Published in final edited form as:

Clin Chem. 2008 December ; 54(12): 2055–2058.

High Resolution Melting Curve Analysis of Genomic and Whole Genome Amplified DNA

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Abstract

Background—High-resolution melting curve analysis is an accurate method for mutation detection in genomic DNA. However, performance in whole genome amplified (WGA) versus genomic DNA has not been directly compared.

Methods—23 amplicons from 9 genes were PCR amplified in 39 paired genomic and WGA samples and analyzed by high-resolution melting curve analysis using the 96-well LightScanner® (Idaho Technology). Genotyping and bidirectional resequencing were used to verify melting curve results.

Results—Melting patterns were concordant between the genomic and WGA samples in 823/863 (95%) of analyzed sample pairs. Of the discordant patterns, there was an overrepresentation of alternate melting curve patterns in the WGA samples suggesting the presence of a mutation (false positives). Targeted resequencing in 135 genomic and 136 WGA samples revealed 43 single nucleotide polymorphisms (SNPs). All SNPs detected in genomic samples were also detected in WGA. Additional genotyping and sequencing allowed the classification of a total of 628 genomic and 614 WGA amplicon samples. Heterozygous variants were identified by non-wild type melting pattern in 98% of genomic and 97% of WGA samples ($P = 0.11$). Wild types were correctly classified in 99% of genomic and 91% of WGA samples ($P < 0.001$).

Conclusions—In whole genome amplified DNA, high-resolution DNA melting curve analysis is a sensitive tool for SNP discovery through detection of heterozygote variants; however, it may misclassify a greater number of wild type samples.

Keywords

Mutation Detection; Single Nucleotide Polymorphism; Genome Sequencing; Candidate Gene

The comprehensive detection of novel DNA variants has traditionally relied on resequencing. However, despite recent advances, resequencing is expensive and time consuming. Methods to screen for variants can reduce the amount of sequencing and potentially increase efficiency and reduce cost. High-resolution DNA melting curve analysis is a sensitive and specific method for variant detection(1,2).

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Relationships to Disclose: We thank Idaho Technology, Salt Lake City, UT, for the use of the LightScanner®.

Categories: Genomics / Technology Development and Molecular Basis of Disorders with Complex Inheritance / Complex diseases

A concern in any genetic or genomic study which includes detection and genotyping of novel variants is the availability of sufficient quantities of DNA. Whole genome amplified (WGA) DNA samples have been reported to have a high reliability in many settings(3); however, few studies have specifically compared the performance of high-resolution DNA melting analysis (HRM) in genomic and whole genome amplified DNA. Our aim was to examine the performance of HRM on WGA DNA as a potential method for high-throughput variant discovery.

We performed whole genome amplification via the multiple displacement amplification (MDA) technique using phi29 DNA polymerase (REPLI-g®, Qiagen) on 39 subjects from the Boston Early-Onset Chronic Obstructive Pulmonary Disease Study (EOCOPD)(4). Participants in the EOCOPD Study gave written informed consent, and the appropriate institutional review boards approved the study.

We chose 23 amplicons from 9 chronic obstructive pulmonary disease candidate genes with at least 1 known polymorphism based on previous genotyping or sequencing studies for analysis (Table S1), resulting in a total of 897 genomic and 897 WGA amplicon samples (Table 1). Amplicons varied from 156bp to 392bp, with GC content from 39.4% to 70.9%. Amplification reactions were performed using Idaho Technology Mastermix with primers added to a final concentration of 0.25μM each. PCR optimization was performed using a 10μl reaction volume. 10ng of either genomic or WGA DNA was provided as an amplification template. Cycling conditions were as follows: 2 minutes at 95°C, followed by 40-45 cycles of: 94°C for 30 seconds, 60°C-72°C annealing temperature gradient for 30 seconds, 72°C for 30 seconds, and a final denaturing and re-annealing step (94°C, 30 seconds ramping to 25°C for 30 seconds, then 4°C at the end of the cycling protocol). Production PCR was performed using a 5μl reaction volume; conditions varied by amplicon. If the region amplified was of high GC content (>60%), DMSO, at a concentration of 10% volume to volume, was included.

PCR products were analyzed by HRM using the 96-well LightScanner® (Idaho Technology, Utah) by heating from 65 to 98°C at 0.3°C/s. Melting data were analyzed by the LightScanner® “Call-It” software by fluorescence normalization and difference curve analysis to optimize detection of heterozygous mutants. Standard sensitivity and autogroup settings were used for all samples. A single operator reviewed normalization and default variant calls.

At least one representative sample from each melting curve, as well as samples with discordant genomic versus WGA melting patterns were selected for resequencing. Samples were purified using the MinElute® PCR Purification Kit (Qiagen), then bi-directionally resequenced on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA), and resulting data were analyzed with Phred/Phrap/Consed and Polyphred software (5-8). In cases where the DNA melting sample was not resequenced, data from prior genotyping and sequencing reactions on genomic samples were used as a reference. For the purposes of analysis, a sample was classified as a heterozygous variant if any SNPs in the amplicon were genotyped or sequenced as heterozygous variant. The sample was classified wild type if sequencing or genotyping data demonstrated that the subject was homozygous wild type for all known SNPs in that amplicon. Homozygous variants were analyzed separately. Statistical analysis was performed using SAS 9.1 (SAS Institute, Cary, NC). Comparisons between WGA and genomic samples were performed using McNemar’s test for paired samples or the exact binomial as appropriate.

Out of 1794 high resolution DNA melting attempts, 1759 melting reactions (resulting in 164 unique melting patterns) were successful, for an overall completion rate of 98%. There were a greater number of failures in the WGA samples (25 failures, 2.8%), versus the genomic (10 failures, 1.1%; P=0.009, Table S2). 863 melting pattern pairs were analyzed in both genomic and WGA samples. Agreement on the presence of a wild type versus heterozygote variant was

seen in 95% of sample pairs. Of the discordant patterns, there were a greater number of melting curve patterns in the WGA samples suggesting the presence of a mutation ($P = 0.004$, Table S3).

Targeted resequencing was performed in 135 genomic and 136 WGA samples (Table 1). Overall, 42 different variants were found; the number of variants per amplicon ranged from 1 to 5. All variants found in the genomic samples were also identified in the WGA samples. A single SNP present in one amplicon sample identified and confirmed through resequencing was not identified by DNA melting pattern in either the WGA or genomic sample. In 132 cases, we were able to compare sequences in both WGA and genomic samples; 3% had discordant results, of which the majority were loss of heterozygosity in the WGA samples.

In addition to resequencing performed specifically for this project, genotyping and sequencing data from other projects were used to assess the accuracy of high-resolution melting, allowing classification of a total of 628 genomic and 614 WGA samples (Table 1). Excluding homozygous variants, the sensitivity for heterozygous variant detection was similar in WGA compared to genomic samples, (257/266, 96.6% versus 269/274, 98.2%; $P = 0.11$) while the specificity (percentage of wild types correctly assigned) was lower in WGA samples (260/287, 90.6% versus 289/293, 98.6%, $P < 0.001$). The positive predictive value and negative predictive value were 90.5% and 96.7% for WGA and 98.5% and 98.3% for genomic samples, respectively, although it should be noted that these values reflect the fact that nearly half the amplicon samples harbored heterozygous variants. Of the homozygous variants, 31/61 (50.8%) of genomic and 32/61 (52.5%) of whole genome amplified samples had variant melting curves. Low sensitivity (~30%) for homozygotes has been reported previously(9); techniques to optimize detection of homozygotes (use of smaller amplicons, mixing with known wild type) were not used for this study(10).

The majority of missed heterozygous variants (7/10, Table S4) occurred in an amplicon in *LTBP4*. This was the longest amplicon tested (392bp), and had 3 SNPs. In addition, the sample selected as representative of a wild type was in fact a heterozygous variant, and the difference curve a priori did not appear to allow clear discrimination of samples. In all cases, a tendency for the WGA melting patterns to have more dispersion was noted, though in general proper classification was possible. A representative run from an amplicon in *SFTPB* is shown in Figure 1. All variants in all samples in this amplicon were called correctly.

The multiple displacement amplification technique using the phi29 polymerase(11) has been shown to have a very low error rate with minimal amplification bias. In general, previous studies have demonstrated a very high rate of genotyping concordance between genomic and whole genome amplified DNA with a variety of methods(3), though others demonstrate evidence of a higher failure rate in WGA samples; loss, or more rarely, gain of heterozygosity; or bias(12-14).

We are aware of two previous studies that used WGA DNA in high-resolution melting curve analysis. Margraf et al (15) identified mutations in *RET*. All known mutations were detected, without false positives; however, these WGA samples were not directly compared via high resolution melting to genomic samples, and wild type controls and smaller amplicons were used. Bastien et al(16) examined *TP53* mutations in breast cancer samples using the LightCycler 480 (Roche Diagnostics), and directly compared WGA and non-WGA samples. Sensitivity and specificity were 86% and 95% for WGA versus 100% and 99% for non-WGA samples, respectively. However, these calculations were based on only 7 mutants out of 294 amplicon samples; in addition, these authors used a PCR-based WGA method, which may result in greater failure rate and sequence-dependant amplification than MDA(3). Our study is

the largest direct comparison of performance of high-resolution DNA melting in WGA versus genomic DNA.

One limitation of our study is that we did not sequence all individuals; thus our sensitivity and specificities represent estimates, and use of incomplete genotyping data could have biased results. However, our choice of sequencing discordant melting patterns likely enriched our sample for finding errors and was otherwise not intentionally selective. In addition, our genotyping and sequencing data were able to verify genotypes in the majority of samples.

In summary, high-resolution DNA melting analysis using the LightScanner® is a sensitive method for variant detection in genomic and whole genome amplified DNA. Whole genome amplified DNA appears to have a slightly higher failure rate and lower specificity than genomic DNA.

Further details on methods and results are available in the online supplement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Glenn Oliveira for his technical work and all the study participants.

This work was supported by U.S. National Institutes of Health (NIH) grants R01 HL075478 (Silverman) and K08 HL74193 (Raby).

We thank Idaho Technology, Salt Lake City, UT, for the use of the LightScanner®.

We thank all the study participants. Work was supported by U.S. National Institutes of Health (NIH) grants R01 HL075478 (Silverman), K08 HL74193 (Raby), and T32 HL07427 (Cho).

Abbreviations

WGA, whole genome amplified; SNP, single nucleotide polymorphism; HRM, high-resolution DNA melting analysis; MDA, multiple displacement amplification; EOCOPD, Boston Early-Onset Chronic Obstructive Pulmonary Disease Study.; Human Genes: LTBP4 latent transforming growth factor-beta-binding protein 4; SFTPB, surfactant, pulmonary-associated protein b.

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Figure 1. High resolution DNA Melt Analysis results for a 269 base pair amplicon containing 2 SNP in *SFTPB*. The melt pattern for the genomic samples is shown on the left, and the pattern for the WGA samples is shown on the right. Difference curve adjusted melt patterns show more scatter in the WGA data.

Table 1

Amplicon Sample Summary

		Genomic DNA	WGA DNA
High-resolution melting curve analysis	Attempted	897	897
	Successful	887	872
Reference data	Direct sequencing	135	136
	Prior genotyping or sequencing*	493	478
	Total	628	614

* Performed on genomic samples in prior experiments; see text.