Reports

Characterization of mitochondrial DNA heteroplasmy using a parallel sequencing system

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BioTechniques 48:287-296 (April 2010) doi 10.2144/000113389
Keywords: mitochondrial DNA; heteroplasmy; parallel sequencing

Supplementary material for this article is available at www.BioTechniques.com/article/113389.

Characterization of human mitochondrial genome sequences is important for the molecular diagnosis of mitochondrial diseases, especially in samples with a low level of mitochondrial DNA (mtDNA) heteroplasmy (≥5%). Currently, no single methodology can simultaneously determine complete mtDNA sequences, identify mitochondrial genome–wide heteroplasmies, and quantify mtDNA heteroplasmy levels. The deep sampling inherent in "next-generation" sequencing approaches should enable the efficient detection of low-level DNA heteroplasmies and address this need. Herein, we used the Illumina Genome Analyzer to re-sequence human mtDNA samples from two subjects that were combined at five different ratios (1:99, 5:95, 10:90, 20:80, and 50:50). We assessed the sensitivity, specificity, and accuracy of this system, and our results show that mtDNA heteroplasmies ≥5% were detected 100% of the time with virtually no false positives and that the estimates of mtDNA heteroplasmy levels were remarkably close to the theoretical values (correlation coefficient = 0.96). Therefore, parallel sequencing provides a simple, high-throughput, and cost-effective platform for mitochondrial genome sequencing with sensitivity and specificity for mtDNA heteroplasmy detection.

Introduction

Mitochondria are the "powerhouses" of human cells and disturbances in mitochondrial functions have been implicated in a wide range of human diseases, including cancer, heart disease, diabetes, Alzheimer's disease, and Parkinson's disease (1). The human mitochondrial genome, which is a circular DNA molecule that consists of 16,569 bp, encodes 13 polypeptides that are components of the electron transport chain (ETC), as well as 22 tRNAs and two rRNAs that contribute to mitochondrial protein synthesis. A variety of human diseases are directly associated with mitochondrial DNA (mtDNA) mutations and hundreds of putative pathogenic mtDNA variants have been identified (2,3).

Mitochondrial DNA is present in hundreds to thousands of copies per cell and also has a very high mutation rate. New mtDNA mutations arise in cells, coexist with wild-type mtDNAs (heteroplasmy), and segregate randomly during cell division (2). The vast majority of deleterious mtDNA point mutations are heteroplasmic and their mutant load can vary significantly among different tissues, even in the same subject. Moreover, different percentages of mutant mtDNA can be associated with completely distinct clinical manifestations (3). Currently, it is challenging to identify all of mutations in the mitochondrial genome and simultaneously quantify the mtDNA heteroplasmy levels. In addition to the molecular diagnosis of mitochondrial diseases, there is a rapidly growing need for methods to analyze mtDNA variants for other applications, including evolutionary and forensic studies (1,4). Therefore, it is critical that mitochondrial genome sequences can be acquired and detected in a reliable, high-throughput, and cost-effective manner, especially in samples with clinically relevant levels of mtDNA heteroplasmy.

Currently, the two most popular complete mitochondrial genome sequencing methods are direct sequencing and the MitoChip. However, these two methods are neither sensitive nor specific enough to detect mtDNA heteroplasmy (5). Methods used for mitochondrial genome–wide heteroplasmic position screening include denaturing HPLC (6), Surveyor Nuclease digestion (7), and high-resolution melt (HRM) profiling (8). Although these methods can be used to detect mtDNA heteroplasmy, they cannot localize or quantify the heteroplasmic position(s). Several other techniques have been developed for the specific quantification of mtDNA heteroplasmy levels. These methods include PCR-RFLP analysis (9), allele-

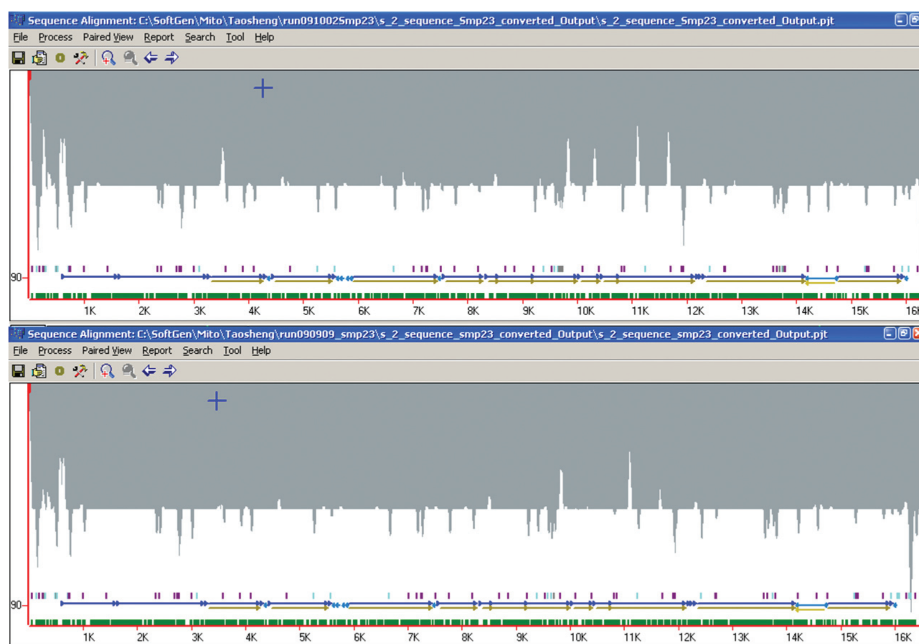

Figure 1. Coverage maps for 5% mixture sample in Test 1 (top) and Test 2 (bottom). The *x* axis represents the position of nucleotide on the mitochondrial genome (rCRS as reference genome, 16.568 kb), and the *y* axis stands for the fold coverage for each nucleotide position.

specific oligonucleotide dot-blot analysis (10), real-time amplification refractory mutation system quantitative PCR (11), and pyrosequencing (12). However, these methods are labor-intensive and can only be used to analyze a known mutation.

Recently developed parallel sequencing methods (13) have the capacity for massive sequencing and offer a highly robust and less labor-intensive approach to genome-wide sequencing. Currently, there are four next-generation sequencing platforms: the Illumina Genome Analyzer (GA; San Diego, CA, USA), the Roche 454 Genome Sequencer FLX system (Indianapolis, IN, USA), the Applied Biosystems SOLiD system (Foster City, CA, USA), and the Helicos True Single Molecule Sequencing system (Cambridge, MA, USA). The small size of the human mitochondrial genome and the resulting high coverage for each nucleotide position generated by parallel sequencing should enable the detection of low levels of mtDNA heteroplasmy. Previously, 454 sequencing was used to generate 34.9-fold coverage of the mtDNA from ~0.3-g bone of a 38,000-year-old Neanderthal individual (14). The Illumina GA, coupled with target microarray-based capture, was successfully employed to re-sequence the entire mitochondrial genome (coverage > 2,900) and the exons of 362 nuclear genes encoding mitochondrial proteins (15). However, neither of these studies investigated the capability of the technologies for heteroplasmy identification and quantification. In the current study, we utilized the Illumina GA system to sequence the entire human mitochondrial genome and determined the sensitivity and specificity of this platform for the analysis of heteroplasmic mtDNA samples.

Materials and methods

Subjects and DNA isolation

NS01 and NS09 are two human subjects who were recruited in our previous studies. The complete mtDNA sequences of NS01 and NS09 were determined previously. Total genomic DNA was extracted from peripheral blood using the QIAamp DNA extraction kit (QIAGEN, Valencia, CA, USA).

Complete mitochondrial genome amplification

The entire human mitochondrial genome was amplified in two overlapping fragments of 9289 bp (fragment I) and 7626 bp (fragment II) in length. The primer pair for amplification of fragment I is hmtF1 569 (5′-AACCAAACCCCAAAGACACC-3′) and hmtR1 9819 (5′-GCCAATAATGACGTGAAGTCC-3′), and the primer pair for amplification of fragment II is htmF2 9611 (5′-TCCCACTCCTAAACACATCC-3′) and hmtR2 626 (5′-TTTATGGGGTGATGTGAGCC-3′). PCR reactions were performed using TAKARA LA *Taq* DNA polymerase (Madison, WI, USA).

Preparation of mtDNA to test the sensitivity of mtDNA heteroplasmy detection

The concentrations of PCR products were measured using a NanoDrop 2000 spectrometer (Thermo Scientific, Wilmington, DE, USA). Equimolar amounts of fragments I and II were pooled for NS01 and NS09 separately. The pooled mtDNA fragments from NS01 and NS09 were then combined in five different ratios (1:99, 5:95, 10:90, 20:80, and 50:50) with a final amount of 500 ng used as starting material for Illumina GA libraries.

Illumina genome analyzer sequencing and data analysis

Parallel DNA sequencing was performed using an Illumina GA at Ambry Genetics Corp. (Aliso Viejo, CA, USA) according to the manufacturer's protocol. Pooled, amplified mtDNA samples were sheared and the resulting fragments were ligated to modified adapters that included 3-bp indexing tags. Following this "barcoding" step, the samples were multiplexed at 16 samples per lane in the Illumina GA flowcell. To assess the reproducibility of the system, each sample mixture was run twice. DNASTAR (Lasergene, Madison, WI, USA) and NextGENe

(Softgenetics, State College, PA, USA) were used to analyze the reads and align against the revised Cambridge reference sequence (rCRS) of human mtDNA (16). NextGENe software was also used to generate two types of SNP reports before (raw report) and after condensation (final report). Positions with SNP percentages <2% were removed from the final SNP report, with the exception of the 1% test mixture.

Comparison with dideoxy-terminator sequencing and conventional PCR-RFLP methods

To compare the sensitivity of parallel sequencing to the widely-used PCR-RFLP and dye terminator sequencing methods for low-level mtDNA heteroplasmy detection, the 1643-bp mtDNA fragment (rCRS positions 15,991–626) was PCR-amplified from the N01 and N09 samples using primers hmtF3 15591 (5′-TTCGCCTACACAATTCTCCG-3′) and hmtR3 626. The purified PCR products from NS01 and NS09 were combined at ratios of 95:5, 90:10, 80:20, 50:50, 20:80, 10:90, and 5:95, and 300 ng of the mtDNA mixtures were digested with *Eco*RV (New England BioLabs, Ipswich, MA, USA) and analyzed on a 1.2% agarose gel. NS09 has a C16278T transition, which creates an *Eco*RV recognition site. Therefore, the 1643-bp PCR product from NS09 spanning rCRS positions 15,991–626 will generate two fragments (956 bp and 687 bp) when cut with *Eco*RV. The same PCR product mixtures (100 ng) were also directly sequenced using the hmtF3 15591 primer with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Results and discussion

Experimental design

When this study was initiated, no single method could simultaneously detect and quantify mitochondrial genome-wide heteroplasmies. To determine whether the parallel sequencing platform could detect and accurately quantify the level of mtDNA heteroplasmy, we analyzed artificial mixtures of NS01 and NS09 mtDNA samples combined at different molar ratios. Mixing a known molar ratio of the pure mitochondrial genome amplicons is superior to having a mixture of genomic DNA for our purpose, since the mtDNA copy number varies substantially in different individuals and thus combining the same amount of genomic DNAs from two individuals does not represent an equal molar mixture of the mitochondrial genomes. A reasonable concern with using PCR amplicons is that amplification may introduce errors that can interfere with the interpretation of results. Mutations generated by polymerase during PCR are not position-specific, and therefore—unless the starting material is very small (for example, only one or two molecules in the template) and the error was introduced very early in the amplification process—noise created by the polymerase will be random and at a frequency of $\sim 10^{-6}$. Since even a single cell has hundreds or thousands of copies of the mitochondrial genome, such mutations should not create any errors that are significant in the final sequencing product. In addition, our results show that detected heteroplasmy levels are very consistent with the theoretical ratios, further proving that errors introduced by PCR should not be a significant problem with this approach.

We selected the NS01 and NS09 samples from a group of subjects with known mitochondrial genome sequences and with biological material available in our laboratory. Pair-wise comparisons of the number of divergent nucleotides among these subjects revealed that the NS01-NS09 pair had the largest number of variant mtDNA positions. Fifty-six nucleotide positions throughout the mitochondrial genome (Supplementary Table S1) differ between NS01 and NS09, enabling both intra- and inter-genome comparisons of mtDNA heteroplasmy detection.

Bioinformatics tools for data analysis

Low-level mtDNA heteroplasmy detection and quantification based on the short reads from parallel sequencing requires powerful analysis tools capable of distinguishing between instrumental errors and true low-frequency mutations. Different in silico algorithms can yield quite divergent results. We first used the DNASTAR algorithm to quantify the heteroplasmic variants and found that the estimated ratios deviated dramatically from the theoretical values and a significant number of false positives were annotated (data not shown). In contrast, NextGENe was highly specific and accurate for mtDNA heteroplasmy analysis. NextGENe employs a condensation tool to solve the three critical problems associated with parallel sequencing: short reads, high system error rates, and large volumes of data. Specifically, the condensation tool clusters similar short reads from

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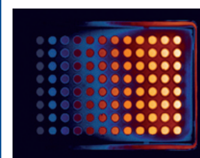

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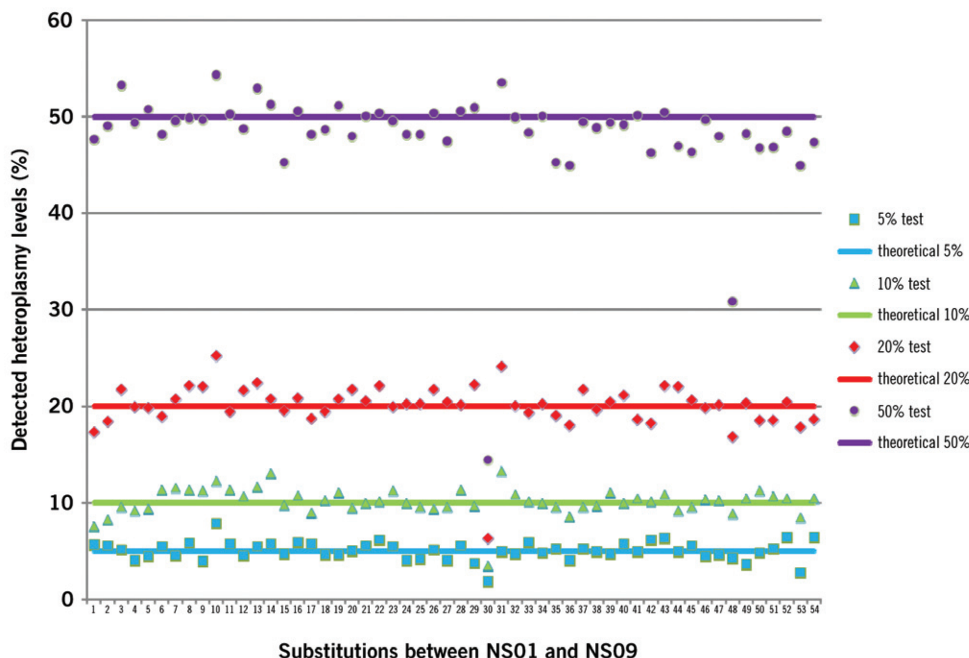

Figure 2. Detected and theoretical heteroplasmy levels for the 54 expected substitutions from Test 1. The mitochondrial genomes of NS01 and NS09 differ in 56 nucleotides, 54 of which are substitutions. The *x* axis represents the 54 individual substitutions in the order of their position in the rCRS reference genome. The lines are the theoretical values (5:95, 10:90, 20:80, and 50:50) and the spots of the same colors denote detected heteroplasmy levels.

the Illumina GA, containing a unique anchor sequence. Therefore, data of adequate coverage are condensed, short reads are lengthened, and instrument errors are filtered from the analysis. The reads used for each condensed read are recorded to maintain allele frequency information.

Depth of coverage by parallel sequencing of mtDNA

Using the barcoding protocol to pool 16 samples in the same lane, each variant nucleotide position was covered by 655–6368 reads (average of 1785; Figure 1). The fold coverage was fairly even across the mitochondrial genome with a few peaks and troughs. The pattern of the coverage map was reproducible in replicate runs of the same samples (Figure 1) and in different samples (data not shown).

Sensitivity of parallel sequencing for mtDNA heteroplasmy detection

To determine the detection threshold of parallel sequencing for mtDNA heteroplasmy, we generated mtDNA mixtures of the two different human samples (NS01 and NS09) at five different ratios (ranging 1–50%). For the 56 known variant positions between NS01 and NS09, all heteroplasmies ≥5% were detected when using this method.

Among the 56 variant positions, 54 were substitutions and the remaining two were insertions/deletions (Supplementary Table S1). Compared with revised Cambridge reference sequence (rCRS), which is the standard reference human mtDNA sequence used by the mitochondrial genetics society (16), the NS01 sample has 523delA and 523delC. The Illumina GA system was able to detect this dinucleotide deletion down to the 5% level, and the estimated variant load was very close to the theoretical ratio (Supplementary Table S1).

In condensed reports for the 54 substitutions, 9 (16.7%, Test 1) or 21 (38.9%, Test 2) substitutions were called in the 1% test mixture (Supplementary Table S1), and all were called in the 5% test mixture, showing that the parallel sequencing system is extremely sensitive to heteroplasmies of 5% and above. Theoretically, 1% mtDNA heteroplasmy is equivalent to 10 variant calls for a position with 1000-fold coverage. With the 1785-fold average coverage in the current study, the parallel sequencing method is expected to be able to detect 1% mtDNA heteroplasmy. Indeed, in the raw report from the 1% test mixture, 49 out of the 54 expected substitutions were identified. However, >100 possible false positive variants were also listed in the raw report from the 1% test mixture. Together, these observations suggested that the parallel sequencing system was sensitive at 1%, but that the specificity was low at this level.

Accuracy of parallel sequencing for mtDNA heteroplasmy detection

To determine the accuracy of the parallel sequencing system, we analyzed the detection levels of all 54 expected substitutions and plotted the observed estimates against the theoretical values. As demonstrated in Figure 2 and Supplementary Table S1, the observed mtDNA heteroplasmy ratios were very consistent with the theoretical levels, except for rCRS positions 9596 and 16,086. We found an excellent correlation between detected and theoretical levels at the different ratios (5:95, 10:90, 20:80, and 50:50) for individual positions, as shown by the almost identical pattern of scattered points for each test mixture (Figures 2). The average detected mtDNA heteroplasmy level in each test mixture was nearly equal to the theoretical ratio, and the mean values from the different test mixtures exhibited a near-perfect linear regression ($R^2 = 0.9997$) with a correlation coefficient of 0.96 for the predicted ratio (Figure 3).

Two outliers, rCRS positions 9596 and 16,086, were associated with significantly lower detected mtDNA hetero-

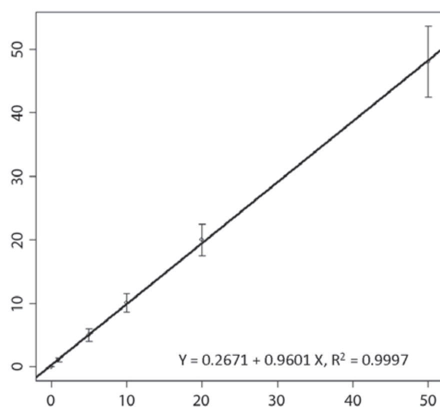

Figure 3. Correlation of expected and observed heteroplasmy levels for 54 substitution positions. For each of the five mixtures of NS01 and NS09 (1:99, 5:95, 10:90, 20:80, and 50:50), the circle and the error bar represent the mean and standard deviation of detected ratio for the 54 positions. The linear regression curve is derived from the mean detected values of the five samples.

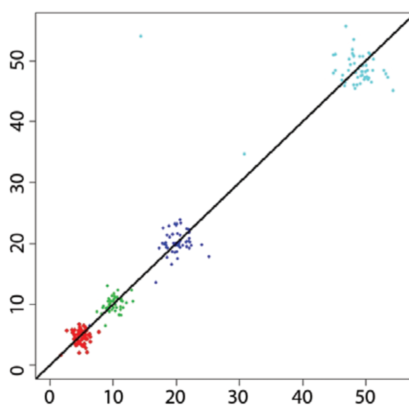

Figure 4. Reproducibility of the parallel sequencing system. *x* and *y* axes stand for the detected heteroplasmy levels in the replicate runs. Red, green, blue, and light blue dots represent the results from mixtures of NS01:NS09 (5:95, 10:90, 20:80, and 50:50, respectively).

plasmy levels than the theoretical values. However, there was no ambiguity in the alignment of the reads covering these two positions. It is possible that fragments with specific nucleotides were preferentially amplified in the GA library and resulted in deviated ratio of the two mtSNPs.

Specificity and reproducibility of parallel sequencing for mtDNA heteroplasmy detection

While detection of all predicted variations is essential, a low number of false positives is also important. We found no false positives in test mixtures ≥5% in the final report. Such low noise levels in the machine-generated results did not require manual input to eliminate noise, which is especially crucial and advantageous for the application of this high-throughput platform in large-scale mtDNA sequencing projects.

To determine the reproducibility of the system for mtDNA heteroplasmy analysis, replicate runs were carried out and analyzed for each test mixture. The results from the two replicate assays were highly reproducible, with the exception of rCRS position 9596 in the 50% test mixture (Figure 4), demonstrating the superior reproducibility of the system.

Comparison to PCR-RFLP and direct sequencing

To compare the sensitivity of parallel sequencing versus PCR-RFLP analysis for mtDNA heteroplasmy identification, we analyzed rCRS position 16,278 using both PCR-RFLP and parallel sequencing. Various factors can affect the detection threshold of heteroplasmy of PCR-RFLP and generally, mutations ≤10% cannot be identified by this methodology (11). Our results showed that parallel sequencing was much more sensitive to low levels of mtDNA heteroplasmy than PCR-RFLP (5% versus 10%; Supplementary Figure S1 and Supplementary Table S2) and did not require the complex signal intensity conversions used for the quantification of PCR-RFLP data.

To compare parallel sequencing with traditional Sanger sequencing for heteroplasmy analysis, we simultaneously analyzed rCRS positions 16,051 and 16,086 using automated sequencing. As shown in Supplementary Figure S2 and Supplementary Table S3, traditional sequencing generated clear double peaks for heteroplasmies ≥20%, but yielded ambiguous and undetectable signals at the lower levels of 10% and 5%, respectively. In contrast, parallel sequencing was able to recognize and reliably estimate 5% mtDNA heteroplasmy at the same two positions, and therefore outperformed direct sequencing in terms of both sensitivity and accuracy of quantification.

Advantages and disadvantages of the Parallel Sequencing Approach

The use of the Illumina GA system in mtDNA analysis as presented in this study allowed us to simultaneously (*i*) characterize the complete nucleotide sequence for the mitochondrial genome, (*ii*) annotate all heteroplasmic positions in the mtDNA (≥5%), and (*iii*) provide estimates of the mutant load at each position. In addition, the parallel sequencing system requires no prior knowledge of the mtDNA sequence and it involves minimal labor. The mtDNA heteroplasmy levels were readily calculated without complex calibration or computation. This technology is cost-effective and the turnaround time is short: the workflow from DNA sample preparation to SNP report can be accomplished in less than one week. Furthermore, this method can be easily standardized and carried out on a large scale. For example, using the indexing protocol in this study, each of the eight lanes in the Illumina GA flow cell can accommodate up to 16 samples, so that a total of 128 samples can be run simultaneously. If necessary, more than 16 samples can be multiplexed in the same lane, which can further reduce the cost of each individual sample. Although the parallel sequencing can provide the most comprehensive information about the sequence of a mitochondrial genome, it is generally not sensitive enough to detect large mtDNA deletions/insertions or to determine mtDNA copy number. For the detection of small deletions/insertions, the analysis parameters can be optimized for the purpose of properly aligning short reads. In addition, a deviation from the theoretical ratio can occur infrequently for specific positions, possibly since fragments with specific nucleotides are preferentially amplified in the GA library.

In summary, the Illumina GA parallel sequencing system provides a high-throughput, accurate, and cost-effective platform for human mitochondrial genome characterization. This system is sensitive in the detection and quantification of genome-wide heteroplasmies ≥5%, with virtually no false positives according to our tests. We expect that parallel sequencing approaches will usher in a new era of opportunity in mtDNA mutation analysis, and will enhance understanding of mtDNA-based disorders.

Acknowledgments

We would like to thank Anja Kammesheidt and Wei Guo at Ambry Genetics for their hard work and efforts in data analysis. This project was supported by the Darren J. Carroll Family. T.H. is also supported by National Eye Institute (NEI; grant no. 1R01EY018876). This paper is subject to the NIH Public Access Policy.

Competing interests

T.H. serves as a consultant/Medical Director in Pharmaceutical Service for Ambry Genetics. He receives compensation from the company. In this study, parallel sequences was run at Ambry Genetics based on fee-for-service. S.T. declares no competing interests.

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Received 3 December 2009; accepted 18 February 2010.

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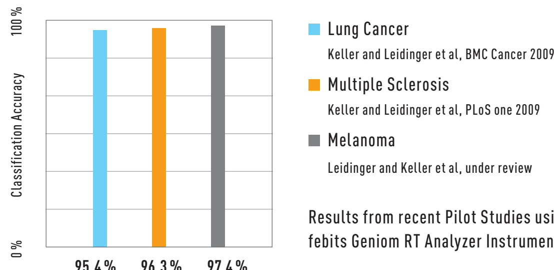

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