°C for 30 s. The cooled plate was then transferred to either a 96- or 384-well LightTyper and slowly heated (0.1°C/s) from 40°C to 80°C. During heating, fluorescence emission from the LCRed640-labeled probe was continuously monitored through a 600LP filter. Genotyping by fluorescent melting curve analysis was done with the LightTyper software.

CFTR gene. Primers and probes for the Δ I507/ Δ F508 and 1717-1 G-to-A mutations in the *CFTR* gene were designed using LightTyper Probe Design (Idaho Technology) and Primer Designer 4 (Scientific and Educational Software, Durham, NC, USA). Primers and probes were received from IT BioChem. A 229-bp region of the *CFTR* gene, containing the Δ I507 and Δ F508 mutation sites, was PCR-amplified using forward (5'-ATTATGGGAGAACTGGAGC-3') and reverse (5'-GGGTAGTGTGAAGGGTTCA-3') primers. Mutations were detected using a single SimpleProbe (5'-ATATCATCT-TTGGTGTTCCTATG-M-F-3') designed to match the wild-type sense sequence. For the 1717-1 G-to-A mutation site, forward (5'-TAGAAGGAAGATGTGCCTT-3') and reverse (5'-AAATGCTTGCTAGACCAAT-3') primers were used to amplify a 205-bp fragment. A SimpleProbe (5'-CTTGGA-

GATGTCCTATTACCAAA-M-F-3') designed to match the wild-type antisense strand was used for mutation detection.

For the *CFTR* mutation sites, each 10- μ L reaction contained 1 U FastStart[™] *Taq* DNA polymerase (Roche Applied Science) diluted in Enzyme Diluent (Idaho Technology), forward and reverse primers (Δ I507/ Δ F508, 0.1 μ M forward and 0.5 μ M reverse; or 1717-1 G-to-A, 0.5 μ M forward and 0.1 μ M reverse), 0.2 μ M SimpleProbe, 0.2 mM each dATP, dCTP, and dGTP (Amersham Biosciences, Piscataway, NJ, USA), 0.6 mM dUTP (Amersham Biosciences), 1 $\times$ PCR buffer with 3 mM MgCl₂, and 25 ng genomic DNA. Eighty-four genomic DNA samples were run in duplicate on a 384-well plate. Wild-type, heterozygous, and no template controls were run for each assay. Samples were overlaid with 10 μ L mineral oil before amplification in a GeneAmp PCR System 9700.

The PCR protocol consisted of an initial denaturation at 94°C for 10 min, followed by 40 amplification cycles at 94°C for 10 s, 55°C for 20 s, and 72°C for 10 s, with a final denaturation at 94°C for 2 min and a final hold at 20°C for 2 min. The 384-well plate was then loaded into the LightTyper and melted at a rate of 0.1°C/s from 40°C to 80°C. Fluorescence of the SimpleProbes was continuously monitored through a 510LP filter.

The Δ I507/ Δ F508 and 1717-1 G-to-A samples were genotyped separately using the analysis subset feature on the LightTyper software. The controls loaded on the plate, including heterozygous controls for both the Δ I507 and Δ F508 mutations, were defined as standards and subsequently used for genotyping all samples with unknown mutation status. The report subset feature was set up to view the results for one sample at both mutation sites.

RESULTS AND DISCUSSION

Temperature Uniformity

The accuracy of genotyping by fluorescent melting curve analysis strongly depends on the temperature uniformity of the reactions during heating. Indeed, differences in heating between reactions may cause the standards and unknowns of the same genotype to have different T_m s, resulting in miscalls.

Fluorescent melting curve data, generated from five 96-well and five 384-well LightTypers, were used to compare instruments for well-to-well temperature uniformity during heating. In each plate, three probe systems were analyzed to provide low ($T_m \approx 43^\circ\text{C}$), intermediate ($T_m \approx 53^\circ\text{C}$), and high ($T_m \approx 79^\circ\text{C}$) melting temperatures. In the 96-well prototypes, the low, intermediate, and high melting probes had a mean cv of 0.36%, 0.25%, and 0.23%, respectively. In the 384-well prototypes, the low, intermediate, and high melting probes had a mean cv of 0.29%, 0.25%, and 0.14%, respectively. The standard errors for the model were all between 0.01% and 0.02%. Overall, the 384-well instruments had a lower well-to-well cv ($P = 0.018$). However, the cvs changed depending on the T_m analyzed ($P < 0.0001$). The 384-well instruments showed lower cvs at the low ($P = 0.008$) and high ($P = 0.006$) T_m s when compared to the 96-well prototypes. There was no significant difference between the two types of

Product Application Focus

instruments for the intermediate temperature ($P = 0.84$).

In general, there was high temperature uniformity across both the 96- and 384-well LightTypers. Differences in reaction well sizes may account for the slightly better temperature uniformity observed in the 384-well plates. The high temperature uniformity in the LightTyper prototypes allows standards to be placed anywhere within the tray without affecting the accuracy of the genotyping. In addition, this level

of uniformity has the potential for future application in mutation scanning since fractional shifts in melting temperatures may be detectable.

Genotyping the Factor V Gene

The majority of inherited thromboembolic disorders are due to defects in the protein C anticoagulant pathway (24).

The most common single cause of activated protein C (APC) resistance in Caucasian populations is due to a G1691A base transition in the Factor V gene (Factor V Leiden). Because of the clinical importance of the Factor V Leiden mutation, diagnostic molecular tests have been developed to detect this variant (25). High-throughput genotyping platforms that provide automatic calling will help meet current demands for the diagnosis of thromboembolic disorders and other common genetic diseases.

Figure 2 illustrates the feature of automatic calling using samples genotyped for the Factor V Leiden mutation. The software allows users to specify standards within a run, use standards from another run, or use automatic genotyping from median average melting curves. Using the default score threshold (0.7) and user-defined temperature range (52°–74°C) for genotyping, the software had no miscalls and no unknowns using the different methods for choosing standards and compared to manual genotyping on the LightCycler® (Roche Applied Science). Scores varied depending on the method by which standards were selected for genotyping. The highest scores for calling each genotype resulted when using automatic genotyping and median average curves for standards (0.96 ± 0.05 ; range 1.00–0.75). User-defined standards selected from within the run gave slightly lower scores (0.95 ± 0.05 ; range 1.00–0.75) than automatic genotyping. Standards generated from the first run of the plate were imported to score the second run. As expected, the imported standards gave the lowest overall genotyping scores (0.94 ± 0.06 ; range 0.99–0.73).

The specificity of genotyping with hybridization probes can be decreased when other variants near the mutation of interest cause a similar T_m shift and result in a false positive. For instance, there are two polymorphisms (G1689A and A1692C) that could result in false positives using the wild-type probe for genotyping the Factor V Leiden (G1691A) mutation. The G1689A mutation has been reported to cause only a 0.8°C T_m shift from the expected Leiden mutation (26). Although no samples tested contained the rare G1689A mutation, the temperature uniformity and accuracy of genotyping on the LightTyper can easily resolve a T_m shift greater than 0.5°C from that expected. Large studies will need to be conducted for further clinical validation.

Figure 2. Hybridization probe chemistry with Factor V Leiden. Automatic genotyping results for the Factor V Leiden mutation assay are presented in the color-coded plate map (A). Three wild-type (G/G-blue), three heterozygous (G/A-yellow), one mutant (A/A-purple), and one negative (Neg-orange) were found in each column. All samples were correctly genotyped, with no unknown (Unk-green) samples found. Representative melting curves (B) and melting peaks (C) for each genotype are shown.

Product Application Focus

Genotyping *CFTR* Mutations and Subset Analysis

Unlike APC resistance in which the majority of disease can be accounted for by a single mutation in the Factor V gene, cystic fibrosis can be caused by hundreds of different mutations in the large (approximately 250 kb) *CFTR* gene (27). Implementing molecular testing for cystic fibrosis poses a challenge for the clinical laboratory because each sample needs to be interrogated for many mutations and the prevalence of different mutations changes with the population studied. Recently, a consensus panel of 25 cystic fibrosis causing mutations, each with a pan-ethnic population frequency of greater than 1 in 1000, has been suggested for clinical testing (28).

The open format of the LightTyper allows laboratories to develop customized assays. Also, the software was designed to genotype different variants under a single probe and to provide an overall scanning profile by reporting on multiple sites for each sample. Figure 3 illustrates both these features, where the Δ F508/ Δ I507 mutations were detected with one probe and the 1717-1 G-to-A mutation was detected with another probe. These mutations are included in the panel recommended for cystic fibrosis carrier screening by the American College of Medical Genetics and American College of Obstetrics and Gynecology (29).

Samples were genotyped in a 384-well plate using a probe for the Δ F508/ Δ I507 mutations in the odd columns and a probe for the 1717-1 G-to-A mutation (data not shown) in the even columns (Figure 3A). The samples were all correctly genotyped, and there were no unknown calls using within-run standards for each genotype and a score threshold of 0.7. When compared to the wild-type DNA duplex T_m , the Δ F508 and Δ I507 deletions caused T_m shifts of approximately 11°C and 5°C (Figure 3B). Results for the two probe sites (Δ F508/ Δ I507 and 1717-1 G-to-A) were linked to provide a genotype report for each sample, with the genotype from each site shown together with the standard (Figure 3C).

Base-pair mismatches shift the stability of a duplex by varying amounts depending on the particular mismatch, the mismatch position, and neighboring base pairs (30,31). Because the Δ F508 and Δ I507 deletions had a large difference in T_m (6°C), a single probe could easily distinguish between mutations. In situations where different mutations under the same probe result in similar melting profiles, a probe designed complementary to the mutation of interest can be used for confirmatory testing.

There are now many real-time PCR instruments that are capable of melting curve analysis,

but many do not provide software for automatic genotyping.

The LightTyper is an end-point genotyping instrument for high-throughput applications. The rate-limiting step in genotyping by fluorescent melting curve analysis is the time re-

Figure 3. SimpleProbe chemistry with cystic fibrosis assays. Genotypes determined for the Δ I507/ Δ F508 assay are shown in the color-coded plate map (A). Eighty-four samples were loaded in rows A–N of a 384-well tray. Samples in columns 1–12 were done in duplicate in columns 13–24. Odd columns contained the Δ IF07/ Δ F508 genotyping probe, and even columns contained the 1717-1 G-to-A genotyping probe (black, data not shown). Row O contained wild-type (wt-blue), Δ I507 heterozygous (h1-orange), and Δ F508 heterozygous (h2-yellow) controls. Row P contained negative (Neg-purple) controls. Representative melting peaks (B) are shown for each genotype group in the Δ I507/ Δ F508 assay. The report subset result (C) for sample 42 (N5 and N6) shows the melting peak of the sample (black line) compared to the standard for the called genotype (red line). Sample 42 was heterozygous for the Δ F508 mutation (h2-yellow box) and wild-type (G/G-blue box) at the 1717-1 G-to-A location.

quired for PCR amplification. Inexpensive thermal block cyclers used at the front end of the LightTyper can significantly increase throughput without having to purchase a real-time PCR instrument. While some available systems require additional enzymatic and wash steps before genotyping, the LightTyper system requires no additional steps for genotyping after amplification.

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